# STUDIES INTO THE REGULATION OF CAAT/ENHANCER BINDING PROTEIN δ EXPRESSION (C/EBPδ) BY CYTOKINES


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Signed ..... (Candidate)

Date 22/1/08

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# ABSTRACT

Inflammation is a physiological response to injury, trauma or infection. The initial phase of inflammation, the acute phase response (APR), is characterised by changes in levels of serum acute phase response proteins (APPs). These APPs are synthesised primarily by hepatocytes in response to several inflammatory cytokines, including interleukin-1 (IL-1) and interleukin-6 (IL-6). Expression of APP genes is known to be regulated by the CCAAT Enhancer Binding Protein (C/EBP) family of transcription factors, which consists of six members (α-ζ). Several studies have implicated C/EBPδ as an important regulator of APP gene transcription during inflammation. Additionally, a number of cytokines and other inflammatory mediators modulate the expression/activity of C/EBPδ during the APR. Unfortunately, the mechanisms by which cytokines regulate the expression of C/EBPδ remain poorly understood. Understanding these mechanisms could potentially aid in the development of therapeutic strategies aimed at combating inflammatory disease. Therefore the objective of this project was to delineate the molecular mechanisms governing C/EBPδ expression in response to IL-1 and to a lesser extent, IL-6.

Initial investigations were designed to determine the effects of various inflammatory mediators on C/EBPδ expression in hepatocytes focusing on IL-1, with the aim of delineating the regulatory elements in the human C/EBPδ gene promoter that were responsible for gene expression in response to this cytokine, and to identify the nuclear factors regulating this response. However, unlike endogenous C/EBPδ mRNA and protein expression, both of which were induced by IL-1, the activity of the human C/EBPδ gene promoter was not stimulated by this cytokine, despite being responsive to IL-6 action. Similar results were also obtained when the actions of IL-1 and IL-6 on the murine C/EBPδ gene promoter were examined. Using the same transfection system, studies were also initiated to determine if the human C/EBPδ gene was subject to auto-regulation, as this mode of control is likely to play a significant role in regulating C/EBPδ gene expression during the APR and because this was a novel area of investigation. Results from these studies showed, for the first time, that the human C/EBPδ gene is subject to auto-regulation, potentially via an indirect mechanism and the *cis*-acting elements responsible for this response were identified to within approximately 200 base pairs of the transcriptional initiation site.

Therefore, as a consequence of our initial findings, we adopted an alternative strategy for determining the mechanisms by which C/EBPδ expression was induced by IL-1. This involved investigating the signal transduction pathways involved in the actions of IL-1 on the expression of this gene and characterising the critical components of these pathways. Simultaneously, some investigations were also carried out to identify the transcription factors regulating endogenous C/EBPδ gene expression by IL-6, given that this was also a novel area of investigation.

With the use of a range of commercially available pharmacological inhibitors, our initial investigations led to the identification of casein kinase 2 (CK2), c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) as potential regulators of IL-1-induced C/EBPδ expression. The exact role of these pathways was then further investigated using several independent approaches. Western blot analysis and associated kinase activation assays revealed that JNK is activated by IL-1 in hepatocytes, a novel finding. With the use of RNA interference, *trans*-activation assays and electrophoretic mobility shift assays, our investigations led to the identification of NF-κB as a key regulator of the IL-1-induced C/EBPδ expression, also a novel finding. In association with this finding, we also investigated a role for CK2 and JNK in the regulation of NF-κB activation by IL-1.

Finally, our studies have also revealed a critical role for the STAT (Signal Transducer and Activator of Transcription) family of transcription factors in the regulation of human C/EBPδ gene expression by IL-6. Consistent with this finding, we also show that IL-6 action leads to the activation of both STAT1 and STAT3 in hepatocytes.

To summarise, the studies presented here have revealed that the human C/EBPδ gene is subject to transcriptional regulation by auto-activation and in response to IL-1 and IL-6 action is induced by NF-κB and STATs, respectively. These studies have provided novel insights into the molecular mechanisms by which C/EBPδ is regulated during the APR.

# ABBREVIATIONS

| ABBREVIATION | FULL TERM |
|---|---|
| AD | Activation domain |
| AGP/EBP | Alpha-1 acid glycoprotein/enhancer binding protein |
| AML | Acute myelogenous leukemia |
| AP-1 | Activator protein-1 |
| ApoE | Apolipoprotein E |
| APP | Acute phase response protein |
| APR | Acute phase response |
| APRE | Acute phase response element |
| APRF | Acute phase response factor |
| APS | Ammonium persulphate |
| ASK | Apoptosis signal-regulating kinase |
| ATF | Activating transcription factor |
| ATP | Adenosine 5'-triphosphate |
| BMP | Bone morphogenetic protein |
| bp | Base pair |
| BRE | TFIIB recognition element |
| BSA | Bovine serum albumin |
| bZIP | Basic-leucine zipper |
| C/ATF | C/EBP-related activating transcription factor |
| C/EBP | CCAAT/enhancer binding protein |
| CA150 | Transcription elongation regulator 1 |
| cAMP | Cyclic adenosine monophosphate |
| CBP | CREB-binding protein |
| CD | Complementarity determinant |
| cDNA | Copy DNA |
| ChIP | Chromatin immunoprecipitation |
| ChIP-on-chip | Chromatin immunoprecipitation-on-chip array |
| CHOP | C/EBP homologous protein |
| CK2 | Casein kinase 2 |
| CMV | Cytomegalovirus |
| COX-2 | Cyclooxygenase-2 |
| CRE | cAMP-responsive element |
| CREB | cAMP-responsive element binding protein |
| CRP | C-reactive protein |
| CTD | Carboxy-terminal domain |
| Da | Dalton |
| $ddH_20$ | Double distilled water |
| DIC | Disseminated intravascular coagulation |
| DMD | Duchenne muscular dystrophy |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulphoxide |
| DN | Dominant negative |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| DPE | Downstream promoter element |
| DRB | 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole |
| dsDNA | Double-stranded DNA |
| dsRNA | Double-stranded RNA |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| ECSIT | Evolutionary conserved signalling intermediate in Toll pathways |
| EDTA | Ethylene diamine tetraacetic acid |
| EGF | Epidermal growth factor |

| | |
|---|---|
| eIF | Elongation initiation factor |
| EMSA | Electrophoretic mobility shift assay |
| ER | Endoplasmic reticulum |
| ERK | Extracellular signal related kinase |
| ES | Embryonic stem |
| FGF | Fibroblast growth factor |
| g | Grams |
| Gab1 | Grb2-associated binder 1 |
| GADD153 | Growth arrest and DNA damage-inducible gene 153 |
| GAPDH | Glyceraldehyde phosphate dehydrogenase |
| GH | Growth hormone |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| gp130 | Glycoprotein 130 |
| Grb2 | Growth factor receptor-bound protein 2 |
| GSK-3 | Glycogen synthase kinase-3 |
| GST | Glutathione S-transferase |
| GTP | Guanosine-5'-triphosphate |
| GTPases | Guanosine triphosphatases |
| h | Hours |
| HAT | Histone acetyltransferase |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HI-FCS | Heat inactivated-fetal calf serum |
| HIV | Human immunodeficiency virus |
| HRP | Horseradish peroxidase |
| IκB | Inhibitor of κB |
| ICAM | Intercellular adhesion molecule |
| ICER | Inducible cAMP early repressor |
| IFN | Interferon |
| Ig/EBP-1 | Immunoglobulin/enhancer binding protein-1 |
| IGF | Insulin-like growth factor |
| IKK | IκB kinase |
| IL | Interleukin |
| IL-1F | Interleukin-1 family |
| IL-1R | Interleukin-1 receptor |
| IL-1Ra | Interleukin-1 receptor antagonist |
| IL-1RAcP | Interleukin-1 receptor accessory protein |
| IL6-DBP | Interleukin-6-dependent DNA binding protein |
| IL-6R | Interleukin-6 receptor |
| IL6-RE | Interleukin-6 responsive element |
| iNOS | Inducible NOS |
| Inr | Initiator element |
| IR | Insulin receptor |
| IRAK | Interleukin-1 receptor associated kinase |
| JAK | Janus kinase |
| JNK | c-Jun amino-terminal protein kinase |
| k | Kilo |
| l | Litre |
| LAP | Liver-enriched activating protein |
| LB | Luria broth |
| LIF | Leukaemia inhibitor factor |
| LIP | Liver-enriched inhibitor protein |
| LPL | Lipoprotein lipase |
| LPS | Lipopolysaccharide |
| LT | Lymphotoxin |
| Luc | Luciferase |
| M | Molar |
| MAPK | Mitogen activated protein kinase |
| MAPKK | Mitogen activated protein kinase kinase |
| MAPKKK/MAP3K | Mitogen activated protein kinase kinase kinase |
| MCP | Monocyte chemoattractant protein |

| | |
|---|---|
| M-CSF | Macrophage-colony stimulating factor |
| MEF | Mouse embryonic fibroblast |
| MEK | Mitogen-activated protein kinase kinase/extracellular regulated kinase |
| min | Minutes |
| MIP | Macrophage inflammatory protein |
| MMLV | Moloney murine leukaemia virus |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger RNA |
| MyD88 | Myeloid differentiation protein 88 |
| MyoD | Myogenic differentiation antigen |
| NCoR | Nuclear receptor corepressor |
| NEMO | NF-κB essential modifier |
| NF-κB | Nuclear factor κB |
| NF-IL6 | Nuclear factor IL-6 |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| OSM | Oncostatin M |
| P/CAF | p300/CBP-associated factor |
| Pa | Pascals |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI | Plasminogen activator inhibitor |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| PEI | Polyethylenimine |
| pen/strep | Penicillin/streptomycin |
| phospho | Phosphorylated |
| PI3K | Phosphoninositide 3-kinase |
| PIAS | Protein inhibitors of activated STATs |
| PIC | Pre-initiation complex |
| Pit-1 | Pituitary specific transcription factor 1 |
| PKA | Protein kinase A |
| PKB | Protein kinase B (Akt) |
| PKC | Protein kinase C |
| PKR | dsRNA-dependent protein kinase/protein kinase R |
| PMA | Phorbol 12-myristate 13-acetate |
| PMSF | Phenylmethanesulphonyl fluoride |
| Pol | Polymerase |
| PPAR | Peroxisome proliferator-activated receptor |
| Pu | Purine |
| PVDF | Polyvinylidene fluoride |
| Rac | Ras-related C3-botulinum toxin substrate |
| Raf | Rous sarcoma associated factor |
| RANK | Receptor activator of NF-κB |
| RANTES | Regulated upon Activation, Normal T-cell Expressed and Secreted |
| Rap | Receptor associated protein |
| Rb | Retinoblastoma protein |
| RD | Regulatory domain |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RPMI-1640 | Roswell Park Memorial Institute-1640 |
| rRNA | Ribosomal RNA |
| RSV | Rous sarcoma virus |
| RT | Reverse transcriptase |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| Runx-2 | Runt-related transcription factor 2 |
| s | Seconds |

| | |
|---|---|
| S6K | Ribosomal protein S6 kinase (p70) |
| SAA | Serum amyloid A |
| SAPK | Stress activated protein kinase |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEK | SAPK/ERK kinase |
| SH2 | Src homology 2 (domain) |
| SHP2 | Src homology 2-containing tyrosine phosphatase |
| siRNA | Small interfering RNA |
| SMC | Smooth muscle cell |
| SOCS | Suppressor of cytokine signalling |
| SOS | Son of sevenless |
| Sp | Specificity protein |
| SRC-1 | Steroid receptor coactivator-1 |
| STAT | Signal transducer and activator of transcription |
| SUMO | Small ubiquitin-related modifier |
| SUV39H1 | Suppressor of variegation 3-9 homolog 1 |
| SV40 | Simian virus 40 |
| SWI/SNF | Switching deficient/sucrose non-fermenting |
| TAB | TAK1-binding protein |
| TAF | TBP-associated factor |
| TAK1 | Transforming growth factor beta activated kinase 1 |
| TANK | TRAF (TNF receptor-associated factor) family member associated NF-κB activator |
| TBB | 4, 5, 6, 7-tetrabromobenzotriazole |
| TBE | Tris-borate-ethylenediaminetetracetic acid |
| TBP | TATA-binding protein |
| TBS | Tris-buffered saline |
| TE | Tris-ethylenediaminetetracetic acid |
| TEMED | N, N, N',N, tetramethylenediamine |
| TF | Transcription factor |
| TGF | Transforming growth factor |
| TIR | Toll-interleukin receptor domain |
| TNF | Tumour necrosis factor |
| Tollip | Toll interacting protein |
| TRAF6 | Tumour necrosis factor-alpha associated factor 6 |
| TRE | TPA responsive element |
| TRF | TBP-related factor |
| tRNA | Transfer RNA |
| Tyk | Tyrosine kinase |
| U | Units |
| UN | Untreated |
| USF | Upstream stimulating factor |
| UV | Ultra-violet |
| V | Volts |
| v/v | Volume/volume |
| w/v | Weight/volume |
| xg | x gravity |

# CHAPTER ONE: GENERAL INTRODUCTION

Inflammation is the physiological response to tissue injury, trauma or infection designed to combat further tissue damage, destroy invading pathogens and activate cellular repair processes. Although inflammation acts as a defence mechanism, aberrant inflammation contributes towards the pathophysiology of many diseases including atherosclerosis, asthma, inflammatory bowel disease and auto-immune conditions such as rheumatoid arthritis. The inflammatory response is orchestrated by a group of regulatory proteins collectively known as cytokines. Cytokines exert multiple actions on a variety of tissues principally by inducing changes in cellular gene expression. Understanding the signalling pathways by which cytokines modulate gene expression could potentially aid the development of therapeutic strategies aimed at combating inflammatory diseases, in addition to providing a more in depth understanding of the molecular mechanisms that govern the regulation of gene expression. Therefore, the focus of this thesis is the investigation of signalling pathways by which the major pro-inflammatory cytokines interleukin 1 (IL-1), and to a lesser extent, interleukin 6 (IL-6) regulate the expression of CCAAT Enhancer Binding Protein δ (C/EBPδ), a key transcription factor implicated in the regulation of gene expression associated with the inflammatory response.

This introduction will start with an overview of eukaryotic gene transcription and its regulation. A description of the C/EBP family of transcription factors, their biological actions and a discussion of their roles in inflammation will follow. Finally, the signal transduction pathways by which the cytokines, IL-1 and IL-6 mediate their affects will be considered.

## 1.1 TRANSCRIPTION

RNA polymerase II is one of three RNA polymerases present in eukaryotic cells. It is responsible for the transcription of messenger RNA (mRNA) and most small nuclear RNAs. As RNA polymerase II regulates the production of mRNAs which are subsequently translated into proteins, this section will be restricted to a discussion of the regulation of transcription, as mediated by this particular polymerase (Latchman 1998).

RNA polymerase II driven gene expression is a tightly controlled process involving several stages, all of which are subject to regulation. These stages include the

transcription of DNA into mRNA, modification of the mRNA, export of the mRNA from the nucleus to the cytoplasm and finally, translation of the mRNA into a specific protein. In addition, it should be noted that regulation of gene expression is subject to the affects of other parameters, such as mRNA and protein stability. It is necessary for gene expression to be a tightly regulated process because it controls the development and differentiation of an organism, it is the terminating point of many signal transduction pathways and it continually reshapes the cell in response to metabolic needs and environmental stimuli.

RNA polymerase II driven gene transcription is dependent on the co-ordinate interaction of specific regulatory proteins (*trans*-acting factors) with numerous DNA sequence elements (*cis*-acting elements). These DNA sequence elements are usually located in the 5' region of a gene upstream from the transcriptional initiation site. The DNA sequences involved include the promoter region, which consists of core promoter and upstream regulatory elements and the enhancer and silencer regions that often function distally from the gene to induce or inhibit gene transcription, respectively (see Roeder 1996; Smale and Kadonaga 2003 for reviews). The influence of enhancer/silencer elements is blocked by boundary/insulator elements (Blackwood and Kadonaga 1998; West *et al.* 2002). *Trans*-acting factors or transcription factors act by binding to specific *cis*-acting elements and subsequently influence transcription by interacting directly or indirectly with RNA polymerase II (or the associated machinery, see section 1.1.3). RNA polymerase II driven gene expression is supported by a group of transcription factors often termed the general transcription factors. These transcription factors function by binding the core promoter of a class II gene (genes transcribed by RNA polymerase II) and associate with RNA polymerase II in initiating transcription. Regulatory transcription factors function by binding *cis*-acting elements located upstream or downstream, proximally or distally from the core promoter and control transcription driven by RNA polymerase II associated with the general transcription factor machinery (see Orphanides *et al.* 1996; Roeder 1996 for reviews).

### 1.1.1 THE RNA POLYMERASE II CORE PROMOTER

The core promoter elements have been defined as 'minimal DNA elements that are necessary and sufficient for accurate transcription initiation by RNA polymerase II in

reconstituted cell-free systems' (Roeder 1996). The core promoter contains the transcriptional start site of a gene and typically extends ~35 nucleotides upstream or downstream from this site. There is a considerable amount of variability in the DNA elements that constitute the core promoter region of many class II genes. The core promoter can contain the TATA box, the TFIIB recognition element (BRE), the initiator (Inr) and the downstream promoter element (DPE) and these sequence elements can be found in various combinations in this region of class II genes (Figure 1.1) (Roeder 1996; Kadonaga 2002; Smale and Kadonaga 2003). Apart from these core promoter elements, other motifs have also been associated with gene promoters such as CpG islands (Smale and Kadonaga 2003).

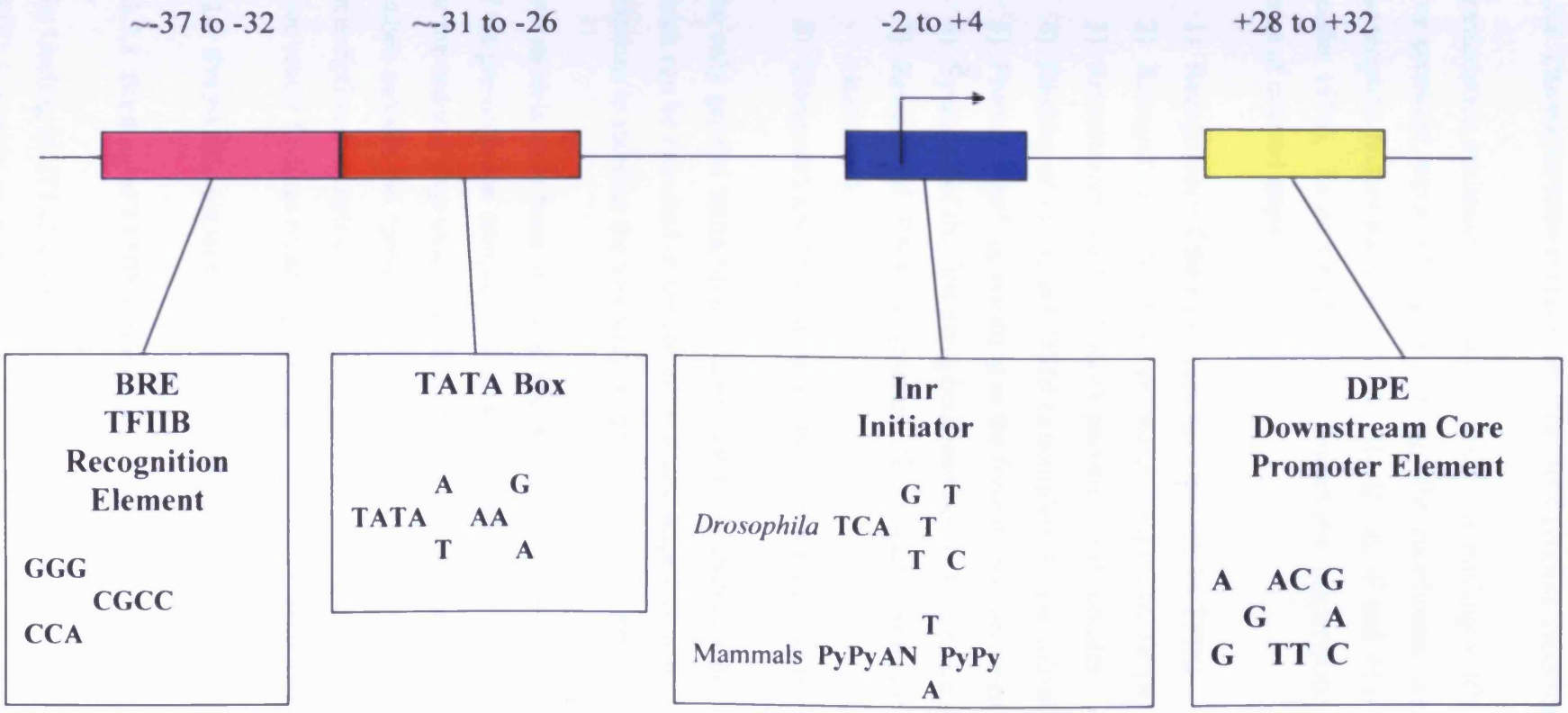



**FIGURE 1.1 A schematic representation of the RNA polymerase II core promoter motifs (adapted from Smale and Kadonga 2003).** The diagram represents some of the sequence elements that can contribute to basal transcription from a core promoter. Each of these sequence motifs can be found only in a subset of core promoters. A particular core promoter may contain some, all, or none of these sequences. The TATA box is functional in the absence of the BRE, Inr and DPE motifs. Conversely, the DPE motif requires the presence of an Inr motif. The BRE is located immediately upstream in a subset of promoters containing a TATA box. The DPE consensus was determined with *Drosophila* core promoters. The Inr consensus is displayed for both mammals and *Drosophila*. The arrow depicts the transcriptional start site, Py indicates a pyrimidine base and N denotes any base.

### 1.1.2 TRANSCRIPTION INITIATION AND THE GENERAL TRANSCRIPTION FACTORS

Transcription initiation is mediated through the binding of RNA polymerase II to the core promoter region of a gene, through the coordinated association of six general transcription factors namely, TFII-A, -B, -D, -E, -F and -H (Orphanides *et al.* 1996; Roeder 1996). In its simplest form, eukaryotic transcription can be divided into a series of ordered steps:

1) Recognition of the core promoter elements by TFIID.
2) Recognition of the TFIID-promoter complex by TFIIB.
3) Recruitment of a TFIIF/RNA polymerase II complex.
4) Binding of TFIIE and TFIIH to complete the pre-initiation complex.
5) Promoter melting resulting in the formation of an 'open' initiation complex.
6) Synthesis of the first phosphodiester bond of the nascent mRNA.
7) Release of RNA polymerase II contacts with the promoter ('promoter clearance').
8) Elongation and termination of the mRNA transcript (Orphanides *et al.* 1996).

The only general transcription factor not to be included in this assembly is TFIIA, which can be recruited to the complex at any stage following the binding of TFIID, it functions to stabilise the assembly (Orphanides *et al.* 1996).

Two models have been proposed and supported by various studies for the formation of the pre-initiation complex (PIC). *In vitro* reconstitution studies support the model for the ordered step-wise assembly of the pre-initiation complex, whilst more recent studies support the 'pre-formed or holoenzyme' model of the RNA polymerase II transcriptional complex. Both these models will be discussed briefly, together with more recent findings regarding RNA polymerase II driven transcription.

### 1.1.3 STEPWISE ASSEMBLY OF THE PRE-INITIATION COMPLEX

#### 1.1.3.1 Binding of TFIID at the core promoter

The binding of TFIID to the TATA box is the first stage in the assembly of the PIC. TFIID is a multi-protein complex; it comprises of TATA-binding protein (TBP) and multiple TBP-associated factors or TAFs. It is the TBP subunit of TFIID that is responsible for TATA box recognition (Hernandez 1993). TFIID (specifically the

$TAF_{II}250$ subunit) possesses both histone acetyltransferase (HAT) and kinase activities (Tansey and Herr 1997). The kinase activity regulates auto-phosphorylation of TFIID. TFIIA and TFIIF are also likely substrates for the TFIID kinase (Dikstein *et al.* 1996; Solow *et al.* 2001). The auto-phosphorylation function of TFIID is likely to play a regulatory role in transcription (Wassarman and Sauer 2001). HAT activity of TFIID may regulate changes in chromatin structure to facilitate binding of other general transcription factors to the PIC (Tansey and Herr 1997).

### 1.1.3.2 Binding of TFIIA and TFIIB

The interaction between TBP and the TATA element is stabilised by the binding of TFIIA and TFIIB to the complex. TFIIA binds to the TBP-DNA complex and increases the affinity of TBP for the TATA box. TFIIB is important in providing a physical link between TFIID and the RNA polymerase II/TFIIF complex at the core promoter. Additionally, some eukaryotic core promoters contain the BRE which is reported to stabilise the interaction between TBP and TFIIB on DNA (Orphanides *et al.* 1996; Smale and Kadonaga 2003).

### 1.1.3.3 Recruitment of RNA polymerase II/TFIIF to the PIC

The binding of TFIID and TFIIB at the core promoter is a prerequisite for RNA polymerase II recruitment to the PIC. However, RNA polymerase II cannot stably associate with the TFIID/TFIIB/TFIIA subassembly without TFIIF. TFIIF increases the specificity and efficiency of RNA polymerase II transcription. Specifically, TFIIF prevents spurious initiation by inhibiting the binding of RNA polymerase II to non-promoter sites on DNA (Orphanides *et al.* 1996; Roeder 1996).

### 1.1.3.4 Binding of TFIIE and TFIIH completes the PIC

RNA synthesis is only initiated when TFIIE and TFIIH are recruited to the PIC. TFIIE can enter the PIC following the recruitment of RNA polymerase II and TFIIF, although it is likely to join the PIC simultaneously with RNA polymerase II and TFIIF. Once TFIIE is stably associated, TFIIH is recruited to the promoter. TFIIH is a complex multi-subunit general transcription factor, with kinase and DNA helicase activity required for transcription initiation (Orphanides *et al.* 1996; Roeder 1996;

Reese 2003). Once TFIIE and TFIIH are recruited to the PIC, RNA polymerase II can initiate transcription.

### 1.1.4 THE HOLOENZYME. A CHALLENGE TO THE STEP-WISE MODEL OF PIC FORMATION

The ordered, step-wise assembly of the PIC in the model described above has been challenged by the discovery of 'holoenzyme' complexes. Essentially, holoenzyme complexes consist of a subset of general transcription factors that can exist in association with RNA polymerase II in a preassembled form. These complexes can potentially bind to a promoter in a single step, rather than in a step-wise fashion detailed above (Bjorklund and Kim 1996; Li *et al.* 1996; Orphanides *et al.* 1996; Malik and Roeder 2005). Holoenzyme complexes were originally identified in yeast by Young and co-workers (Koleske and Young 1994; Koleske and Young 1995) and Kim *et al.* (1994). A multi-protein complex termed 'Mediator,' was found in association with RNA polymerase II and TFIIF.

The discovery of yeast holoenzyme complexes prompted extensive research in this field and it is now generally accepted that the Mediator complex is universally required for the expression of almost all genes expressed in yeast (Holstege *et al.* 1998). Further research eventually led to the discovery of mammalian holoenzyme and Mediator complexes also (see Orphanides *et al.* 1996; Malik and Roeder 2005 and references therein). The mammalian Mediator complex is believed to consist of at least 28 subunits, many of which are orthologs of yeast Mediator subunits. Like the yeast Mediator complex, little is known about the detailed mechanism by which mammalian Mediator regulates RNA polymerase II gene transcription. However, what is clear is that the mechanism involves a complex network of interactions between components of the Mediator, transcriptional activators (and repressors; see section 1.1.8.1), RNA polymerase II, the general transcription factors and co-factors (Section 1.1.8.2) (Malik and Roeder 2005).

### 1.1.5 CELL AND TISSUE TYPE-SPECIFIC PICS

Until recently, the components of the basal transcription machinery (that make up the PIC) described above, were thought to be unvarying between different cell types of an organism. However, recent studies have challenged this theory and have resulted in

the identification of cell type-specific components of the core transcriptional machinery. Specifically, cell type-specific and in some cases gene-specific homologs of basal transcription factors have been identified in higher eukaryotes. Investigations have led to the characterisation of TATA box-binding protein (TBP)-like proteins, known as TBP-related factors (TRFs) and a number of tissue-specific homologs of the TAF components of TFIID (see Hochheimer and Tjian 2003 and references therein). Therefore, it is now evident that complex eukaryotes contain cell/tissue-specific components of the basal transcriptional machinery, increasing diversity and complexity of transcription.

### 1.1.6 TRANSCRIPTION INITIATION

The first stage of transcriptional initiation requires the separation of the DNA double helix, thus permitting RNA polymerase II access to the necessary promoter sites of the gene to be transcribed. This process is described as 'promoter melting' and is followed by the formation of the first phosphodiester bond of the nascent mRNA. The continuing extension of the RNA transcript eventually disrupts RNA polymerase II contacts at the site of PIC formation, in the process of 'promoter clearance.' Promoter clearance is followed by the elongation stage of transcription, which involves the continual extension of the mRNA transcript along its DNA template. The transition from the 'initiation' stage to the 'elongation' stage of transcription is accompanied by covalent modification of RNA polymerase II. Specifically, the carboxy-terminal domain (CTD) of RNA polymerase II is a target for phosphorylation. Initiation of RNA synthesis is accompanied by extensive phosphorylation of the CTD of RNA polymerase II. The unphosphoylated form of RNA polymerase II is preferentially recruited to the PIC, whilst RNA polymerase II engaged in elongation is heavily phosphorylated (Orphanides *et al.* 1996; Roeder 1996). As mentioned briefly before, TFIIH possesses an ATP-dependent helicase activity and a protein kinase activity specific for the CTD of RNA polymerase II. The helicase activity of TFIIH is responsible for the unwinding of DNA in the 'promoter melting' stage of initiation, although this also requires the presence of TFIIE and ATP (Orphanides *et al.* 1996; Svejstrup *et al.* 1996). Both TFIIE and TFIIH have also been implicated in 'promoter clearance,' although the precise role of these general transcription factors in both 'promoter clearance' and 'promoter melting' has been disputed (see Orphanides *et al.* 1996 and references therein).

### 1.1.7 RNA POLYMERASE II ELONGATION AND TERMINATION

In recent years it has become apparent that transcript elongation is not simply a process that describes the addition of ribonucleoside tri-phosphates to the growing mRNA chain. On the contrary, the process is a dynamic, highly regulated stage of transcription which can control multiple stages required for the maturation of mRNA. These processes include; pre-mRNA capping, splicing, 3'-end processing (addition of a poly-A tail), and mRNA export. Pre-mRNA processing events are believed to be coupled with the elongation process itself, for which the CTD of RNA polymerase II is required (Shilatifard 1998; Sims *et al.* 2004; Hirose and Ohkuma 2007).

Transcriptional termination is defined as cessation of RNA synthesis, where the RNA polymerase II elongation complex dissociates from the DNA template to release the newly transcribed mRNA. Transcription termination is necessary for maintaining active cellular RNA polymerase II pools and to prevent erroneous transcription of downstream genes from the elongating RNA polymerase II. The exact molecular mechanisms governing the termination process are still under investigation (Sims *et al.* 2004; Gromak *et al.* 2006).

### 1.1.8 DNA SEQUENCE ELEMENTS AND TRANSCRIPTION FACTORS INVOLVED IN THE REGULATION OF TRANSCRIPTION

As described previously (Section 1.1.1), 'the core promoter sequence elements are sufficient to direct accurate transcription initiation by RNA polymerase II in reconstituted cell-free systems' (Roeder 1996). This type of transcription is not dependent on the presence of regulatory transcription factors (e.g. activators; section 1.1.8.1) and is often referred to as 'basal' transcription (Orphanides *et al.* 1996). However, *in vivo* the situation is quite different. This is mainly because in a cell, DNA is packed into chromatin and this generally results in transcriptional repression. Transcription is triggered by regulatory transcription factors that bind to specific DNA sequences (distinct from the core promoter) and interact with the RNA polymerase II general transcription factor machinery (bound at the core promoter) to induce the expression of a gene. Therefore transcription that occurs in a cell is referred to as 'activated transcription'. Hence, 'basal' transcription does not occur *in vivo*, but is a term used to describe transcription *in vitro* (Orphanides *et al.* 1996).

The additional DNA sequence elements, also briefly mentioned in section 1.1, involved in the regulation of transcription are represented in Figure 1.2. It is worthy of note that these sequences bind transcription factors that have both positive and negative influences on transcription.

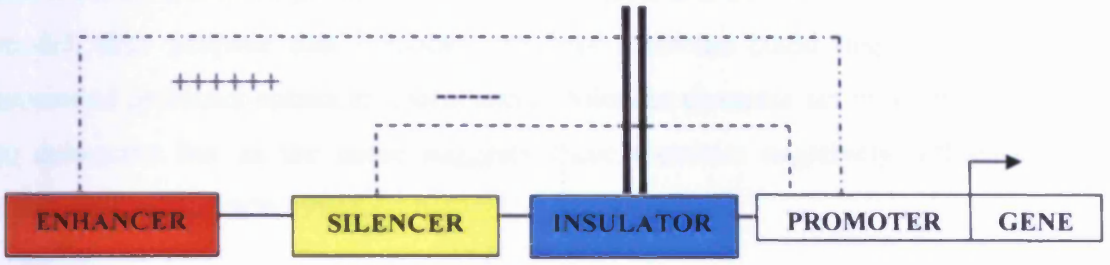



**FIGURE 1.2 Regulatory *cis*-acting elements involved in the control of eukaryotic gene transcription.** Broken lines indicate activating (+++) or repressive (---) influence on gene transcription by enhancer or silencer elements respectively, often located distally from the promoter of a class II gene. Insulator/boundary elements are thought to function as transcriptionally neutral DNA elements that insulate/block (indicated by thick vertical lines) the spreading of the positive or negative influences of enhancers and silencers respectively. The arrow indicates gene transcription from the transcriptional initiation site.

Proximal to the transcriptional start site, a number of sequence elements make up the promoter region of a gene. This region contains the core promoter elements and also upstream promoter elements. The upstream promoter elements (approximately -50 to -200bp relative to the transcriptional start site), contain multiple binding sites for a range of different transcription factors. By binding to their specific sequence elements, these transcription factors regulate gene transcription by influencing the formation of the PIC at the core promoter (see below, section 1.1.8.1) (Latchman 1998).

Enhancers were originally identified as DNA sequence elements that positively influence gene transcription in a manner that is independent of their orientation and distance, relative to the transcriptional start site of the gene (Khoury and Gruss 1983). Like the promoter region, enhancers are composed of DNA motifs that bind transcription factors. Regulatory transcription factors that bind to enhancer elements are able to interact with protein complexes formed at the promoter and thus positively influence gene transcription (Latchman 1998). Interactions between enhancer-bound transcription factors and those bound at the promoter are important because they influence whether the enhancer acts at the correct gene promoter, as enhancers often act over a large stretches of DNA. These interactions are particularly significant

when the enhancer must only act on one of many promoters in its immediate vicinity. Blackwood and Kadonaga (1998) proposed two possible mechanisms by which enhancer-promoter selectivity may be achieved. In the first model, it was suggested that there may be selective interactions between enhancer bound transcription factors and those bound at the promoter of the gene which is to be transcribed. In the second model, they propose that boundary/insulator elements could negatively influence unwanted promoter-enhancer interactions. Silencer elements act in a similar fashion to enhancers but as the name suggests these elements negatively influence gene transcription (Jackson 1991).

### 1.1.8.1 Regulatory transcription factors-activators and repressors

The general transcription factors and their role in RNA polymerase II-mediated transcription have been discussed in some detail above. Although the general transcription factors are important in regulating RNA polymerase II-mediated gene transcription, other transcription factors also known as transcriptional activators and repressors are vital for the control of cell/tissue-type specific and regulated gene transcription.

Activator proteins can regulate the synthesis of an mRNA molecule by a number of different mechanisms (Orphanides *et al.* 1996);

1) By removing repressor molecules from promoter DNA.
2) By recruiting general transcription factors/RNA polymerase II to a promoter.
3) By inducing conformational changes within the PIC.
4) By inducing covalent modifications of proteins in the PIC.
5) By stimulating promoter clearance and elongation.

Transcriptional repressors can be classified as 'active' or 'passive' repressor proteins (Thiel *et al.* 2004). Active transcriptional repressor proteins directly target chromatin assembly/organisation. Specifically, active repressors (e.g. retinoblastoma protein) can recruit histone deacetylases to a gene promoter and histone deacetylation results in subsequent gene silencing. Others can induce gene silencing via histone methylation by histone methyltransferases (e.g. SUV39H1) and heterochromatin formation. By contrast, passive repressor proteins do not possess an intrinsic repressive activity. Rather, these proteins inhibit RNA synthesis by competing with

activators for DNA binding. They can act as dominant-negative inhibitors (e.g. inducible cAMP early repressor-ICER) by forming inactive heterodimers with transcriptional activators, essentially neutralising the positive effect of the activator by inhibiting its DNA binding or *trans*-activation potential. Passive repressor proteins can also sequester co-activator molecules, preventing them from binding to the cognate activator proteins. Hence, passive repressor proteins act by regulating DNA- or protein-protein interactions (see Thiel *et al.* 2004 and references therein).

#### 1.1.8.2 Co-factors. Accessory proteins to transcription factors

Co-factors are accessory proteins that interact with DNA-bound transcription factors and play a critical role in the action of these transcription factors. Transcriptional co-factors can be subdivided into co-activators, which mediate gene activation and co-repressors, which inhibit gene transcription (Latchman 1998). A variety of other co-activator molecules exist to facilitate activator-dependent transcription such as CBP (CREB-binding protein). CBP was originally defined as a co-activator pivotal for cyclic AMP-mediated activation of the transcription factor, CREB (cAMP responsive element binding protein). Subsequently, it has been demonstrated that CBP and p300 (a close relative of CBP) are essential co-activators for a variety of other transcription factors such as myogenic differentiation antigen (MyoD), activator protein-1 (AP-1), p53 and the C/EBPs (Latchman 1998; Kovacs *et al.* 2003). CBP is believed to activate gene transcription by recruiting basal transcription machinery (TFIIB, TBP and RNA polymerase II), through modifying chromatin structure via histone acetylation and also by recruiting other histone acetyltransferases such as steroid receptor coactivator-1 (SRC-1) and p300/CBP-associated factor (P/CAF) (see Kovacs *et al.* 2003 and references therein). Additionally, a variety of co-repressor molecules have also been identified and characterised. NCoR is an example of a nuclear receptor co-repressor and is involved in recruiting histone deacetylases to induce gene silencing (Burke and Baniahmad 2000).

### 1.1.9 CLASSIFICATIONS OF TRANSCRIPTION FACTORS

On the basis of the structure and their DNA-binding domains, it has been possible to classify transcription factors into families (Harrison 1991; Pabo and Sauer 1992; Latchman 1998). The DNA-binding motifs that define these families are the helix-turn-helix motif (e.g. λ repressor, catabolite activator protein), the related POU

domain (e.g. Pit-1, Oct-1 and Oct-2), the zinc finger motif (e.g. TFIIIA, steroid receptors, Sp1), the helix-loop-helix motif (e.g. E12/E47 and myogenic regulatory factors including MyoD) and the basic leucine zipper motif or bZIP (e.g. C/EBP, AP-1 and CREB). DNA-binding motifs can be used to classify major groups of transcription factors, however not all transcription factors can be categorised this way. For example, the major transcription factors p53 and NF-κB contain similar DNA-binding motifs that are structurally distinct from those mentioned above (Latchman 1998).

This next section will discuss the family of regulatory transcription factors belonging to the bZIP classification, namely the C/EBPs. Their structure, function and regulation will be detailed, with particular emphasis on their role in the regulation of gene expression during inflammation, before establishing the aims of this study.

## 1.2 CCAAT ENHANCER BINDING PROTEINS

The first C/EBP family member was identified by McKnight and co-workers in 1988 (Landschulz *et al.* 1988). Two members of his team were working to purify what was then believed to be two independent transcription factors, isolated from rat liver tissue. As the projects matured, it emerged that both these transcription factors co-purified to the level of a single polypeptide (Graves *et al.* 1986; Johnson *et al.* 1987). Interestingly, what was thought to be the discovery of two very different transcription factors, one of which bound to *cis*-acting elements containing a CCAAT motif, common to many RNA polymerase II promoters and the other that bound to enhancer core elements of SV40, murine sarcoma virus and polyomavirus, led to the identification of the same protein, now designated C/EBPα (Cao *et al.* 1991; McKnight 2001). A further five other members of the C/EBP family were identified by 1992, all of which contained the conserved bZIP domain at their C-terminus (Akira *et al.* 1990; Chang *et al.* 1990; Descombes *et al.* 1990; Poli *et al.* 1990; Roman *et al.* 1990; Cao *et al.* 1991; Williams *et al.* 1991; Ron and Habener 1992). The discovery of these transcription factors prompted extensive investigations into their function and it has now emerged that the C/EBPs regulate various cellular responses including; growth and differentiation, aspects of metabolism, immune and inflammatory processes.

There are six members of the C/EBP family designated C/EBPα-C/EBPζ by Cao and co-workers (Cao *et al.* 1991). However, because these transcription factors have been identified and characterised in a number of different species, from different laboratories, they have often been designated alternative names. To avoid confusion, the detail of alternative nomenclature for each C/EBP family member is listed in Table 1.0, together with the different species from which the genes have been cloned.

**TABLE 1.0 Alternative nomenclature of C/EBPs and the species from which they have been cloned.**

| C/EBP FAMILY MEMBER | ALTERNATIVE IDENTITY | SOURCE AND REFERENCES |
|---|---|---|
| C/EBPα | C/EBP, RcC/EBP-1 | **rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Rana catesbeiana*, zebrafish, *Podocoryne carnea***<br><br>(Landschulz *et al.* 1989; Cao *et al.* 1991; Williams *et al.* 1991; Xu and Tata 1992; Chen *et al.* 1994; Antonson and Xanthopoulos 1995; Calkhoven *et al.* 2000; Lyons *et al.* 2001; Seipel *et al.* 2004; Lo *et al.* 2007) |
| C/EBPβ | NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP | **rat, mouse, human, chicken, bovine, *Xenopus laevis*, *Aplysia*, zebrafish, *Paralichthys olivaceus***<br><br>(Akira *et al.* 1990; Chang *et al.* 1990; Descombes *et al.* 1990; Poli *et al.* 1990; Cao *et al.* 1991; Williams *et al.* 1991; Katz *et al.* 1993; Alberini *et al.* 1994; Kousteni *et al.* 1998; Lyons *et al.* 2001; Tucker *et al.* 2002; Lo *et al.* 2007) |
| C/EBPγ | Ig/EBP-1 | **rat, mouse, human, chicken, zebrafish**<br><br>(Roman *et al.* 1990; Lyons *et al.* 2001) |
| C/EBPδ | NF-IL6β, CRP3, CELF, RcC/EBP2 | **rat, mouse, human, *Rana catesbeiana*, bovine, ovine, zebrafish, *Xenopus laevis***<br><br>(Cao *et al.* 1991; Kageyama *et al.* 1991; Williams *et al.* 1991; Kinoshita *et al.* 1992; Chen *et al.* 1994; Davies *et al.* 2000; Lyons *et al.* 2001; Ikuzawa *et al.* 2005; Lo *et al.* 2007) |
| C/EBPε | CRP-1 | **rat, mouse, human, ovine, *Paralichthys olivaceus***<br><br>(Williams *et al.* 1991; Antonson *et al.* 1996; Chumakov *et al.* 1997; Yamanaka *et al.* 1997a; Sabatakos *et al.* 1998a; Tucker *et al.* 2002) |
| C/EBPζ | CHOP-10, GADD153 | **rat, mouse, human, hamster**<br><br>(Luethy *et al.* 1990; Park *et al.* 1992; Ron and Habener 1992) |

(adapted from Ramji and Foka 2002)

### 1.2.1 STRUCTURAL FEATURES OF C/EBPS

The DNA-binding motif of bZIP family members is bipartite, consisting of a dimersiation interface 'leucine zipper' and a DNA binding region or 'basic region.' A consensus sequence for C/EBP binding has been determined as **R**TTGCG**Y**AA**Y** (**R**=A or G and **Y**=C or T) (Osada *et al.* 1996). All C/EBP proteins contain a C-terminal bZIP domain and with the exception of C/EBPγ, also contain activation and/or negative regulatory domains at their N-terminus. The C-terminus leucine zipper consists of a heptad of leucine repeats that interdigitate with the equivalent repeats of the dimer partner, together assuming a coiled-coil configuration of α-helices. It is the electrostatic interactions between amino acids along the dimerisation interface that determine the specificity of dimer formation. Importantly, dimerisation is a prerequisite of DNA binding and DNA binding is mediated through a basic stretch of amino acids (approximately 20), immediately upstream of the leucine zipper, which also adopt an α-helical configuration (Figure 1.3). Specificity of DNA binding is mediated by three amino acids that lie along the DNA-protein contact surface (see Lekstrom-Himes and Xanthopoulos 1998; Ramji and Foka 2002 and references therein). The spacing between the basic and leucine zipper regions is critical for binding activity, with alterations abolishing activity (Agre *et al.* 1989).

In contrast to the highly conserved C-terminal bZIP domain, the N-termini of C/EBP proteins are quite divergent (less than 20% sequence identity in common), except for certain regions identified as activation domains. Functionally, the activation domains interact with various components of the basal transcription apparatus and stimulate transcription (Nerlov and Ziff 1994; Williams *et al.* 1995; Williamson *et al.* 1998; Ji *et al.* 2003). N-termini of the C/EBP proteins have also been proposed to contain 'negative regulatory domains,' the precise details of which are still under investigation (Pei and Shih 1991; Williamson *et al.* 1998; Angerer *et al.* 1999; Tang and Koeffler 2001). However, a more recent study by Kim *et al.* (2002a) suggests negative regulatory domains of C/EBPs are targets for sumoylation (see section 1.2.5).

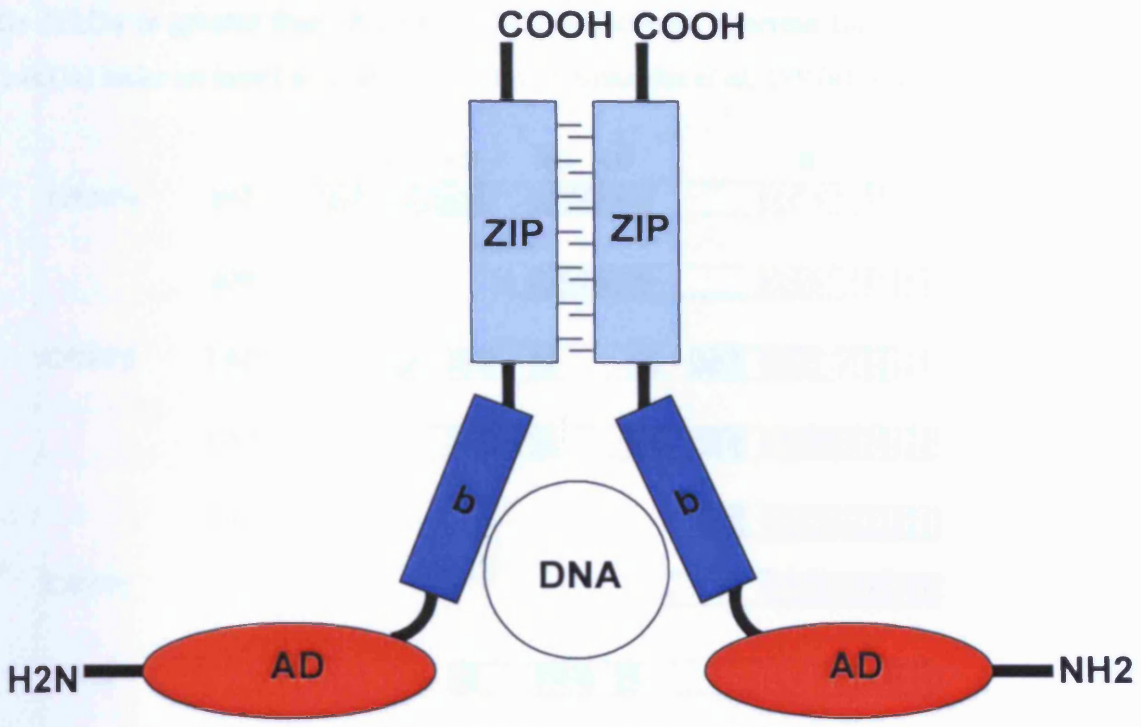



**FIGURE 1.3 Schematic representation of C/EBP dimer formation (adapted from Wedel and Ziegler-Heitbrock 1995).** ZIP represents the leucine zipper domain; b indicates the basic DNA-binding domain and AD the activation domain.

### 1.2.2 C/EBP HOMO- AND HETERODIMERISATION AND INTERACTIONS WITH OTHER bZIP TRANSCRIPTION FACTORS

The numbers of transcription factors containing a C/EBP component in various tissue/cell types can be considerably diverse. There are a number of contributing factors that allow this phenomenon to occur. Firstly, this is partly because certain C/EBPs may exist as several isoforms, as is the case with C/EBPα, C/EBPβ and C/EBPε (see section 1.2.6 and Figure 1.4). C/EBPα can exist as two isoforms of 42kDa and 30kDa in size, with the 42kDa protein possessing a higher activation potential than the shorter 30kDa protein (Lin *et al.* 1993; Ossipow *et al.* 1993). Similarly, C/EBPβ exists as three isoforms; 38kDa (LAP*-liver-enriched transcriptional activator protein), 35kDa (LAP) and 20kDa (LIP-liver-enriched inhibitory protein), with LAP and LIP predominating in cells. LAP contains both bZIP domain and activation domains, whereas LIP only possesses the bZIP domain (Descombes and Schibler 1991; Xiong *et al.* 2001). C/EBPε exists as at least four different isofoms (32kDa, 30kDa, 27kDa and 14kDa) and the activation potential of

the 32kDa is greater than that of the 30kDa isoform, whereas the smallest isoform (14kDa) lacks an intact activation domain (Yamanaka *et al.* 1997a) (Figure 1.4).

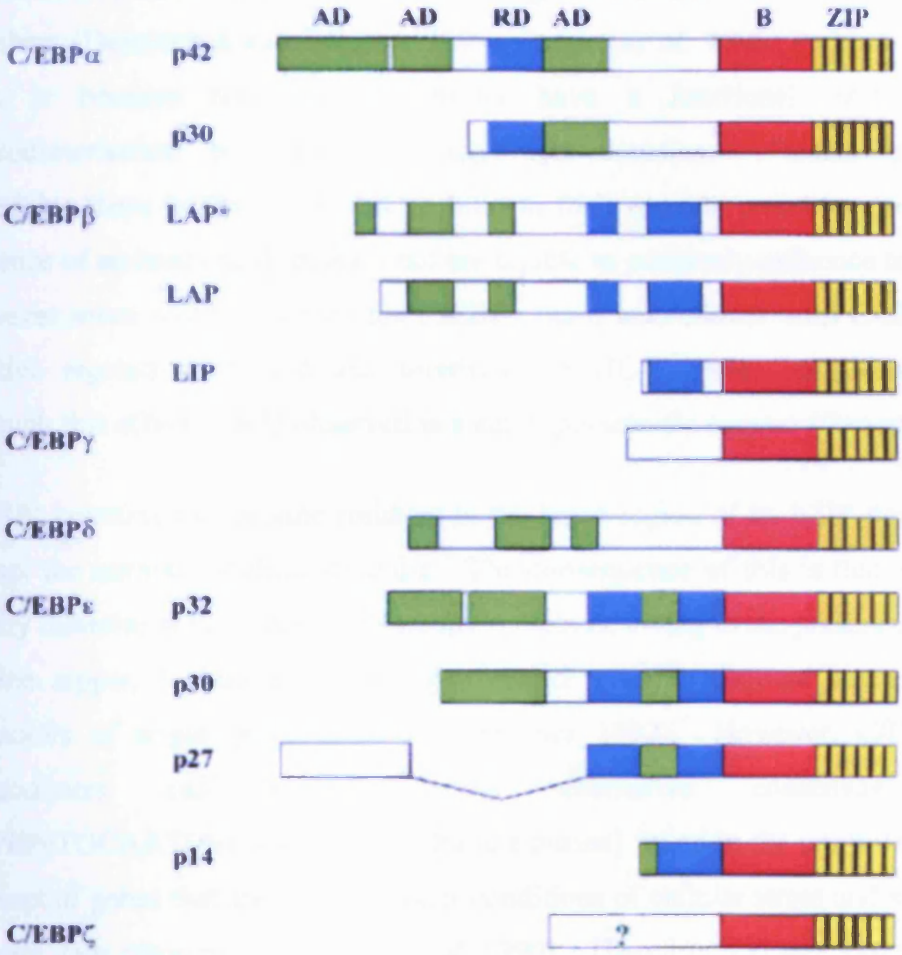



**FIGURE 1.4. A schematic representation of the C/EBPs with their various isoforms (taken from Ramji and Foka 2002).** The leucine zipper of the bZIP domain is depicted in yellow, with black vertical lines representing the leucine residues. The basic region of the bZIP domain is blocked in red. The positions of the activation domains (AD) and negative regulatory domains (RD) are shown in green and blue respectively. A RD is also suggested to exist in C/EBPδ (Kim *et al.* 2002a), although this domain is not depicted in the above figure. ? indicates that the N-terminus of C/EBPζ is reported to contain an activation domain but its exact position remains to be determined (Ron and Habener 1992; Ubeda *et al.* 1996). The mechanisms by which the various polypeptides of C/EBPα –β and –ε are formed are discussed in section 1.2.6.

Secondly, given that C/EBPs must dimerise in order to regulate gene transcription, it is not surprising that they are capable of forming dimers in all interfamilial combinations. This generates combinations of family members with varying properties of DNA binding and *trans*-activation potential (Akira *et al.* 1990; Chang *et al.* 1990; Descombes *et al.* 1990; Poli *et al.* 1990; Roman *et al.* 1990; Cao *et al.* 1991;

Williams *et al.* 1991; Ron and Habener 1992; Rorth and Montell 1992; Ramji and Foka 2002). For example, C/EBPγ and the LIP form of C/EBPβ act as dominant-negative inhibitors of C/EBP function through heterodimerisation with other family members (Descombes and Schibler 1991; Cooper *et al.* 1995; Parkin *et al.* 2002). This is because both these isoforms have a functional bZIP permitting heterodimerisation but lack *trans*-activation domains. Hence, heterodimers containing these isoforms are able to bind to their cognate promoters, owing to the presence of an intact bZIP domain but are unable to positively influence transcription. However more recently, a role for C/EBPγ (as a heterodimer with C/EBPβ) in the positive regulation of IL-6 and interleukin 8 (IL-8) genes has been suggested, although this effect is only observed in a cell type-specific manner (Gao *et al.* 2002).

C/EBPζ contains two proline residues in the basic region of its bZIP domain, which disrupt the normal α-helical structure. The consequence of this is that, C/EBPζ can readily dimerise with other C/EBP family members, owing to the presence of an intact leucine zipper, but these heterodimers cannot bind to cognate sequences in the promoters of target genes (Ron and Habener 1992). However, C/EBPζ/C/EBP heterodimers can bind to an alternative consensus sequence [PuPuPuTGCAAT(A/C)CCC, where Pu is a purine] found in the promoter regions of a subset of genes that are induced under conditions of cellular stress and subsequently activate gene transcription (Ubeda *et al.* 1996). Therefore, C/EBPζ can either act as an activator or inhibitor of gene transcription, depending on the cellular state.

C/EBPδ readily heterodimerises with C/EBPα and C/EBPβ and has a *trans*-activation potential comparable to C/EBPα and C/EBPβ (Cao *et al.* 1991; Kinoshita *et al.* 1992). C/EBPε is also reported to heterodimerise with other C/EBP family members, including C/EBPδ and -ζ (Chumakov *et al.* 1997; Chumakov *et al.* 2007).

Furthermore, C/EBP proteins can also heterodimerise with other bZIP transcription factors. The existence of heterodimers formed between members of the C/EBP family and the CREB/ATF family of bZIP transcription factors (e.g. ATF-2, ATF-3, ATF-4, C/ATF and CREB) has been reported (Vallejo *et al.* 1993; Shuman *et al.* 1997; Wolfgang *et al.* 1997; Ross *et al.* 2001). For example, C/EBPγ and C/EBPε dimerise with ATF4 (Vinson *et al.* 1993; Chumakov *et al.* 2007) and C/EBPα and -β can dimerise with C/ATF (Vallejo *et al.* 1993). Cross-dimerisation of this nature results

in changes in the binding specificity of these transcription factors. C/EBP-ATF heterodimers bind to a 'C/EBP-ATF composite site' in promoters of regulated genes. Heterodimers bind to an asymmetric sequence composed of one consensus half-site for each monomer (Shuman *et al.* 1997). Heterodimerisation between C/EBPs and members of the Fos/Jun family of bZIP transcription factors has also been reported. Dimerisation of C/EBPβ with Fos or Jun directs binding away from conventional C/EBP consensus sites towards AP-1 sites (Hsu *et al.* 1994). Additionally, an interaction between c-Jun and C/EBPβ is required for the regulation of the tumor necrosis factor-α (TNF-α) gene expression in myelomonocytic cells (Zagariya *et al.* 1998).

### 1.2.3 INTERACTIONS BETWEEN C/EBPS AND NON BZIP TRANSCRIPTION FACTORS

C/EBP proteins have also been reported to interact with a number of different non-bZIP transcription factors and various co-activators. Protein-protein interactions between C/EBPα, -β, -δ, -ε and NF-κB proteins (p50, p65/RelA) have been reported previously (LeClair *et al.* 1992; Matsusaka *et al.* 1993; Stein and Baldwin 1993; Stein *et al.* 1993; Chumakov *et al.* 2007). Interactions between C/EBPβ and the glucocorticoid receptor have also been reported to synergistically activate the $\alpha_1$-acid glycoprotein gene promoter (Nishio *et al.* 1993). More recently, it has been reported that C/EBPβ and C/EBPδ physically interact with the SMAD transcription factors during a transforming growth factor β (TGF-β)-mediated signalling pathway in NIH3T3 cells (Choy and Derynck 2003). Liu and co-workers (2003) have revealed there to be a physical interaction between Sp1 with C/EBPβ or C/EBPδ that is enhanced upon lipopolysaccharide (LPS) treatment of the murine macrophage cell line RAW264.7. This interaction between Sp1 with C/EBPδ has also recently been confirmed (Chiang *et al.* 2006). Additionally, the product of the proto-oncogene c-myb (c-Myb) has been demonstrated to interact with C/EBPβ (Tahirov *et al.* 2002). Furthermore, the zinc finger transcription factors GATA-2 and GATA-3 have been shown to interact with C/EBPα and –β. The interaction between GATA and C/EBP factors is critical for GATA-mediated suppression of adipocyte differentiation (Tong *et al.* 2005). A physical association between the p53 tumour suppressor and C/EBPβ has been shown to be important for regulating negative cross-talk between these transcription factors during the differentiation of human endometrial stromal cells (Schneider-Merck *et al.* 2006).

The C/EBPs also interact with various transcriptional co-factors. C/EBPα and C/EBPβ recruit the chromatin remodelling complex SWI/SNF as a mechanism of mediating transcription (Kowenz-Leutz and Leutz 1999; Pedersen *et al.* 2001). Furthermore, the co-activators p300 and CBP are involved in regulating C/EBPα-, C/EBPβ- and C/EBPδ-driven transcription (Oelgeschlager *et al.* 1996; Mink *et al.* 1997; Erickson *et al.* 2001; Schaufele *et al.* 2001; Kovacs *et al.* 2003). C/EBP recruitment of CBP and p300 has also been shown to induce the phosphorylation of these co-factors through a yet undetermined mechanism (Kovacs *et al.* 2003; Schwartz *et al.* 2003). Recently, p300-mediated acetylation of C/EBPβ has been suggested to be important for its transcriptional activation in 3T3-F442A preadipocytes (Cesena *et al.* 2007). Additionally, p300-mediated acetylation of C/EBPδ is reported to be one of the mechanisms by which the expression of the cyclooxygenase-2 (COX-2) gene is regulated in response to epidermal growth factor (EGF) (Wang *et al.* 2006). Moreover, the elongation factor CA150 has been shown to act as a transcriptional co-repressor of C/EBPs. Over-expression of CA150 inhibits *trans*-activation mediated by C/EBPα and reverses the enhancing effect of p300 on C/EBPβ-mediated *trans*-activation (McFie *et al.* 2006).

### 1.2.4 PHOSPHORYLATION OF C/EBPS

The phosphorylation status of the C/EBP proteins can not only determine their function, but their *trans*-activation potential, DNA-binding and translocation properties. Therefore, understandably, phosphorylation of C/EBP proteins is key in their regulation.

Mahoney *et al.* (1992) were the first to hypothesise that phosphorylation of C/EBPα may regulate its DNA binding activity. Specifically, they observed that C/EBPα was efficiently phosphorylated by protein kinase C (PKC) but not protein kinase A (PKA). PKC-mediated phosphorylation of serine$^{299}$ attenuated the sequence-specific DNA binding activity of C/EBPα by 80%, at least *in vitro*. Additionally, insulin action has been implicated in regulating the phosphorylation status of C/EBPα. Insulin sensitive phosphorylation sites in C/EBPα have been identified and insulin action was suggested to result in the dephosphorylation of C/EBPα (Ross *et al.* 1999). C/EBPα phosphorylation sites were identified as threonine$^{222}$, threonine$^{226}$ and serine$^{230}$ (Ross *et al.* 1999). Phosphorylation of these threonine residues is regulated by glycogen

synthase kinase 3 (GSK3). As GSK3 activity is inhibited by insulin action, a possible mechanism for the insulin-mediated dephosphorylation of C/EBPα was suggested (Ross *et al.* 1999; Roesler 2001). However, results from a recent study suggest that phosphorylation of threonine$^{222}$ and threonine$^{226}$ in C/EBPα is not inhibited by insulin action, at least in hepatocytes-H411E cells (Liu *et al.* 2006d). Results from this study also revealed that mutating threonine$^{222}$ and threonine$^{226}$ phosphorylation sites in C/EBPα has no effect on C/EBPα activity in hepatocytes, questioning the functional significance of this phosphorylation. Furthermore, data also suggested that C/EBPα was a poor substrate for GSK3 and that insulin does not regulate threonine$^{222}$ and threonine$^{226}$ phosphorylation through the inhibition of GSK3 in hepatocytes. Nevertheless, preliminary results suggested that phosphorylation of threonine$^{222}$ and threonine$^{226}$ residues in C/EBPα may negatively regulate the activity of this transcription factor in 3T3L1 pre-adipocytes, and that the phosphorylation status of these residues may be regulated through GSK3 as modulated by insulin, consistent with the prediction by Ross *et al.* (1999). Collectively, these results suggest that phosphorylation of C/EBPα may regulate its activity in a cell/tissue-specific manner.

Phosphorylation of C/EBPα is also associated with its inactivation in acute myelogenous leukemia (AML) cell lines. Thirty percent of AML cases are associated with mutations in the FLT3 receptor tyrosine kinase gene. Mutations result in constitutive activation of the receptor and aberrant activation of downstream pathways, including the extracellular signal-regulated kinase (ERK1/2) mitogen-activated protein kinase (MAPK). C/EBPα expression/activity is important for normal myeloid cell differentiation (Section 1.3) and its inactivation is associated with various types of leukemia, including AML. It has recently been shown that FLT3-ERK1/2-mediated phosphorylation of C/EBPα (serine$^{21}$) inactivates C/EBPα in AML patients and cell lines, blocking granulocytic differentiation (see Radomska *et al.* 2006 and references therein).

Phosphorylation of CEBPβ plays a key role in modulating the activity of this transcription factor. PKC regulates the phosphorylation of C/EBPβ in its activation domain (serine$^{105}$) and this increases its *trans*-activation potential (Trautwein *et al.* 1993). By contrast, phosphorylation of C/EBPβ (serine$^{240}$) by PKC inhibits its DNA binding activity *in vitro* (Trautwein *et al.* 1994). In an effort to identify the region in C/EBPβ important for gene activation by phosphorylation of serine$^{105}$, further

experiments were carried out by Trautwein and colleagues (Trautwein *et al.* 1995). Results from these experiments indicate that an acidic region located between amino acids 21 and 105 of C/EBPβ is important for *trans*-activation.

It has also been proposed that C/EBPβ is a 'repressed' transcription factor with concealed activation potential. Phosphorylation is believed to play a unique role to 'derepress' rather than to enhance the *trans*-activation potential of C/EBPβ. Negative regulatory domains normally mask the *trans*-activation domains of C/EBPβ, but residues in the repression domain can be phosphorylated to unmask the activation domains and alleviate repression (Kowenz-Leutz *et al.* 1994; Williams *et al.* 1995).

Phosphorylation of murine C/EBPβ at serine$^{276}$ by a $Ca^{2+}$-calmodulin-dependent protein kinase (CaMKII), increased its *trans*-activation potential (Wegner *et al.* 1992). The Ras/MAPK pathway has also been shown to phosphorylate human C/EBPβ (threonine$^{235}$), leading to significant activation of this transcription factor (Nakajima *et al.* 1993). Another study has demonstrated that treatment of 3T3-F442 fibroblasts with growth hormone rapidly induces binding of C/EBPβ to its cognate site on the *c-fos* gene promoter. This effect seemed to be mediated through the dephosphorylation of C/EBPβ, through a unidentified residue (Liao *et al.* 1999). Phosphorylation of C/EBPβ has also been implicated in the control of other cellular activities. For example, phosphorylation of C/EBPβ (threonine$^{271}$) by p90 ribosomal S kinase has been found to be critical for rescuing stellate cells from apoptosis in a model of liver injury (Buck *et al.* 2001b).

Regulation of C/EBPβ translocation (cytoplasm-nuclear shuttling) is mediated, at least in part through phosphorylation. Forskolin treatment of rat PC12 cells causes phosphorylation of C/EBPβ and its translocation into the nucleus from the cytoplasm (Metz and Ziff 1991). PKA-dependent phosphorylation of C/EBPβ is also responsible for its nuclear translocation in antioxidant-treated DKO-1 cells (Chinery *et al.* 1997). TNF-α action in hepatocytes is believed to regulate translocation of C/EBPβ and there is some evidence to suggest that this process is regulated through phosphorylation of this factor (Yin *et al.* 1996; Buck *et al.* 2001a).

Streams of recent studies implicate the ERK1/2 MAPK pathway as a regulator of C/EBPβ phosphorylation by a variety of extracellular mediators, including the cytokine interferon-gamma (IFN-γ). ERK1/2-mediated phosphorylation of C/EBPβ is

reported to regulate both *trans*-activation and translocation of this transcription factor in a variety of cellular systems, depending on the target residue of phosphorylation (Piwien-Pilipuk *et al.* 2002; Meng *et al.* 2005; Ghosh *et al.* 2006; Raymond *et al.* 2006; Bezy *et al.* 2007; Koria and Andreadis 2007).

Phosphorylation of C/EBPδ also plays a role in modulating its DNA binding and activation function. Ray and Ray (1994a) showed that phosphorylation enhances activation of C/EBPδ, with only the phosphorylated form able to bind to the C/EBP site of the serum amyloid A promoter during the acute-phase response (APR) (Section 1.3.1). Dephosphorylation severely impaired the DNA binding activity of C/EBPδ. Preliminary data indicate a potential role of ERK1 MAPK in phosphorylation-mediated regulation of C/EBPδ (Ray and Ray 1994a). Similar results were obtained in a separate study where expression of the α1-acid glycoprotein gene was analysed. C/EBPδ *trans*-activates this gene in response to inflammatory stimuli and its phosphorylation enhances its activating potential (Ray and Ray 1994b). In a model of turpentine-induced APR, both C/EBP-β and -δ were found to bind the promoter region of rat α-2-macroglobulin gene and binding was dependent on their phosphorylation status (Milosavljevic *et al.* 2002).

Data presented by Osada *et al.* (1996) also suggested that C/EBPδ is indeed a phospho-protein. The group showed that phosphorylation of C/EBPδ by casein kinase 2 (CK2) increased the DNA binding capability of this transcription factor by approximately 3-fold. Previous studies have shown that CK2 phosphorylates serine residues located in acidic regions of proteins (Krebs *et al.* 1988). Five serine residues exist in the DNA binding domain of C/EBPδ, with serine$^{227}$ located within an acidic stretch of amino acids. However, it remains to be elucidated as to whether serine$^{227}$ is indeed phosphorylated by CK2 *in vivo* and *in vitro* (Osada *et al.* 1996).

Recent evidence suggests that C/EBPε is also a phospho-protein. C/EBPε is phosphorylated on multiple serine and threonine residues and can be a target for phosphorylation by a number of kinases. Threonine$^{75}$ is part of a consensus MAPK site within the *trans*-activation domain of C/EBPε, and is phosphorylated by p38 MAPK. Phosphorylation of this residue results in enhanced transcriptional activity and increased DNA-binding of this transcription factor (Williamson *et al.* 2005).

During cellular stress, C/EBPζ undergoes inducible phosphorylation of serine[79] and serine[81], via a p38 MAPK pathway. This in turn enhances the *trans*-activation potential of the factor (Wang and Ron 1996). It has also been shown that association between C/EBPζ and CK2 results in the phosphorylation of its amino terminal *trans*-activation domain. This phosphorylation results in impaired transcriptional activity of the factor (Ubeda and Habener 2003).

### 1.2.5 SUMOYLATION OF C/EBPS

A novel post-translational modification, termed sumoylation has more recently been described. Covalent modification of cellular proteins by a ubiquitin-like protein SUMO (small ubiquitin-related modifier) regulates various cellular processes, including nuclear transport, signal transduction, stress response and cell cycle progression (Muller *et al.* 2001).

Sequence analysis revealed that C/EBPα, -β, -δ and –ε all contain a conserved transcriptional regulatory region that can act as a negative regulatory domain. This domain contains a five-amino acid motif, of which a conserved lysine residue is a target for sumoylation. Sumoylation of C/EBPε is reported to relieve the inhibitory effect of the negative regulatory domain and improves its *trans*-activating capability. Moreover, mutation of the conserved lysine into an alanine residue in C/EBPε also ablates transcriptional repression, indicating that this conserved residue is important for negative regulatory domain function (Kim *et al.* 2002a). In a similar study, Eaton and Sealy (2003) demonstrated that the largest isoform of C/EBPβ, C/EBPβ-1 (equivalent to LAP*) was also sumoylated, however the related isoform C/EBPβ-2 (equivalent to LAP) was not. These results indicate that the integrity of the N-terminal amino acids present in C/EBPβ-1 but absent in C/EBPβ-2, are important for targeting C/EBPβ-1 for sumoylation. A conserved lysine residue [lysine[173] - equivalent to the lysine residue as specified by Kim *et al.* (2002a)] was determined as the target for sumoylation in C/EBPβ-1. Previous studies from this laboratory demonstrate that C/EBPβ-2, but not C/EBPβ-1 can activate the cyclin D1 promoter (Eaton *et al.* 2001). As the Kim *et al.* (2002a) study suggested that the conserved lysine residue in the C/EBPs is critical for negative regulatory domain function, the authors assessed whether mutation of the equivalent residue, lysine[173] of human C/EBPβ, was responsible for preventing C/EBPβ-1 from activating the cyclin D1

promoter. Mutation of this site in C/EBPβ-1 caused this isoform to behave similarly to the C/EBPβ-2 isoform, with respect to its ability to activate the cyclin D1 promoter (Eaton and Sealy 2003).

C/EBPδ is also a target for sumoylation and sumoylation of this transcription factor plays an important role in the regulation of COX-2 gene expression in A431 epithelial cells (Wang *et al.* 2006). C/EBPδ regulates both basal and EGF-stimulated COX-2 gene expression. Sumoylation at lysine$^{120}$ of human C/EBPδ attenuates the activation of the COX-2 gene promoter, whereas acetylation at the same residue (as mediated by p300) increases COX-2 gene promoter activation. Therefore, it seems that sumoylated-C/EBPδ and acetylated-C/EBPδ are involved, respectively, in the silencing and activation of COX-2 gene transcription (Wang *et al.* 2006).

### 1.2.6 MOLECULAR BIOLOGY OF THE C/EBPS

#### 1.2.6.1 C/EBPα

The human C/EBPα gene is a single copy, intronless gene and its locus assigned to chromosome 19q13.1 (Hendricks-Taylor *et al.* 1992). Expression patterns for C/EBPα are similar in both mouse and human tissues and cell types, with measurable levels in the liver, adipose tissue, intestine, lung, adrenal gland, peripheral blood mononuclear cells and placenta (Cao *et al.* 1991; Williams *et al.* 1991; Antonson and Xanthopoulos 1995; Lekstrom-Himes and Xanthopoulos 1998). In the liver and adipose tissue, increased levels of C/EBPα mRNA are present in the terminally differentiated cells of the tissue (Cao *et al.* 1991; Williams *et al.* 1991; Lekstrom-Himes and Xanthopoulos 1998).

Transcriptional control of the C/EBPα gene is important for its regulation. Although various transcription factors have been implicated in regulating C/EBPα gene expression, auto-regulation of the C/EBPα gene plays a significant role in modulating its expression (Legraverend *et al.* 1993; Timchenko *et al.* 1995; Kockar *et al.* 2001). Both C/EBPα and to a lesser extent, C/EBPβ are involved in auto-regulation of the murine C/EBPα gene (Legraverend *et al.* 1993). A mechanism of indirect auto-activation is proposed for the regulation of the human C/EBPα gene (Timchenko *et al.* 1995). Unlike its murine counterpart, the human C/EBPα gene promoter does not contain a C/EBP *cis*-acting element. Instead, C/EBPα stimulates upstream stimulating

factor (USF) to bind to a consensus element within the C/EBPα promoter, thereby activating gene transcription. Therefore, C/EBPα indirectly regulates the expression of its own gene by recruiting USF. In contrast, the C/EBPα gene from *Xenopus laevis*, is subject to direct auto-regulation (Kockar *et al.* 2001).

Two isoforms of C/EBPα are generated from a single mRNA by a 'leaky ribosomal scanning mechanism.' As mentioned in section 1.2.2 and depicted in Figure 1.4, the full length protein is 42kDa in size. However, a fraction of ribosomes are unable to recognise the first two AUG codons from the mRNA and instead initiate translation at the third AUG, 351 nucleotides downstream of the first initiation codon. This generates a protein of 30kDa that retains its dimerisation and DNA binding domains but has a shorter N-terminus compared to its 42kDa counterpart (Lin *et al.* 1993; Ossipow *et al.* 1993; Calkhoven *et al.* 1994).

### 1.2.6.2 C/EBPβ

The human C/EBPβ gene, like C/EBPα is intronless, and has been assigned to chromosome 20 between q12-q13.1 (Hendricks-Taylor *et al.* 1992). Constitutive expression of C/EBPβ is highest in liver, intestine, lung and adipose tissue but is also detectable in kidney, heart and spleen (Descombes *et al.* 1990; Cao *et al.* 1991; Lekstrom-Himes and Xanthopoulos 1998).

Transcriptional control of the C/EBPβ gene has also been described and CREB is one of the transcription factors known to regulate its expression (Niehof *et al.* 1997). The murine C/EBPβ gene is subject to direct auto-regulation during the APR (Chang *et al.* 1995) and two cAMP responsive elements within the rat C/EBPβ gene promoter play an important role in auto-regulation (Niehof *et al.* 2001a). Interactions between C/EBPβ and members of the CREB or NF-κB family enhances C/EBPβ gene auto-regulation in response to different stimuli in a tissue specific manner (Niehof *et al.* 2001a). Auto-regulation of chicken and *Xenopus laevis* C/EBPβ genes has also been described (Mink *et al.* 1999; Foka *et al.* 2001). The transcription factor STAT3 (signal transducers and activators of transcription-3) is reported to regulate the transcriptional induction of the C/EBPβ gene during the APR by IL-6 (Niehof *et al.* 2001b). Transcriptional induction of C/EBPβ can also be regulated by an enhancer element found 3' downstream of the protein coding region of the human C/EBPβ gene in response to amino acid deprivation (Chen *et al.* 2005b).

As with C/EBPα, C/EBPβ proteins are translated from a single mRNA and at least three isoforms are generated by alternative use of translation initiation codons (Descombes and Schibler 1991; Ossipow *et al.* 1993). The initiation of translation at the first or second AUG, results in the generation of LAP* and LAP isoforms, both of which contain activation and bZIP domains. LIP however, is translated from the third in frame AUG and possesses only the bZIP domain (Xiong *et al.* 2001) (Section 1.2.2 and Figure 1.4). A mechanism involving proteolytic cleavage of full length C/EBPβ has also been proposed to generate various isoforms (Welm *et al.* 1999).

Post-transcriptional/translational regulation of C/EBPβ has also been described. LPS-induced APR in mouse liver leads to an increase in expression of the LIP isoform of C/EBPβ. The 5' region of C/EBPβ mRNA has been shown to be involved in the regulation of LIP translation, through the action of RNA-binding proteins. One of these proteins (a CUG repeat binding protein), CUGBP1 has been shown to physically interact with the 5' region of C/EBPβ mRNA. The binding activity of CUGBP1 is induced upon LPS stimulated APR in mouse liver and this is accompanied by increased binding of this protein to polysomes. It seems that this RNA-binding protein regulates the translation of LIP during the APR, as CUGBP1 immunoprecipitated from LPS-treated mouse livers can sustain translation of LIP in an *in vitro* cell-free translation system. Increased production of LIP through this mechanism has been suggested to mediate inhibition of C/EBPα gene transcription during the APR (Timchenko *et al.* 1999; Welm *et al.* 2000), although these results should be interpreted with some caution (see section 1.3.1.3 for details).

Although transcriptional regulation of C/EBPβ gene expression has been described, the main mode of regulation by which C/EBPβ activity is controlled is post-translational and as described in section 1.2.4, phosphorylation is key in its regulation.

### 1.2.6.3 C/EBPγ

The C/EBPγ gene itself is short, intronless and generates an mRNA that is ubiquitously expressed. The highest expression levels have been noted in non-differentiated progenitor cells. The protein encoded by C/EBPγ is 16.4kDa, possesses an intact dimerisation and DNA binding domain, although it lacks *trans*-activation domains (Figure 1.4) (Cooper *et al.* 1995).

### 1.2.6.4 C/EBPδ

The mouse C/EBPδ gene has been assigned to chromosome 16, with the human counterpart at 8q11 (Cleutjens *et al.* 1993; Jenkins *et al.* 1995). Putative binding sites for AP-2, USF, Sp1, STAT, NF-κB and CREB transcription factors have been identified in the promoter region of the gene (Yamada *et al.* 1997; Davies *et al.* 2000). Like previous members of the C/EBP family, the C/EBPδ gene is intronless (Kinoshita *et al.* 1992; Cleutjens *et al.* 1993). Constitutive expression of C/EBPδ has been detected in lung, adipose tissue, intestine, spleen, with lower levels in the liver and brain (Cao *et al.* 1991; Alam *et al.* 1992; Kinoshita *et al.* 1992).

Transcriptional regulation of the C/EBPδ gene is well documented. The Runt domain factor (Runx)-2 is implicated in C/EBPδ gene transcription in osteoblasts. Mutation of a Runx-2 binding sequence in the rat C/EBPδ gene promoter dramatically reduces promoter activity. Moreover, co-transfection experiments with an anti-sense Runx-2 expression plasmid attenuated C/EBPδ gene promoter activity, whereas over expression of wild-type Runx-2 activated the promoter (McCarthy *et al.* 2000). Recently, a role for NF-κB in the transcriptional regulation of C/EBPδ expression has been demonstrated in response to LPS and peptidoglycan in murine macrophages (Liu *et al.* 2006b; Huang *et al.* 2007).

Transcription of C/EBPδ is also induced in growth-arrested mammary epithelial cells, the involuting mammary gland and in IL-6-induced, growth-arrested LNCaP prostate cancer cells (Sabatakos *et al.* 1998b; Hutt *et al.* 2000; Sivko *et al.* 2004; Sanford and DeWille 2005). Activation of C/EBPδ gene transcription is mediated by the transcription factor STAT3 in growth-arrested mammary epithelial cells and in IL-6-induced growth-arrested LNCaP cells, as demonstrated by transfection analysis using C/EBPδ gene promoter-reporter DNA constructs (Hutt *et al.* 2000; Sivko *et al.* 2004; Sanford and DeWille 2005), although a role of STAT3 in the regulation of endogenous C/EBPδ gene transcription is yet to be confirmed. However, a detailed analysis of the murine C/EBPδ gene promoter in mammary epithelial cells revealed several insights into the factors regulating C/EBPδ transcription in growth-arrested and exponentially growing epithelial cells. Chromatin-immunoprecipitation (ChIP) assays demonstrated that phosphorylated-STAT3 and Sp1 interact and bind to the transcriptionally active C/EBPδ promoter. ChIP assays also showed that in

exponentially growing (C/EBPδ non-expressing) cells, the C/EBPδ gene promoter was bound with the factors; Sp1, CREB, TBP and RNA Polymerase II. In contrast, under growth-arrested (C/EBPδ expressing) conditions ChIP analysis detected phosphorylated-STAT3, Sp1, SRC1, CBP/p300, phosphorylated-CREB, TBP and phosphorylated RNA polymerase II, at the C/EBPδ gene promoter. Furthermore, chromatin remodelling experiments demonstrated that the ATPase component of the SWI/SNF was also required for C/EBPδ transcription. Additionally, C/EBPδ expression was shown to be repressed in proliferating mammary epithelial cells by c-Myc (Zhang *et al.* 2007b). STAT3 is also required for the IL-6-mediated induction of mouse and rat C/EBPδ gene transcription during the APR (Yamada *et al.* 1997; Cantwell *et al.* 1998). Furthermore, IL-6 action induces the expression of myelin gene expression in rat embryonic dorsal root ganglia cultures and in murine melanoma cells through its induction of C/EBPδ, possibly through a similar pathway involving STAT3 as mentioned above (Kamaraju *et al.* 2004).

Activation of STAT3 by IL-6 for example, is short-lived. However, C/EBPδ expression is prolonged in comparison with levels of C/EBPδ detected for up to 24h following IL-6 stimulation of hepatocytes (Ramji *et al.* 1993a; Yamada *et al.* 1997; Cantwell *et al.* 1998). In response to LPS, IL-1 or IFN-γ, C/EBPδ expression is also induced and similar to its induction by IL-6, LPS, IL-1 or IFN-γ treatment of various cell types leads to prolonged expression of C/EBPδ over several hours (Alam *et al.* 1992; Juan *et al.* 1993; Cantwell *et al.* 1998; Granger *et al.* 2000; Tengku-Muhammad *et al.* 2000). This is achieved, at least in part, through auto-activation of the gene (Yamada *et al.* 1998; O'Rourke *et al.* 1999a; Davies *et al.* 2000). Species-specific mechanisms have been described to account for auto-activation of the C/EBPδ gene. Auto-regulation of the rat C/EBPδ gene is regulated by two C/EBP binding sites found at the 3' end of the gene (Yamada *et al.* 1998), whereas the 5' promoter region of the gene is sufficient for regulating auto-activation of the murine and ovine genes (O'Rourke *et al.* 1999a; Davies *et al.* 2000). Ovine C/EBPδ gene auto-activation is proposed to be mediated by an indirect mechanism as no putative C/EBP binding site was identified in the promoter region of this gene (Davies *et al.* 2000). In contrast, the murine gene is thought to be regulated by a direct mechanism of auto-activation as putative C/EBP binding sites were identified in the region of C/EBPδ gene promoter. Presently, no study has sought to determine whether the human C/EBPδ gene promoter is also subject to auto-regulation.

### 1.2.6.5 C/EBPε

The human gene (localised to chromosome 14q11.2) contains two introns, two exons and five AUG initiation codons. Isoforms are produced by use of alternative promoters in combination with differential splicing of mRNA (Yamanaka *et al.* 1997a; Yamanaka *et al.* 1997b; Yamanaka *et al.* 1998; Tang and Koeffler 2001). Expression of C/EBPε has been reported to be primarily limited to cells of the myeloid and lymphoid lineages (Chumakov *et al.* 1997; Yamanaka *et al.* 1997a; Yamanaka *et al.* 1997b; Lekstrom-Himes 2001). Isoforms are translated into at least four proteins (32kDa, 30kDa, 27kDa and 14kDa), all of which harbour identical bZIP domains but differ in their *trans*-activation domains (Figure 1.4). The C/EBPε gene is transcribed by two alternative promoters pα and pβ. This promoter area is similar to those of several other myeloid-cell-specific genes in that it has no TATAA box, but contains a number of purine-rich stretches, harbouring multiple sites for the factors of the Ets family of transcriptional regulators (Chumakov *et al.* 1997). The promoter also contains a functional retinoic acid-response element that regulates inducibility by the corresponding ligand (Park *et al.* 1999).

### 1.2.6.6 C/EBPζ

C/EBPζ was originally cloned from hamster as a gene that was induced by DNA damage and then aptly named growth arrest and DNA damage inducible gene (GADD153) (Fornace *et al.* 1989). The human gene has been cloned and its location assigned to 12q13.1-q13.2 (Park *et al.* 1992). The gene contains four exons and is expressed ubiquitously. Sequence analysis indicated that the human promoter region is relatively GC-rich and contains putative binding sites for multiple transcription factors, including recognition sites for TATA- and CAAT-binding proteins, six Sp1-binding sites, an AP-1 recognition element, an E-26-specific sequence-binding protein-1 DNA binding site and four IL-6 response elements (Park *et al.* 1992). C/EBPζ gene expression is induced by a variety of growth-arresting, oxidative stress and DNA-damaging agents. The C/EBPζ gene promoter is strongly activated by methyl methanesulfonate, hydrogen peroxide and ultra-violet (UV) irradiation (Luethy *et al.* 1990). The AP-1 element in the C/EBPζ gene promoter is necessary for C/EBPζ gene transcription in response to UV-C or oxidant treatment (Guyton *et al.* 1996). Messenger RNA and protein levels of C/EBPζ are also induced by glucose

and amino-acid deprivation (Carlson *et al.* 1993; Bruhat *et al.* 1997). C/EBPζ gene transcription is also induced in response to endoplasmic reticulum stress (ER-stress) and the promoter region responsible for conferring this response has been mapped (Ubeda and Habener 2000).

## 1.3 BIOLOGICAL ROLES OF THE C/EBPS

The action of the C/EBPs has been investigated extensively, leading to the identification of pivotal roles of family members in a number of biological processes. These include the differentiation and proliferation of various cell types, aspects of metabolism and of particular relevance to this thesis, the inflammatory response. Since a discussion of the C/EBPs in the context of biological processes other than inflammation is beyond the scope of this thesis, the reader is directed to a number of informative reviews covering various aspects of C/EBP function, as presented in Table 1.1.

**TABLE 1.1 Major biological roles of the C/EBPs**

| BIOLOGICAL ROLE | REFERENCE(S) |
|---|---|
| Adipocyte differentiation | Darlington *et al.* (1998), Rosen *et al.* (2000), Ramji and Foka (2002) |
| Myeloid cell differentiation | Yamanaka *et al.* (1998), Ramji and Foka (2002), Rosmarin *et al.* (2005) |
| Epithelial cell differentiation | Zahnow (2002), Ramji and Foka (2002) |
| Cell cycle control | Nerlov (2007) |
| Liver cell regeneration | Diehl (1998), Ramji and Foka (2002), Schrem *et al.* (2004) |
| Metabolic regulation | Croniger *et al.* (1998), Roesler (2001), Ramji and Foka (2002) |

### 1.3.1 INFLAMMATION AND THE ACUTE PHASE RESPONSE

During the aftermath of injury, trauma or infection of a tissue a complex series of reactions are carried out by the host in an effort to prevent ongoing tissue damage, destroy invading pathogens and activate repair processes which are necessary to return the organism back to normal function. Collectively, these series of reactions can be defined as the inflammatory response, with the early and immediate set of inducible reactions known as the acute phase response or APR (Baumann and Gauldie 1994). Characteristic features of the systemic acute phase response are; fever, neutrophilia, alterations in lipid metabolism, decreased serum levels of zinc and iron,

increased gluconeogenesis, increased muscle protein catabolism and amino acid transfer from muscle to liver, activation of the complement and coagulation pathways, hormonal changes and production of acute phase response proteins (Moshage 1997).

The APR is part of the body's innate immune defence against microbial infection or injury. Innate immune responses are initiated by chemical structures that are presented by invading microbes (e.g. endotoxin, teichoic acid, polysaccharides and mannans) or revealed by damage to the host. These structures are recognised by the body's 'pattern recognition' molecules or receptors. Engagement of these receptors initiates the inflammatory response that is the APR (Suffredini *et al.* 1999). Table 1.2 displays details of pattern recognition molecules of the innate immune response.

**TABLE 1.2 Major pattern recognition molecules of the innate immune system**

| TYPE | NAME | LOCATION | LIGAND | FUNCTION |
|---|---|---|---|---|
| Humoral | C-reactive protein | Primarily synthesised in the liver; part of APR | Microbial polysaccharides | Activates complement; enhances phagocytosis |
| | Serum amyloid P | Primarily synthesised in the liver | Extracellular matrix proteins; microbial cell wall saccharides | Enhances phagocytosis; stabilises extracellular matrix components |
| | Mannose binding protein | Primarily synthesised in the liver | Microbial cell wall saccharides | Activates complement; promotes phagocytosis; regulates CD14-induced cytokine production |
| | LPS binding protein | Primarily synthesised in the liver; induced production by APR | Catalytically transfers LPS to CD14 and from CD14 to serum lipoproteins | Enhances sensitivity to LPS; system for LPS inactivation |
| | soluble CD14 | Plasma protein; presumed shed from myelomonocytic cells | LPS and numerous microbial cell wall components | Enhances sensitivity to LPS; complex with LPS binds to receptor on endothelial cells and leukocytes, including macrophages |
| | Complement factor 3 (C3) | Primarily synthesised in the liver; induced by APR | Forms ester linkage to OH | Attachment of ligands for |

| | | | | |
|---|---|---|---|---|
| | | | groups on carbohydrates and proteins | complement receptors CD21and CD35 |
| Cellular | Mannose receptors (macrophage mannose receptor and CD205) | Tissue macrophages; hepatic endothelial cells; dendritic cells; thymic epithelium | Carbohydrates and glycoproteins | Potentially targets antigens to class II loading compartment |
| | Scavenger receptors | Tissue macrophages; endothelial cells | Bacteria and yeast cell wall | Clearance of LPS and microbes |
| | LPS receptor (CD14) | Monocyte-macrophages and other leukocytes | LPS and other microbial cell wall components | LPS sensitivity; clearance of microbes; pro-inflammatory cytokine production |
| | Toll receptors | Lymphoid tissue and monocyte-macrophages | LPS; heat shock proteins; viral dsRNA; flagellins | Induce production of cytokines including IL-1, IL-6 and IL-8 |
| Complement receptors | CD35 | Monocyte-macrophages; lymphocytes | C3b, C4b | Enhances C3b and C4b cleavage |
| | CD21 | B lymphocytes; follicular dendritic cells | iC3b, C3dg, C3d | Augments B cell activation by antigen |
| | CD11b/CD18 | monocyte-macrophages; natural killer cells and other leukocytes | iC3b, LPS and fibrinogen | Adhesion and LPS clearance |

(Adapted from Suffredini *et al.* 1999)

Activated immune cells including macrophages, release a variety of inflammatory mediators such as IL-1, IL-6 and TNF-α that are essential for regulating the APR. In response to inflammatory stimuli, major changes in liver physiology occur. This is characterised by dramatic changes in the expression and synthesis of liver APR proteins (APPs). Examples and functions of APPs are given in Table 1.3.

**TABLE 1.3 Acute phase response proteins and their functions**

| EXAMPLE(S) | FUNCTION(S) |
|---|---|
| C-reactive protein | Enhances phagocytosis; complement activation; immunomodulation |
| Serum amyloid A | Leukocyte activation; chemotaxis; phagocytosis; cholesterol transport |
| α1-Antitrypsin | Serine protease inhibitor; major inhibitor of elastase and trypsin |
| α2-Antiplasmin | Serine protease inhibitor; major inhibitor of fibrinolysis |
| Ceruloplasmin | Copper transport |
| Transferrin | Iron transport |
| Haptoglobin | Haemoglobin scavenger |
| Complement C2-C5 and C9 | Complement activation |
| Prothrombin, fibrinogen, von Willebrand Factor, Factor VIII, antithrombin III | Clot formation |
| Plasminogen | Activation of fibrinolysis, complement and clotting |
| Plasminogen activator inhibitor-1 | Inhibits fibrinolysis |
| Fibronectin | Cell attachment; fibrin clot |
| α1-Acid glycoprotein | Transport protein; steroid binding |
| α1-Antichymotrypsin | Serine protease inhibitor; inhibits chymases and cathepsin G |
| Hemopexin | Haem scavenger |
| α2-Macroglobulin | serine-, cysteine-, aspartic- protease inhibitor; inhibitor of coagulation |
| Transthyretin (prealbumin) | Carrier of thyroxine and retinol |

(Adapted from Suffredini *et al.* 1999)

The APPs can be distinguished into two major groups depending on whether their expression is induced or reduced during the APR. The so-called positive APPs (e.g. hemopexin, C-reactive protein (CRP), haptoglobin and α1-acid glycoprotein) whose plasma levels increase during inflammation and the negative APPs (e.g. prealbumin

and transferrin) whose concentrations decrease. Serum concentrations of some positive APPs can increase up to 1000-fold with onset of the APR, as is the case for CRP. For other positive APPs (e.g. α1-antitrypsin), the induction is more modest with a two- to threefold increase in concentration (Ramji *et al.* 1993b; Gruys *et al.* 2005).

Four major classes of factors regulate APP production; the IL-6-type cytokines (e.g. IL-6 and oncostatin M), IL-1-type pro-inflammatory cytokines (e.g. IL-1 and TNF), growth factors (e.g. insulin and hepatocyte growth factor) and glucocorticoids. The cytokines act as primary stimulators of APP production by inducing APP gene transcription, whereas the glucocorticoids and growth factors act as regulators of cytokine action (Baumann and Gauldie 1994).

Natural termination of the APR involves up-regulation of cytokines such as IL-4, IL-10 and IL-13, enhanced expression of soluble decoy receptors of TNF and IL-1 or IL-1-receptor antagonist, and endogenous production of glucocorticoids (Koj 1998).

### 1.3.1.1 Cytokines

Cytokines are small, regulatory proteins (<30kDa in size) with pleiotropic actions, particularly in the regulation of the inflammatory response. Leukocytes secrete numerous cytokines, although the vast majority of other cells in the body are also capable of synthesising these proteins (Dinarello 2000). Typically (but not always), cytokines act over short distances and their binding to specific cell surface receptors triggers signalling events that ultimately result in alterations in the pattern of gene expression in target cells.

On the basis of their structural features it is possible to group cytokines into various families. Families include: interleukins (e.g. IL-1 and IL-18); interferons (e.g. IFN-α, IFN-β, IFN-ω and IFN-γ); chemokines (chemotactic cytokines e.g. IL-8); tumour necrosis factors (e.g. TNF-α); transforming growth factor-β family (e.g. TGF-β and bone morphogenetic proteins); and other growth factors [e.g. platelet-derived growth factor (PDGF) and EGF] (Table 1.4) (Vilcek 2003).

Functional redundancy among the different cytokines is common and even those that are structurally very distinct often exhibit similar or overlapping effects [e.g. TNFα and IL-1 (Zhao *et al.* 2003)]. There are also some examples of cytokines acting

synergistically [e.g. TNF-α and IFN-γ in the regulation of lipoprotein lipase gene expression (Tengku-Muhammad *et al.* 1998); IL-6 and TNF-α or IL-1 in the induction of serum amyloid A gene expression (Hagihara *et al.* 2004)] or antagonistically [e.g. IL-6 and TNF-α in the regulation of haptoglobin gene expression (Mackiewicz *et al.* 1991); TNF-α and TGF-β in the regulation of type I collagen gene expression (Verrecchia and Mauviel 2004)].

**TABLE 1.4 Categorisation of cytokines into families based on structural features**

| FAMILY | EXAMPLES |
|---|---|
| IL-1 | IL-1α<br>IL-1β<br>IL-1 receptor antagonist<br>IL-18<br>IL-1F5-F10*<br>IL-33* |
| IL-10 | IL-10<br>IL-19<br>IL-20<br>IL-22<br>IL-24 |
| IL-17 | IL-17<br>IL-25 |
| IL-2/IL-4 | IL-2<br>IL-4<br>IL-5<br>GM-CSF |
| IL-6/IL-12 | IL-6<br>IL-12 |
| Interferons | IFN-α<br>IFN-β<br>IFN-ω<br>IFN-γ<br>IFN-τ<br>IFN-λ(1-3) |
| TGF-β | TGF-β<br>bone morphogenetic proteins<br>Inhibins<br>Activins |
| Tumor necrosis factors | TNF-α<br>LT-α (TNF-β)<br>LT-β<br>Fas ligand<br>CD40 ligand |
| Chemokines | IL-8<br>MCP-1<br>MIP-1α |

(Adapted from Vilcek 2003; Harvey 2006)

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; LT, lymphotoxin; MIP-1α, macrophage inflammatory protein 1 alpha; IL-1F, interleukin-1 family. * IL-1F5-F10 and IL-33 represent newest members of the IL-1 family of cytokines (Barksby *et al.* 2007).

Another way to group cytokines is in terms of their action during the inflammatory response. Some cytokines clearly promote inflammation and are thus termed pro-inflammatory cytokines (e.g. IL-1 and TNF-α), whereas other cytokines attenuate the activity of pro-inflammatory cytokines and are therefore named anti-inflammatory (e.g. IL-4 and IL-10). However, dividing cytokines into these two broad categories should be done with some caution, because cytokines can function differentially depending on the biological process. For example, IL-4, IL-10 and IL-13 are all potent activators of B lymphocytes, but by virtue of their ability to suppress genes for pro-inflammatory cytokines such as IL-1, they are regarded as anti-inflammatory (Dinarello 2000). Another example of a pleiotropic cytokine is IL-6, which has long been considered as pro-inflammatory because it is induced by IL-1, TNF-α and LPS. In addition, this cytokine is also a potent activator of the APR. However, there is also considerable evidence to suggest that IL-6 has anti-inflammatory properties also, as studies with IL-6 knock-out mice have revealed (Xing *et al.* 1998). Furthermore, IL-6 has the ability to inhibit the synthesis of a number of pro-inflammatory cytokines including IL-1, TNF, GM-CSF and IFN-γ and it promotes the expression of IL-1 receptor antagonist and soluble TNF receptor, inhibitors of IL-1 and TNF action respectively (see Opal and DePalo 2000 and references therein).

#### 1.3.1.2 C/EBPs and the liver APR

The C/EBPs are one of the major groups of transcription factors responsible for regulating APP gene expression. Numerous APP genes contain functional C/EBP binding sites in their gene promoters. These include, hemopexin, haptoglobin, α1-acid glycoprotein, α2-macroglobin, serum amyloid A1, A2 and A3, complement C3, CRP, plasminogen and plasminogen activator inhibitor-1 (PAI-1) (see Poli 1998 and references therein; Milosavljevic *et al.* 2002; Bannach *et al.* 2004; Dong *et al.* 2005).

APP genes can be divided into two classes according to their pattern of responsiveness to cytokines. Class I genes require a combination of both IL-1 and IL-6, and sometimes the addition of glucocorticoids, for maximal induction (e.g. hemopexin and haptoglobin). In contrast, class II genes are responsive to IL-6 and related cytokines, either alone or in combination with dexamethasone (e.g. α2-macroglobulin). Functional C/EBP binding sites [originally known as type I IL-6-responsive elements (IL-6REs)] have been identified in most promoters of class I

genes, often in association with IL-1-responsive elements (IL-1REs), containing binding sites for NF-κB for example. Conversely, genes responsive to IL-6 (class II) often contain type II IL-6REs in their promoters, which are characterised as binding sites for the cytokine-inducible STAT1 and STAT3 transcription factors. Type II IL-6-REs are also found in gene promoters of class I genes (Poli 1998).

In addition, the activity and/or expression levels of C/EBP family members is regulated by a number of inflammatory stimuli including LPS and a variety of cytokines during the APR (see Ramji and Foka 2002 and references therein). Indeed, C/EBPβ was originally discovered as an IL-6 and IL-1 inducible factor in human hepatoma cells and in a glioblastoma cell line respectively (Akira *et al.* 1990; Poli *et al.* 1990). Subsequently, it was discovered that C/EBPδ as well as C/EBPβ were induced by numerous inflammatory stimuli not only in hepatocytes, but in a range of cell types (Alam *et al.* 1992; Ramji *et al.* 1993a; Magalini *et al.* 1995; Cardinaux *et al.* 2000; Granger *et al.* 2000; Tengku-Muhammad *et al.* 2000; Milosavljevic *et al.* 2003; Dinic *et al.* 2004). Conversely C/EBPα expression is down-regulated under inflammatory conditions (Alam *et al.* 1992; Tengku-Muhammad *et al.* 2000; Milosavljevic *et al.* 2003). C/EBPζ is also induced during the APR by C/EBPβ, although its precise role in the process is yet to be fully elucidated, but it may be involved in curtailing the inflammatory response (Sylvester *et al.* 1994).

Several studies have investigated the transaction between the C/EBPs at the promoters of liver APP genes. A general trend has emerged, where in the resting state; the majority of promoters are 'loaded' with C/EBPα homodimers or C/EBPα:C/EBPβ heterodimers. However, upon APR induction, the C/EBPα dimers are rapidly replaced by C/EBPβ and –δ homo-/heterodimers, although the exact composition of these complexes varies with different experimental models used for study (Alam *et al.* 1993; Chen and Liao 1993; Juan *et al.* 1993; Ray and Ray 1994a; Ray and Ray 1994b; Poli 1998; Milosavljevic *et al.* 2003). These results collectively suggest that C/EBPβ and C/EBPδ are principally involved in regulating APP gene expression during the inflammatory response and that C/EBPα is involved in regulating constitutive expression of these genes, although a critical role for C/EBPα in the regulation of neonatal APR has been shown (see section 1.3.1.5).

### 1.3.1.3 Regulation of the C/EBPs in hepatocytes during the APR

As mentioned previously in section 1.2.2 to 1.2.6 the C/EBPs are regulated at multiple levels and the situation is quite the same during the APR.

Transcriptional suppression of the murine C/EBPα gene occurs in response to LPS during the APR (Welm *et al.* 2000). As mentioned in section 1.2.6.2 this transcriptional inhibition is suggested to occur in response to increased binding of the inhibitory LIP form of C/EBPβ to the promoter region of the murine C/EBPα gene (Welm *et al.* 2000). However, a study by Baer and Johnson (2000) has shown that truncated C/EBPβ isoforms can be produced by *in vitro* proteolysis during the isolation of tissue/cellular extracts, thereby suggesting that any data generated in relation to gene regulation by LIP should be interpreted with caution and confirmed using *in vivo* studies. In human hepatoma cells, suppression of C/EBPα gene transcription occurs by a novel mechanism whereby auto-activation of the C/EBPα gene is suppressed in the presence of IL-6 (Foka *et al.* 2003).

Regulation of C/EBPβ can occur at a number of levels; including gene transcription, translation, protein-protein interactions and phosphorylation-mediated changes in DNA-binding activity, activation potential and nuclear localisation (see sections 1.2.2-1.2.4 and 1.2.6.2). Furthermore, sumoylation of C/EBPβ has been described and this novel post-translational modification has also been implicated in the regulation of C/EBPβ activity (Section 1.2.5).

In relation to the APR, CRE-like sequences in the promoter region of rat C/EBPβ gene are required for IL-6 mediated induction of C/EBPβ transcription in hepatocytes by a mechanism involving the tethering of STAT3 to a DNA-bound complex. Additionally, C/EBPβ mRNA levels were increased in liver extracts from mice treated with IL-6, compared to controls, indicating transcriptional regulation of this gene *in vivo* (Niehof *et al.* 2001b). Auto-regulation of the C/EBPβ gene may also regulate its transcriptional expression during the APR (see section 1.2.6.2 and Niehof *et al.* 2001a).

As previously mentioned, post-transcriptional regulation of LIP translation has also been described as a mechanism of C/EBPβ regulation during the APR (Welm *et al.* 2000). Furthermore, TNF-α stimulation of hepatocytes results in rapid accumulation

of C/EBPβ in the nucleus. However, this cytokine was reported to have no effect on the expression levels of this transcription factor, indicating that TNF-α regulates C/EBPβ at the post-transcriptional/-translational level (Yin *et al.* 1996).

Regulation of C/EBPβ is mainly controlled in a post-translational manner. Early studies first indicated this because it was observed that IL-6 treatment of human hepatoma cells, resulted in increased DNA binding and enhanced *trans*-activation potential of C/EBPβ, but this cytokine had a minimal effect on the expression levels of this transcription factor (Poli and Cortese 1989; Poli *et al.* 1990; Ramji *et al.* 1993a). Phosphorylation-mediated changes in C/EBPβ activity play a predominant role in C/EBPβ regulation (Section 1.2.4). For example, regulation of the rat α2-macroglobulin gene during the APR is dependent on the phosphorylation status of C/EBPβ (Milosavljevic *et al.* 2002). As detailed in section 1.2.4 a number of protein kinases have been implicated in controlling the phosphorylation status of C/EBPβ. In murine hepatocytes, IL-6-mediated regulation of plasminogen gene expression is mediated by C/EBPβ as determined by electrophoretic mobility shift assays (EMSAs) and this response was suggested to be regulated by the ERK1/2 MAPK (Bannach *et al.* 2004). Although, no experiments were carried out in this study to determine at what level the ERK1/2 kinase was involved in the regulation of C/EBPβ, a plethora of studies have suggested a role for this pathway in the regulation of the phosphorylation status of C/EBPβ (see section 1.2.4). More recently, it has been shown that IL-1 induces the phosphorylation of C/EBPβ and its subsequent activation by a ceramide- and ERK1/2-dependent pathway in primary rat hepatocytes (Giltiay *et al.* 2005). Furthermore, the orphan nuclear receptor-liver receptor homolog 1 has been shown to act as a negative regulator of the hepatic APR, by antagonising C/EBPβ action potentially through regulating its post-translational modification (Venteclef *et al.* 2006).

A number of different transcription factors have been shown to physically and functionally interact with C/EBPβ and this dictates regulation through protein-protein interactions (see sections 1.2.2 and 1.2.3). Of particular relevance to the APR, are interactions between C/EBPβ and members of the NF-κB family (Agrawal *et al.* 2001). Adjacent C/EBP and NF-κB binding sites have been located in the promoters of many APP class I genes, which require both the actions of IL-1 and IL-6 for maximal induction. Synergistic activation of APP genes, mediated through C/EBP

and NF-κB activity are well documented. Of the genes regulated in this manner, serum amyloid A1, A2, A3 and α1-acid glycoprotein are of particular relevance during the APR (see Poli 1998 and references therein).

Furthermore, in relation to the regulation of the APR, protein-protein interactions between peroxisome proliferator activated receptor α (PPARα) and C/EBPβ may also be significant (Mouthiers *et al.* 2005). Recently, a role of PPARα in the hepatic APR has been associated with a decrease of APP gene expression. Exposure of rodents to peroxisome proliferator ligands results in the down-regulation of many positive APR genes including fibrinogen, α1-acid glycoprotein, α1-antitrypsin, ceruloplasmin and serum amyloid A (Corton *et al.* 1998; Anderson *et al.* 1999; Mouthiers *et al.* 2005). This effect seems to be mediated mainly by PPARα as transcriptional repression of these genes is abolished in PPARα knock-out mice (Anderson *et al.* 1999). More recent studies have shown that PPARα regulates transcriptional repression of liver APP genes by interfering with C/EBP action. For example, PPARα has been shown to repress fibrinogen gene expression by interfering with C/EBPβ action in hepatocytes (Gervois *et al.* 2001). Similarly, PPARα inhibits dexamethasone-induced α1-acid glycoprotein gene expression by physically associating with C/EBPβ and preventing its binding to the α1-acid glycoprotein gene promoter (Mouthiers *et al.* 2005).

Regulation of APP genes by C/EBPδ is to a large degree dependent on transcriptional activation of the gene itself (Ramji *et al.* 1993a; Yamada *et al.* 1997; Cantwell *et al.* 1998). However post-translational modifications, of which phosphorylation-mediated changes in C/EBPδ activity are particularly significant during the APR, have also been described (Ray and Ray 1994a; Ray and Ray 1994b; Milosavljevic *et al.* 2002).

Although little is known about the exact mechanisms by which the C/EBPδ gene is regulated during the APR, work by Yamada *et al.* (1997) and Cantwell *et al.* (1998) has successfully identified transcription factors that induce C/EBPδ expression in response to IL-6 in cultured hepatocytes. These studies have implicated the transcription factors STAT3 in regulation of C/EBPδ gene expression, mainly with the use of transfection based assays utilising C/EBPδ gene promoter-reporter constructs and electrophoretic mobility shift assays (EMSA). Similar findings have now been confirmed in relation to the regulation of the human C/EBPδ gene promoter in

response to IL-6, albeit in a prostate cancer cell line, LNCaP (Sanford and DeWille 2005). Cantwell *et al.* (1998) determined the positions of STAT3 and Sp1 binding sites in the mouse C/EBPδ gene promoter and demonstrated that both sites were necessary for IL-6 responsiveness. It was concluded that Sp1 synergises with STAT3 in regulating C/EBPδ gene promoter activity, induced by IL-6. Interestingly, STAT3 also confers IL-6 responsiveness in the C/EBPβ gene (see above), implicating it as major transcription factor in the regulation of IL-6 signalling pathways. However, Alonzi and co-workers (2001) have shown that overall induction of C/EBPβ and C/EBPδ genes (as mediated by IL-6 or LPS) in mouse livers was only minimally defective in STAT3 deficient mice. This suggests that other signalling pathways, independent of STAT3 activation, can lead to the transcriptional activation of C/EBPβ and C/EBPδ genes during the IL-6 induced APR *in vivo*.

Several studies have reported that C/EBPδ expression is induced and maintained several hours following treatment of cells with pro-inflammatory stimuli, including in hepatocytes (Alam *et al.* 1992; Juan *et al.* 1993; Ramji *et al.* 1993a; Cantwell *et al.* 1998; Granger *et al.* 2000; Tengku-Muhammad *et al.* 2000). It is generally accepted that this effect is attributed to auto-activation of the C/EBPδ gene as discussed in section 1.2.6.4 (Yamada *et al.* 1998; O'Rourke *et al.* 1999a; Davies *et al.* 2000; Tanabe *et al.* 2000). Auto-regulation of the C/EBPδ gene is therefore likely to bare significance in modulating its expression during the APR.

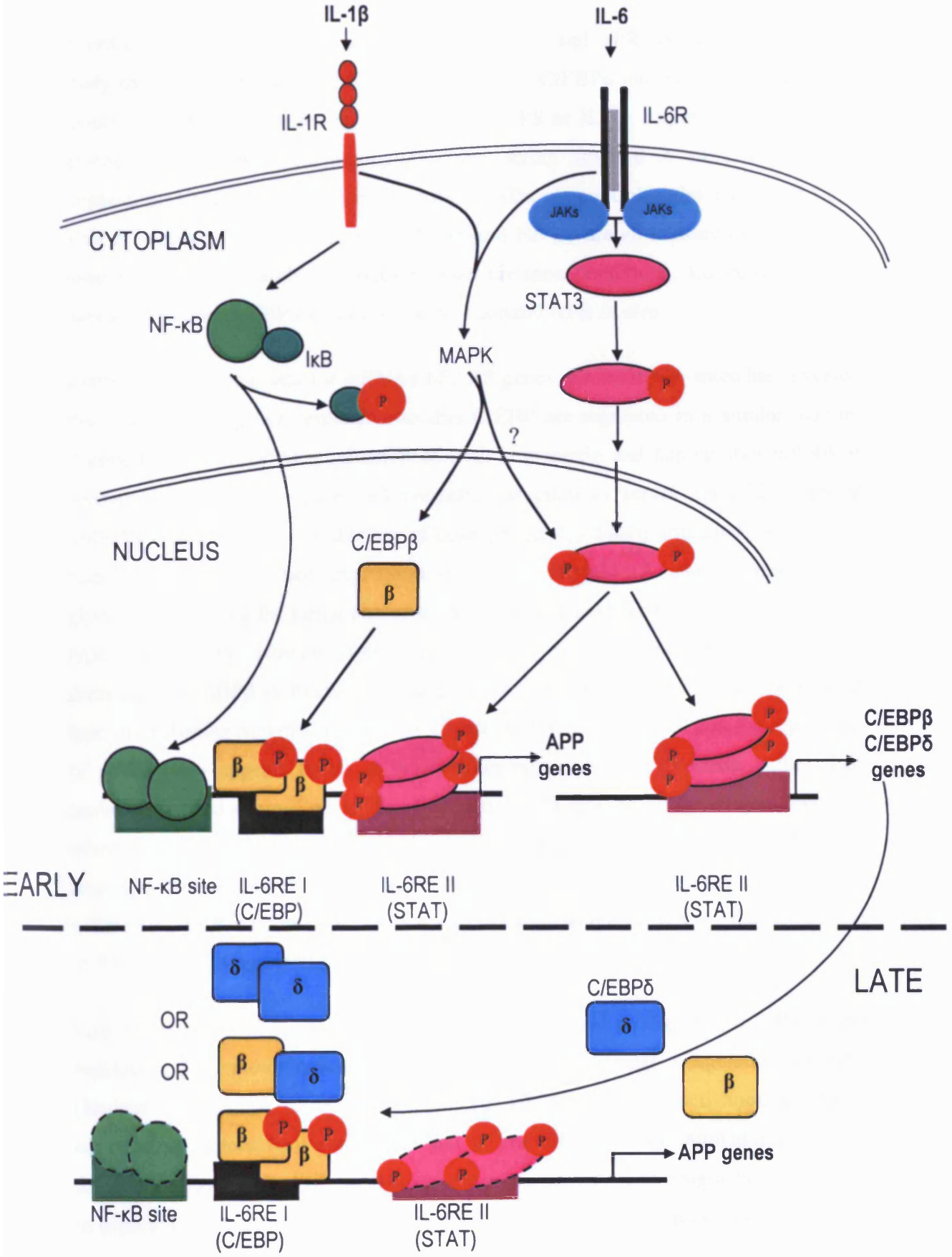
Phosphorylation mediated changes in C/EBPδ activity have also been suggested to contribute to its regulation during the APR (Section 1.2.4). Additionally, synergistic interactions between C/EBPβ and C/EBPδ has been demonstrated to control the expression of the IL-6 gene and similar interactions are likely to be involved in regulating expression of a number of acute phase genes (Kinoshita *et al.* 1992; Ray and Ray 1994a; Ray and Ray 1994b; Poli 1998).

#### 1.3.1.4 A model of action for C/EBPβ and –δ in APP gene regulation

A sequential model of APP gene induction involving C/EBPβ and C/EBPδ was proposed by Valeria Poli (1998). The model proposed that cytokine-activated transcription factors like NF-κB and STAT3 (activated rapidly but transiently in response to inflammatory stimuli) mediate initial transcription of APP genes. Their activity is then, at least partially, replaced by the action of C/EBPβ and C/EBPδ, in

the later stages of the APR. In this scheme of events, inflammatory stimuli such as IL-1 and IL-6 would first trigger the activation of NF-κB and STAT3 respectively. Both STAT3 and NF-κB would initiate transcription of class I and class II APP genes. STAT3 and other yet unidentified transcription factors would also induce transcription of C/EBPβ and C/EBPδ genes. Synthesis of new C/EBPβ and C/EBPδ proteins would lead to *trans*-activation of additional class I APP genes, substituting the activity of STAT3, NF-κB and other 'early' transcription factors. As the expression of C/EBPβ and C/EBPδ is maintained sometime after their initial induction, it is likely that this gene transcription is continued through auto-regulation of these genes. The increasing pools of C/EBPβ and C/EBPδ would then act to support APP gene transcription during the later stages of the APR. Constitutive C/EBPβ pools may also be activated by phosphorylation during the initial stages of inflammation and therefore contribute to 'early' and 'late' APP gene transcription. A schematic representation of this model is shown in Figure 1.5.

**FIGURE 1.5. A model for the induction of APP genes by IL-1 and IL-6 (adapted from Poli 1998).** Inflammatory cytokines (IL-1 and IL-6) bind to cognate receptors and activate respective signalling pathways. IL-1 is known to activate MAPK pathways and the translocation of NF-κB (see section 1.4 for details). Activated NF-κB can induce the transcription of class I APP genes. IL-1 is also known to increase the expression of C/EBPβ and C/EBPδ genes, although the exact mechanisms are still unclear and therefore this is not depicted in the schematic. IL-6 activates the JAK (Janus kinases)-STAT pathway and also MAPK pathways. JAK stimulation results in STAT3 activation but MAPK may also be required for the process (flagged with ?) (see section 1.5 for details). Activation of STAT3 leads to the transcription of class I and II APP genes. At the same time activated STAT3 also induces transcription of C/EBPβ and C/EBPδ genes. MAPK-dependent phosphorylation of C/EBPβ can also activate constitutive pools of this transcription factor, resulting in transcription of class I APP genes. This phase is represented as 'EARLY.' Newly synthesised C/EBPβ and C/EBPδ may be able to maintain their expression during the APR through auto-regulation mechanisms (although this is not depicted in the schematic). C/EBPβ and C/EBPδ can bind and *trans*-activate class I APP gene promoters (in various combinations), either functionally replacing other 'early' transcription factors or synergising with them, depending on the gene promoter. This is the latent phase of APP gene induction (LATE). It is worthy of note that this scheme only illustrates a model proposed for the coordinated role of the transcription factors NF-κB, STAT3, C/EBPβ and –δ in APP gene regulation. This is not meant as an exhaustive description of all possible activators of these factors. 'P' denotes phosphate group. See text for further details.


IL-1β
IL-6
IL-1R
IL-6R
JAKs
JAKs
CYTOPLASM
STAT3
NF-κB
IκB
MAPK
P
?
NUCLEUS
C/EBPβ
β
APP
genes
C/EBPβ
C/EBPδ
genes
EARLY
NF-κB site
IL-6RE I
(C/EBP)
IL-6RE II
(STAT)
IL-6RE II
(STAT)
LATE
δ
OR
OR
C/EBPδ
NF-κB site
IL-6RE I
(C/EBP)
IL-6RE II
(STAT)
APP genes

### 1.3.1.5 Abnormalities of the APR in C/EBP knock-out mice

A critical role for C/EBPα in the regulation of neonatal APR was demonstrated in a study by Burgess-Beusee and Darlington (1998). C/EBPα null neonatal mice fail to mount a hepatic APR reaction in response to LPS or IL-1. Expressions of α1-acid glycoprotein, γ-fibrinogen, haptoglobin and serum amyloid A were all severely impaired in C/EBPα-/- mice compared to C/EBPα +/+ controls, stimulated with LPS. This is in spite of the fact that C/EBPβ, -δ and NF-κB are all induced in C/EBPα -/- mice at a level comparable to controls under the same conditions. Collectively, these data suggest that C/EBPα is critical for the neonatal APR *in vivo*.

Expression profiling data for mRNAs of APP genes in C/EBPβ -/- mice has revealed that not all APP genes responsive to this C/EBP are regulated in a similar manner (Cappelletti *et al.* 1996). Induction of both hemopexin and haptoglobin mRNA is normal in C/EBPβ -/- mice. Conversely, induction of serum amyloid A and P mRNAs is reduced, with induction of complement C3 totally impaired. C/EBPβ-/- mice are able to induce expression of serum amyloid (A and P) and $\alpha_1$-acid glycoprotein during the initial phases of the APR, with levels comparable to their wild type counterparts. However following the initial induction, mRNA levels begin to decrease in C/EBPβ -/- mice, dropping to basal levels within 24h at which point wild type mice display maximal induction. Therefore, the role of C/EBPβ in the regulation of such genes appears to be in the maintenance of their expression, rather than initiating the induction (Poli 1998). Furthermore, to account for the normal levels of other APP gene mRNAs in C/EBPβ -/- mice, it is possible that other C/EBP family members functionally compensate for its loss. Indeed redundancy amongst the C/EBPs has been demonstrated in their regulation of IL-6 and MCP-1 gene expression in P388 lymphoblasts (Hu *et al.* 1998).

Results from a recent study in C/EBPδ-/- mice reveal a role for C/EBPδ in the regulation of endotoxin-induced disseminated intravascular coagulation or DIC (Slofstra *et al.* 2007). DIC is characterised by systemic activation of blood coagulation factors that eventually lead to the formation of intravascular micro-thrombi and impaired organ perfusion. Ongoing activation of coagulation can result in depleted levels of platelets and coagulation factors, resulting in excessive bleeding.

Of relevance, the liver is one of the key organs that is susceptible to- and responsible for the initiation of organ failure during DIC or related sepsis (Slofstra *et al.* 2007).

One of the triggers for DIC is the presence of excessive amounts of pro-inflammatory cytokines such as IL-6 and TNF-α in the system. Interestingly, systemic levels of IL-6 and TNF-α were significantly reduced in LPS-challenged C/EBPδ-/- mice compared to their equivalent controls. Markers for kidney and liver failure were also compared between the two groups. Markers for kidney injury (creatinine and blood urea nitrogen) were significantly lower in LPS-challenged C/EBPδ-/- mice compared to controls, indicating that C/EBPδ deficient mice were protected against kidney damage mediated by endotoxin-induced DIC. In contrast, makers for liver injury were increased in LPS-challenged C/EBPδ-/- mice compared to controls, suggesting that absence of C/EBPδ may actually aggravate hepatic damage in these mice.

Therefore, consistent with its role during the inflammatory response and given that C/EBPs have been implicated in the regulation of cytokine gene expression (see below), the observation that TNF-α and IL-6 are present in reduced amounts in C/EBPδ-/- LPS-challenged mice, emphasises a key role for C/EBPδ in the regulation of the inflammatory response *in vivo*. The fact that C/EBPδ deficiency may cause endotoxin-induced liver dysfunction is consistent with its role in the APR. As mentioned above, many of the APP genes are induced by C/EBPδ, including haptoglobin, C3 and CRP. These proteins are known to affect the pro-coagulant and anti-fibrinolytic state and function to limit tissue damage. C/EBPδ deficiency could impair the production of these proteins and therefore make the liver more susceptible to injury induced by potent inflammatory stimuli (Dhainaut *et al.* 2001; Slofstra *et al.* 2007). The observation that C/EBPδ-/- mice are protected against DIC-mediated kidney damage is more difficult to explain, although discrepant responses between organs are a recurring theme in sepsis and may be reflected by varying sensitivities and responses to injury-inducing stimuli in different cell types (Mahidhara and Billiar 2000; Slofstra *et al.* 2007).

Given the significance of both C/EBPβ and C/EBPδ activity during the APR and the potential existence of functional redundancy amongst these family members, it would be of interest to create C/EBPβ and C/EBPδ double knock-out mice for study.

Although such a strain has been generated, mice are perinatal lethal and therefore prove difficult to study (Tanaka *et al.* 1997).

### 1.3.1.6 C/EBP regulation of other genes relevant to inflammation

The C/EBPs play a significant role in controlling hepatic APP gene expression but they also regulate the expression of a plethora of other genes relevant to the inflammatory response. Far from being liver specific, the expression of C/EBPα, -β and –δ is modulated during the inflammatory response in a range of cell types, including cells of the myelomonocytic lineages, mesangial cells and astroglial cells. Notably, the expressions of various C/EBPs are also differentially modulated during myeloid cell differentiation (Table 1.1). Therefore, it is not surprising that the C/EBPs are able to regulate multiple genes expressed in these cell types. Of note, genes for multiple cytokines and their receptors (e.g. IL-6, IL-1β, TNF-α, IL-8, IL-12, MCP-1 and GM-CSF receptor) and genes encoding proteins necessary for macrophage or granulocytic functions (e.g. COX-2, inducible nitric oxide synthase, lysozyme, myeloperoxidase and neutrophil elastase), all contain C/EBP binding motifs in their regulatory regions, many of which have been shown to be necessary for their *trans*-activation (see Poli 1998; Ramji and Foka 2002 and references therein).

A role for the C/EBPs in the inflammatory response is further confirmed by studies in knock-out mice. Defects in the inflammatory response and in the development and function of various immune cells are apparent in knock-out mouse models for C/EBPα, -β and –ε. Some of the experimental findings from published studies are summarised in Table 1.5.

**TABLE 1.5 C/EBP knock-out mice with phenotypic abnormalities relevant to the inflammatory response**

| KNOCK-OUT MODEL | PHENOTYPIC ABNORMALITIES | REFERENCE(S) |
|---|---|---|
| **C/EBPα** | Lack mature granulocytes<br>Unresponsive to G-CSF | Zhang *et al.* (1997) |
| | Disrupted macrophage development | Heath *et al.* (2004) |
| **C/EBPβ** | Major defects in bactericidal and tumoricidal activities of macrophages | Tanaka *et al.* (1995) |
| | Lymphoproliferative disorder associated with defects in macrophage activation<br>Impaired IL-12 production | Screpanti *et al.* (1995) |
| | Curtailed inflammatory response in reaction to cerebral ischemia | Kapadia *et al.* (2006) |
| **C/EBPε** | Immunodeficient<br>Produce morphologically atypical granulocytes | Yamanaka *et al.* (1997b) |
| | Lack mature macrophages<br>Impaired production of MCP-3, CD14, PAI-2, IL-6 and IL-12 | Tavor *et al.* (2002) |

Abbreviations: G-CSF, granulocyte-colony stimulating factor; ICAM1, intercellular adhesion molecule 1; MCP-3, monocyte chemotactic protein-3; PAI-2, plasminogen activator inhibitor-2.

The above sections have highlighted the critical role of the C/EBPs in regulation of the inflammatory response, in particular during the APR. It is evident from the discussion that aspects of C/EBP regulation in response to cytokine action remain largely unknown. In particular, the mechanisms by which C/EBPδ is transcriptionally up-regulated during the inflammatory response are elusive. As already mentioned, IL-1 and IL-6 are major inflammatory cytokines that regulate several key aspects of the APR and are known to induce C/EBPδ expression. Therefore, this next section aims to provide an account of the signalling pathways by which these cytokines are known to mediate their effects before establishing the aims of this study.

## 1.4 IL-1 SIGNALLING PATHWAYS

IL-1 is a potent, pro-inflammatory cytokine with the ability to produce countless biological effects. Its effects include induction of fever and the APR (as mentioned above), leukocyte activation and stimulation of cytokine production (e.g. IL-6). The three primary members of the IL-1 gene superfamily are IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra) (Table 1.4). Studies in knock-out mice have revealed that IL-1β is the critical IL-1 isoform required to mediate the APR (Zheng *et al.* 1995). IL-1α and IL-1β are both agonists, whereas IL-1Ra is a specific receptor antagonist for IL-1α and IL-1β. When IL-1Ra occupies the IL-1 receptor, it prevents both IL-1α and IL-1β from binding the receptor and thus there is no biological response to IL-1. IL-1α and IL-1β are both synthesised as precursors and their conversion to 'mature' forms requires enzymatic processing. The IL-1α precursor is functionally active, although the IL-1β precursor must be processed into its mature form to be biologically active. IL-1Ra can exist as a secreted (sIL-1Ra) protein or as an intracellular form in cells (Loppnow *et al.* 2001; Dinarello 2002).

### 1.4.1 IL-1 RECEPTORS

The two IL-1 isoforms and IL-1Ra bind two receptors, aptly named type I and type II IL-1 receptors (IL-1RI and IL-1RII). These receptors are encoded by two separate genes and belong to the immunoglobulin (Ig)-like receptor family. They contain three extracellular Ig-like domains, a transmembrane domain and a cytoplasmic portion. The cytoplasmic segment of IL-1RI contains a Toll-IL-1R (TIR) domain that is common to the superfamily of Toll-like receptor/ IL-1 receptors whose other members are also involved in regulating aspects of defence and immunity. The IL-1RII is also part of this superfamily of receptors although it does not contain the TIR domain. Its cytoplasmic segment is substantially shorter than that of IL-1RI and therefore does not accommodate this domain. All three Ig-domains are required for high affinity binding to the IL-1 ligand, whereas only two of these domains are required for IL-1Ra binding. Owing to the absence of the TIR domain in IL-1RII, that is required for IL-1 signalling, IL-1RII cannot transduce a signal by IL-1, despite being able to bind the ligand. It therefore serves as a decoy receptor and together with IL-1Ra probably serves to limit IL-1 action. Both receptors can also be found as soluble forms, produced as a result of enzymatic shedding from cell surfaces and these soluble

receptors also serve to neutralise the action of IL-1. For IL-1 responsiveness another TIR containing 'receptor' is required for IL-1 signalling, the IL-1R-accessory-protein (IL-1RAcP). Despite sharing a similar structure with IL-1RI, IL-1RAcP does not physically bind IL-1 on its own and thus is not a true IL-1 receptor. Rather, it appears to function to increase the affinity of IL-1RI for IL-1 by forming a heterodimeric complex with this receptor. IL-1 binding may induce a conformational change in IL-1RI that recruits IL-1RAcP to the complex (Dinarello 1996; Loppnow *et al.* 2001; Dinarello 2002; Martin and Wesche 2002; Dunne and O'Neill 2003). Figure 1.6 illustrates the various IL-1 receptors and their functions.

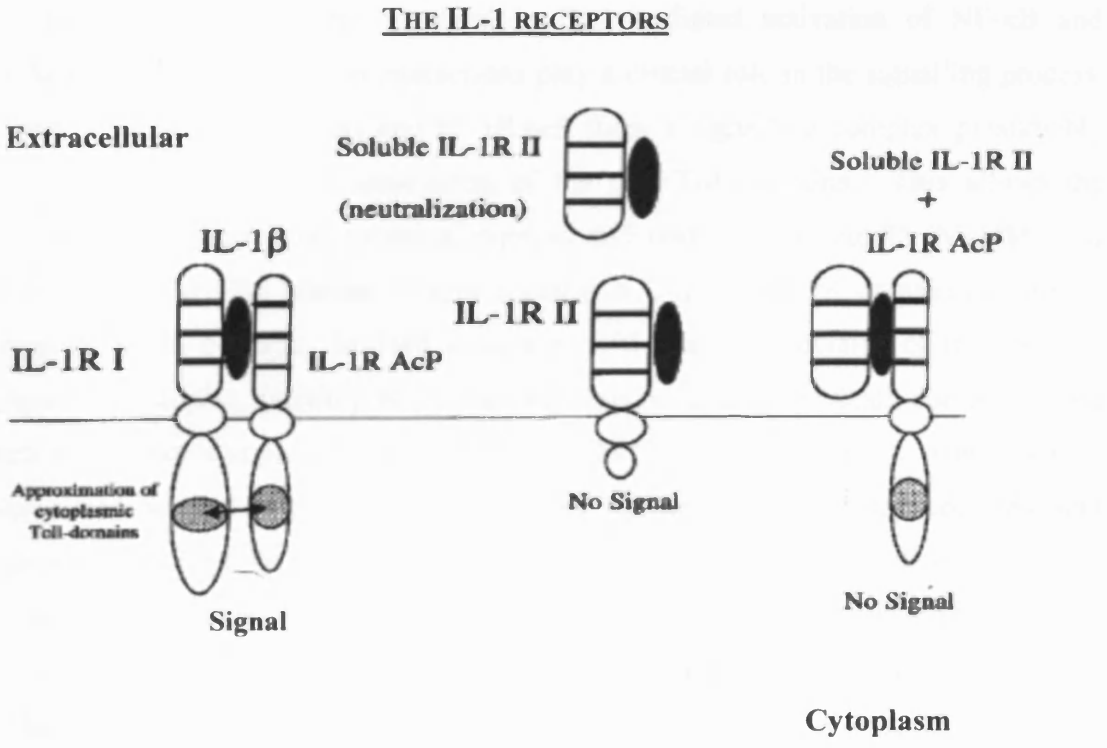



**FIGURE 1.6 The IL-1 receptors (adapted from Dinarello 2002).** IL-1RI binds IL-1β and this complex recruits IL-1RAcP and signalling is initiated. Conversely, when IL-1β binds IL-1RII, there is no signal transduction as it lacks a complete cytoplasmic domain comparable to IL-1RI. Soluble IL-1RII can also bind IL-1β and this interaction serves to neutralise IL-1 action. Additionally, the soluble IL-1RII-IL-1β complex may bind to the IL-1RAcP, depriving the IL-1RI of its co-receptor and thus also dampening IL-1 action.

IL-1-mediated gene transcription is known to be regulated by the activity of the transcription factor, NF-κB. Therefore, NF-κB has been the focus of much attention in studies attempting to delineate IL-1-dependent signalling pathways. Furthermore, studies have concentrated on the regulation of NF-κB activity because it is well established that NF-κB is a key regulator of inflammatory gene expression (O'Neill 2000; O'Neill 2002). In addition to the activation of NF-κB, the other major signalling pathways activated by IL-1 are dependent on MAPK activation. Signalling events leading to the activation of both NF-κB and the MAPKs are discussed below.

### 1.4.2 IL-1-DEPENDENT SIGNALLING EVENTS LEADING TO THE ACTIVATION OF NF-κB AND MAPKS

A number of proteins are involved in IL-1-mediated activation of NF-κB and MAPKs, and protein:protein interactions play a crucial role in the signalling process. Upon binding IL-1, IL-1RI and IL-1RAcP form a signalling complex presumably through the close spatial association of the two TIR domains. This allows the recruitment of the adapter proteins, myeloid differentiation protein 88 (MyD88) and Tollip. The MyD88 adapter protein contains a C-terminal TIR domain and an N-terminal death domain. MyD88 associates with the TIR domains of the receptor signalling complex, possibly as a dimer and thus introduces the death domains at the active receptor complex. The IL-1R-associated kinase-1 (IRAK-1), which is pre-associated with Tollip, rapidly translocates to the active receptor complex and interacts with MyD88 with its N-terminal death domain. This interaction is likely to cause dissociation of Tollip from IRAK-1, thus liberating IRAK-1, which is anchored to the active receptor via death domain protein:protein interactions with MyD88 (Martin and Wesche 2002; Dunne and O'Neill 2003). Subsequently, IRAK-1 becomes initially phosphorylated by another IRAK, IRAK-4 as more recently described (Suzuki *et al.* 2002; Li *et al.* 2002a), although the significance of IRAK-4 in IL-1 signalling has been disputed (Qin *et al.* 2004). In the present model, IRAK-4 is believed to be recruited to the active receptor complex via an adapter protein, possibly by Tollip (Qin *et al.* 2004).

Following the initial phosphorylation, IRAK-1 can catalyse multiple auto-phosphorylations and this event may trigger its release into the cytoplasm (Martin and Wesche 2002). It has been reported that at this stage IRAK-1 can interact with novel

scaffold proteins called Pellinos, although their precise role in the signalling process is still unclear (Schauvliege *et al.* 2007). IRAK-1 also interacts with the downstream adapter protein TNF-α receptor associated factor 6 (TRAF6) and in parallel IRAK-1 also makes transient contact with the membrane-associated TAB2, an adapter protein for the MAPKKK, TGF-β-activated kinase 1 (TAK1). TAB2 serves as an adapter by linking TRAF6 to TAK1 and its other adapter protein TAB1 (Martin and Wesche 2002; Dunne and O'Neill 2003). More recently however, a new TAK1 adapter protein has been identified, TAB3 (Cheung *et al.* 2004). Two distinct TAK1 containing complexes were identified in cells, where TAK1 is complexed with TAB1 and either TAB2 or TAB3, with both these forms activated by IL-1.

Chen and co-workers (Deng *et al.* 2000; Wang *et al.* 2001) showed that protein ubiquitinylation plays a key role in TRAF6-mediated TAK1 and inhibitor-κB kinase (IKK) activation. TRAF6 has been shown to function as an E3 ubiquitin ligase and TRAF6 itself is a target for ubiquitinylation. Of relevance, IL-1 stimulation triggers ubiquitinylation and oligomerisation of TRAF6, indicating that this process is required for signal transduction events (Martin and Wesche 2002; Dunne and O'Neill 2003; Yao *et al.* 2007). By a yet unresolved mechanism, polyubiquitinylated TRAF6 directly facilitates full activation of TAK1 which can activate itself by auto-phosphorylation in its activation loop (Kishimoto *et al.* 2000). TAK1 activation can lead to the activation of NF-κB and/or the subsequent activation of the MAPKs, both of these processes will be discussed separately below (Sections 1.4.3 and 1.4.4).

The molecular switch to turn off the signalosome is the proteolytic degradation of hyperphosphorylated IRAK-1 (Yamin and Miller 1997). It has been speculated that degradation of IRAK-1, results in the dissociation of the proteins centring around TRAF6 and this limits the duration of the IL-1 response (Martin and Wesche 2002).

The sequence of events detailed above is essentially the scheme of events described during IL-1 signalling as reviewed by Martin and Wesche 2002 and Dunne and O'Neill 2003. However, it should be noted that although other groups have proposed very similar schemes, some models of IL-1 signalling deviate slightly from the one described above, particularly in terms of describing the translocation of various signalling components involved in the process (Qin *et al.* 2004; Yao *et al.* 2007).

### 1.4.3 IL-1-DEPENDENT ACTIVATION OF NF-κB

There are five proteins of the NF-κB family; NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100) and the Rel subfamily, RelA/p65, RelB and c-Rel. All members contain a conserved Rel-homology domain which consists of a DNA-binding motif, a dimerisation region and a nuclear localisation signal. Additionally, members of the Rel subfamily contain a *trans*-activation domain that is required for target gene activation (Wietek and O'Neill 2007).

Pro-inflammatory cytokines like IL-1 activate the 'classical' or 'canonical' NF-κB pathway which results in the activation of mainly the p50:p65 NF-κB heterodimer. This involves the phosphorylation and subsequent ubiquitinylation-mediated degradation of inhibitor κB proteins (IκB), which are bound to NF-κB protein dimers in the cytoplasm of unstimulated cells. Degradation of IκB (normally IκBα), unmasks the nuclear localisation signal on NF-κB proteins and allows translocation of NF-κB from the cytoplasm to the nucleus where it can regulate the transcription of target genes (Wietek and O'Neill 2007).

The regulatory event in this pathway is the phosphorylation of IκBs. This process is regulated by the IκB-kinases (IKKs). IKK is a large multimeric complex consisting of two kinase subunits, IKKα (IKK1), IKKβ (IKK2) and a regulatory subunit IKKγ or NEMO (for NF-κB essential modifier). In the classical pathway, IKKβ phosphorylates IκBα at two key residues serine$^{32}$ and serine$^{36}$, marking it for ubiquitinylation and subsequent degradation. IKKs also have substrates outside the IκB family, most notably the p65 subunit of NF-κB. Activation of the IKK complex is regulated by its phosphorylation. Multiple upstream kinases have been suggested to act as IKK-kinases (Dunne and O'Neill 2003; Hayden and Ghosh 2004). In IL-1 signalling, both TAK1 and the extracellular signal-regulated kinase kinase kinase 3 (MEKK3) have been implicated in this process (Wang *et al.* 2001; Yao *et al.* 2007). Additionally, the Toll/IL-1 pathway specific adapter protein ECSIT (evolutionary conserved signalling intermediate in Toll pathways) has been described to interact and activate MEKK1 resulting in NF-κB activation (Kopp *et al.* 1999).

NF-κB (p50:p65), activation is also regulated by phosphorylation-mediated changes in *trans*-activation potential of mainly the p65 subunit and by its recruitment of co-factors such as p300 or CBP. p65 NF-κB is phosphorylated at a number of serine

residues in its *trans*-activation domain and generally phosphorylation has been shown to enhance the *trans*-activation potential of p65 (Wietek and O'Neill 2007). IL-1 stimulates the phosphorylation of p65 as a number of studies have indicated. Kinases involved in this reaction have been reported as casein kinase 2 (CK2), IKKα, -β and the two non-canonical kinases IKKε (IKKi) and TANK-binding kinase 1 (Bird *et al.* 1997; Buss *et al.* 2004).

### 1.4.4 IL-1-DEPENDENT ACTIVATION OF MAPKS

IL-1 is reported to activate all three MAPK modules; the ERK1/2 MAPK and the stress activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 (α, β, γ and δ) (Dunne and O'Neill 2003). MAPKs modulate gene expression by regulating the activity of a wide range of transcription factors (Yang *et al.* 2003) and by regulating mRNA stability (particularly in the case of p38 MAPK) (Clark *et al.* 2003). MAPKs denote an entire class of protein kinases that share several fundamental features. Principally they are regulated by threonine and tyrosine phosphorylation and are organised into a hierarchical cascade of kinases. The MAPKs are phosphorylated at a threonine-x-tyrosine motif. Both threonine and tyrosine phosphorylations are catalysed by dual-specificity protein kinases generically termed MAPK kinases (MAPKKs). These enzymes are in turn phosphorylated (at serine/threonine residues) and activated by a third class of enzymes the MAPKK kinases (MAPKKKs) (Kyriakis and Avruch 2001).

Precise details of the upstream regulation of the MAPKs by IL-1 are not fully determined, although some details have been established. As mentioned previously, IL-1 activates TAK1. TAK1 is a MAPKKK and is reported to activate the MAPKK, MKK6 and this in turn activates both the p38 and JNK MAPK modules (Wang *et al.* 2001). Raf-1 is another upstream kinase of the MAPK cascade and it too is activated by IL-1 (Huwiler *et al.* 1996). IL-1 is also reported to activate the MAPKKs, MKK4 (Guan *et al.* 1999) and MKK7 (Tournier *et al.* 1999), which lie upstream of the JNK MAPK. In addition, many studies report that MEK1 and MEK2, which are upstream MAPKKs for the ERK1/2 MAPK, are necessary in IL-1 signalling with the use of pharmacological inhibitors and dominant negative constructs (e.g. Laporte *et al.* 1999; Jiang *et al.* 2004). Details of how these protein-kinase cascades are activated during IL-1 signalling are less well documented. However, roles of the small guanosine

triphosphatases (GTPases) have been identified. IL-1 triggers increased GTP binding and hydrolysis in membranes and evidence for Rho and Rac1 GTPase activation has been presented. Furthermore, dominant negative mutants for Rac1 and Cdc42 impair activation of both JNK and p38 MAPK in response to IL-1. Additionally, the Ras GTPase has been shown to be necessary for p38 MAPK activation and has been positioned downstream of TRAF6 in the IL-1 pathway (see Dunne and O'Neill 2003 and references therein). A summary of the prototypal signalling events leading to the activation of MAPKs and NF-κB by TAK1 are presented in Figure 1.7 below.

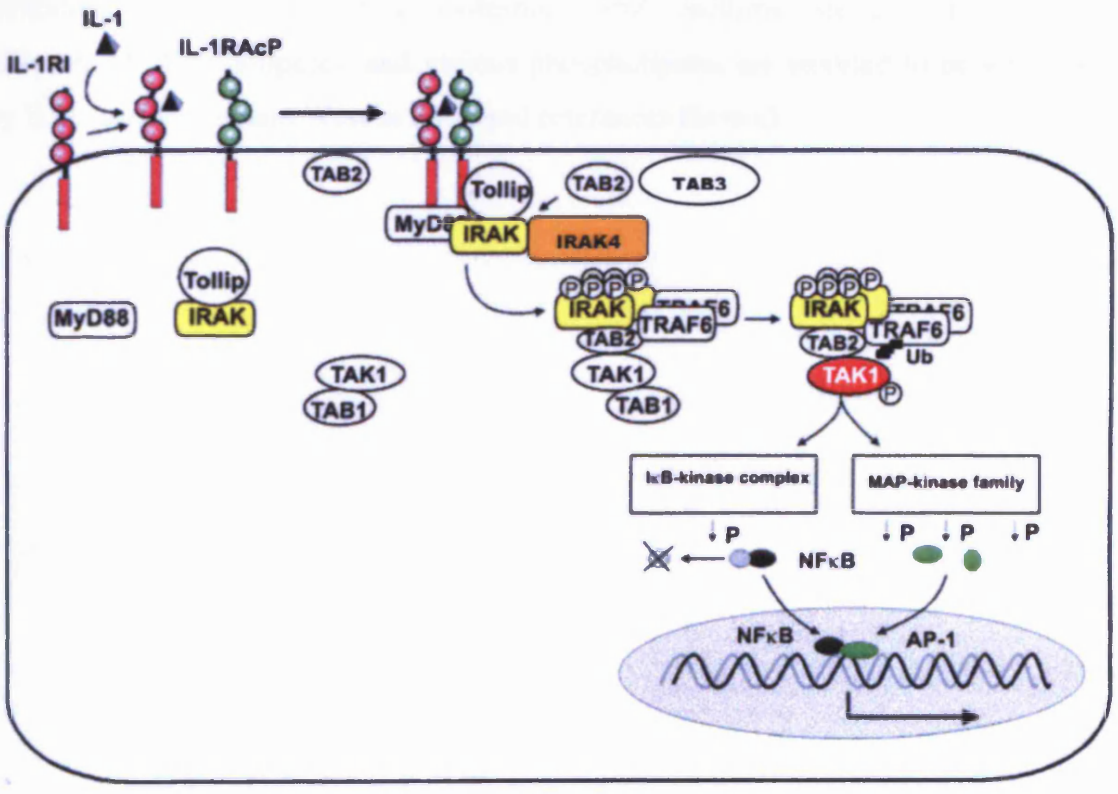



**FIGURE 1.7 A summary of the signalling events leading to the activation of MAPKs and NF-κB through TAK1 activation by IL-1 (adapted from Martin and Wesche 2002).** IL-1 binding to its receptor stimulates the formation of a receptor complex with IL-1RAcP. This results in the recruitment of MyD88, Tollip and IRAK-1 to the active receptor. In parallel, IRAK-4 is also recruited to the receptor complex where it initially phosphorylates IRAK-1 and then promotes its auto-phosphorylation. Hyperphosphorylated IRAK-1 dissociates from the receptor and may transiently interact with Pellinos (although not depicted here). The adapter protein TAB2 or possibly TAB3, is transported by IRAK1 from the membrane to the developing signalling complex. IRAK1 activates TRAF6 which becomes ubiquitinylated (Ub-TRAF6) and may polymerise. TAK1 is recruited to the complex with TAB1 and associates with TAB2/3. This leads to auto-phosphorylation of TAK1 which subsequently phosphorylates various regulatory kinases leading to the activation of NF-κB- and MAPK-dependent signalling pathways. 'P' denotes phosphate group.

### 1.4.5 OTHER SIGNALLING PATHWAYS ACTIVATED BY IL-1

Apart from the signalling pathways mentioned above, IL-1 is also known to activate a number of other signalling events but the coupling of these to the IL-1 receptor complex is not fully understood. One of these pathways involves Phosphatidylinositol-3 kinase (PI3K) and its downstream effector protein kinase B (PKB). IL-1 can also induce ceramide production by stimulating neutral and acid sphingomyelinases in various cell types. Protein kinase C isoforms have also been implicated in IL-1 signalling cascades, mainly with the use of pharmacological inhibitors targeting the various isoforms. PKC isoforms are also downstream effectors of phospholipases, and various phospholipases are reported to be activated by IL-1 (see Martin and Wesche 2002 and references therein).

## 1.5 IL-6 SIGNALLING PATHWAYS

IL-6 has many diverse functions; it not only regulates immune and inflammatory responses, but also plays a role in the regulation of hematopoiesis, neural development and bone metabolism (Stein and Kung Sutherland 1998). The human IL-6 protein contains 212 amino acids of which a 28 amino acid signal peptide is cleaved off resulting in the generation of a mature protein of 184 amino acids that can be post-translationally modified (Keller *et al.* 1996).

### 1.5.1 IL-6 RECEPTORS

There are two components to the IL-6 receptor complex, the non-signalling α-receptor (IL-6Rα) and the signal-transducing receptor component, gp130. IL-6 first binds to the IL-6Rα subunit, and only then can it recruit the gp130 signalling receptor subunits (Heinrich *et al.* 1998). A schematic representation the IL-6-bound receptor complex is shown in Figure 1.8.

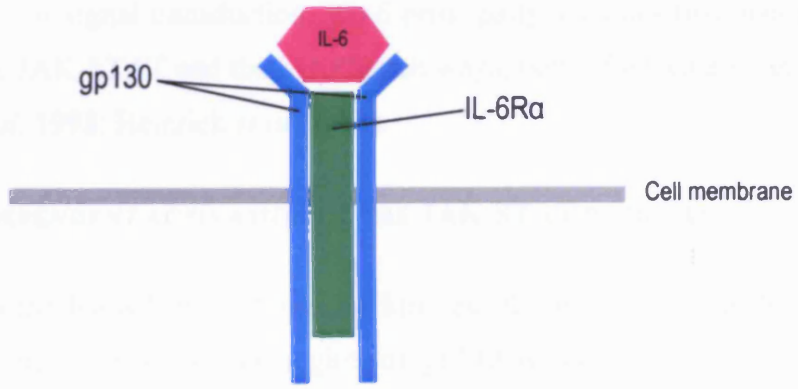



**FIGURE 1.8 A schematic representation of the IL-6 receptor complex.**

It seems that the function of the IL-6Rα subunit is to render cells sensitive to IL-6 because even though gp130 is ubiquitously expressed, the number of cell types that respond to IL-6 action is more limited. In accordance, the expression of IL-6Rα is restricted, limited to the cell membranes of leukocytes and hepatocytes in humans (Heinrich *et al.* 1998; Gabay 2006). However, IL-6 action can be potentiated with soluble receptors. Like the IL-1 receptors, the IL-6Rα can also exist as a soluble form, which lacks transmembrane and cytoplasmic segments. Soluble IL-6Rα can be shed from neutrophil membranes by limited proteolysis, for example. In contrast to

the soluble IL-1 receptors, that function to neutralise IL-1 action, soluble IL-6 receptors (sIL-6Rα) can act to enhance IL-6 action. The sIL-6Rα can combine with IL-6 and bind gp130 on cell membrane to activate cells by a mechanism termed '*trans*-signalling' (Gabay 2006). However, sIL-6Rα action can also be limited by a soluble form of gp130, which like sIL-6Rα is also detectable in human serum. Therefore, the balance between levels of IL-6 and soluble forms of IL-6Rα and gp130 can dictate the strength and duration of the IL-6-mediated response (Heinrich *et al.* 1998; Heinrich *et al.* 2003).

The extracellular domains of both IL-6Rα and gp130 contain fibronectin type III domains and an immunoglobulin-like domain. The fibronectin type III domains constitute the ligand-binding module. In each receptor the immunoglobulin-like domain is located N-terminally in close proximity with the ligand-binding module. In contrast with the IL-6Rα, gp130 contains three additional fibronectin type III domains and these domains are required for coupling ligand binding and signalling. The intracellular domain of gp130 contains binding/docking sites for various proteins that are necessary for signal transduction. IL-6 principally activates two major signalling pathways, the JAK-STAT and the MAPK pathways, both of which are detailed below (Heinrich *et al.* 1998; Heinrich *et al.* 2003).

### 1.5.2 IL-6-DEPENDENT ACTIVATION OF THE JAK-STAT PATHWAY

IL-6 activates the Janus family of tyrosine kinases otherwise known as the JAKs. The intracellular, membrane-proximal region of gp130 is bound to any of three JAKs; JAK1, JAK2 or Tyk2. The first event in the activation of the JAK-STAT pathway by IL-6 is cytokine-mediated homodimerisation of gp130 receptor subunits (Figure 1.8). Ligand-induced receptor dimerisation is believed to bring the gp130-associated JAKs into close proximity with one another, leading to their activation by phosphorylation. Their activation results in the subsequent phosphorylation of target tyrosine residues in the cytoplasmic tail of gp130. Phosphorylated tyrosine residues serve as docking sites for STATs, to which they are recruited and anchor via their src-homology 2 (SH2) domains. Subsequently, STATs also become tyrosine-phosphorylated, they dimerise (homo- or heterodimerisation) and translocate to the nucleus to their target genes. IL-6 action results in the predominant activation of STAT3 and to as lesser extent, STAT1. The phospho-tyrosine residue that is indicative of STAT1 activation

is tyrosine$^{701}$ and for STAT3 it is tyrosine$^{705}$. Additionally, both these transcription factors are also serine-phosphorylated (serine$^{727}$), in their C-terminal *trans*-activation domains and generally this is believed to be required for maximum transcriptional activation. A number of kinases have been suggested to regulate the serine-phosphorylation of STAT1 and STAT3 (Heinrich *et al.* 1998; Heinrich *et al.* 2003). In response to IL-6 action, STAT3 serine phosphorylation is reported to be regulated by components of the JNK MAPK pathway, specifically MKK4 and there is also evidence for PKCδ involvement (Jain *et al.* 1999; Schuringa *et al.* 2000; Schuringa *et al.* 2001). Figure 1.9 shows the sequence of events resulting in JAK-STAT pathway activation as mediated by IL-6.

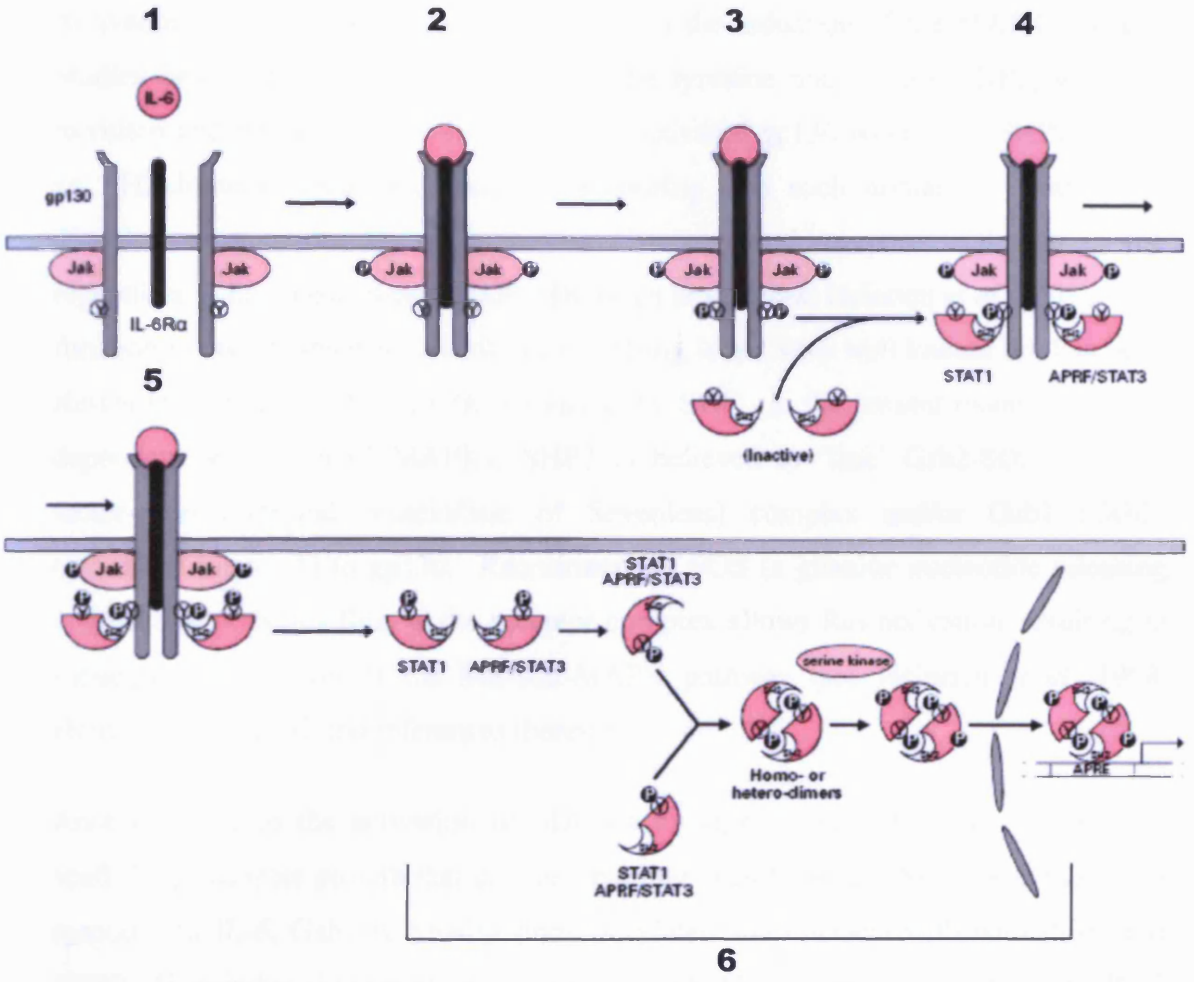



**FIGURE 1.9 IL-6 signalling via the JAK-STAT pathway (adapted from Heinrich *et al.* 1998).** IL-6 binding leads to the homodimersiation of gp130 receptor subunits and subsequent JAK activation (1-2). JAKs phosphorylate conserved tyrosine residues in the cytoplasmic tail of gp130, creating docking sites for the STATs (3). STATs are recruited to the receptor complex via their SH2 domains, which they use to bind the tyrosine-phosphorylated gp130 (4). Their binding results in their tyrosine-phosphorylation (5), subsequent dimerisation and translocation into the nucleus where they regulate gene expression (6). STATs are also substrates for serine kinases and both STAT1 and 3 are known to be phosphorylated at serine[727]. Abbreviations: APRF, acute-phase response factor; APRE, acute-phase response element; Y, tyrosine; P, phosphate group.

Negative regulation of the JAK-STAT pathway is controlled by two groups of inhibitory proteins called suppressors of cytokine signalling (SOCS) and protein inhibitors of activated STATs (PIAS). In addition, protein phosphatases are also involved in the negative regulation of the JAK-STAT pathway, of which the action of SHP2 (SH2-domain-containing tyrosine phosphatase) has been reported (see Heinrich *et al.* 2003 and references therein).

### 1.5.3 IL-6-DEPENDENT ACTIVATION OF MAPK PATHWAYS

Activation of the IL-6R-complex also leads to the induction of the MAPK cascade. Studies have indicated a requirement for the tyrosine phosphatase SHP2, which is recruited and phosphorylated (by JAK1) at activated gp130 receptors. SHP2 is also an SH2-domain containing protein, harbouring two such domains. Both these domains are required for its recruitment to activated receptors and also in the regulation of its phosphatase activity (Heinrich *et al.* 1998; Heinrich *et al.* 2003). The function of its phosphatase activity in signalling is not very well known but has been shown to be required for MAPK activation by EGF. In the present model of SHP2-dependent activation of MAPKs, SHP2 is believed to 'link' Grb2-SOS (growth-factor-receptor-bound protein/Son of Sevenless) complex and/or Gab1 (Grb2-associated binder-1) to gp130. Recruitment of SOS (a guanine nucleotide releasing protein that activates Ras) to the receptor complex allows Ras activation, resulting in subsequent activation of the Ras-Raf-MAPK pathway (see Heinrich *et al.* 1998; Heinrich *et al.* 2003 and references therein).

Another route to the activation of MAPKs by IL-6 is through Gab1. Gab1 is a scaffolding adapter protein that contains binding sites for Grb2, SHP2 and PI3K. In response to IL-6, Gab1 is tyrosine phosphorylated and interacts with both PI3K and SHP2. IL-6-induced association of Gab1 with SHP2 leads to the activation of ERK2 MAPK and Gap1 deficiency impairs MAPK activation by IL-6 in fibroblasts (Itoh *et al.* 2000; Heinrich *et al.* 2003). IL-6 action also leads to the activation of both p38 MAPK and upstream activators of the JNK MAPK module, although the pathways leading to their activation are less clear (Heinrich *et al.* 2003). Figure 1.10 summarises the signalling pathways associated with IL-6R activation.

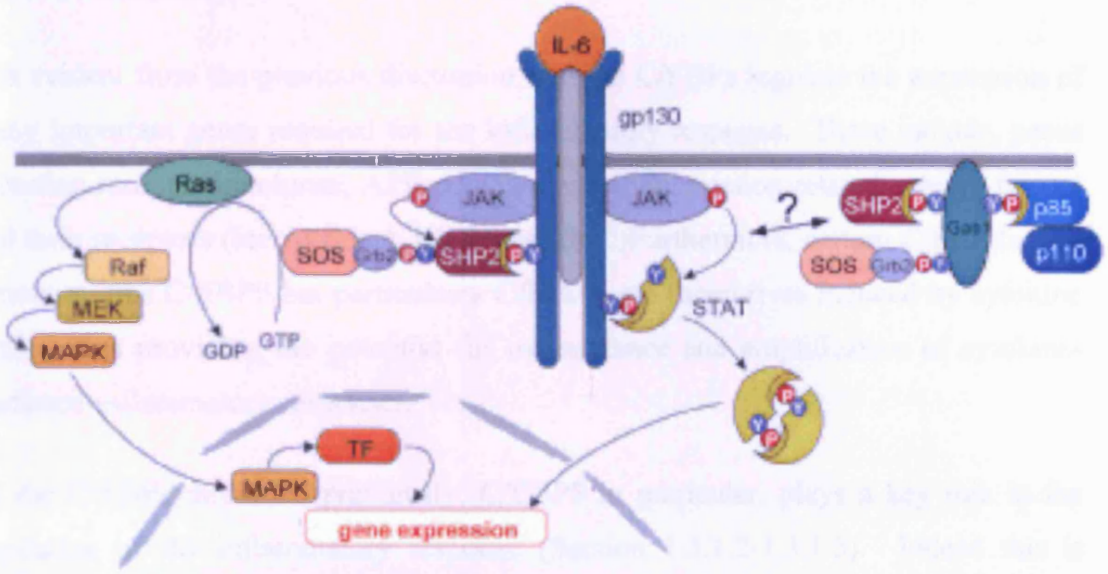



**Figure 1.10 A schematic representation of the known signalling pathways activated by IL-6 (taken from Heinrich *et al.* 2003).** IL-6 activates two principal pathways, the JAK-STAT pathway (detailed previously) and the MAPK cascade. Activation of JAKs can switch on both pathways, as detailed in the figure. A number of regulatory proteins are involved in the activation of the MAPK cascades by IL-6. Activation of this pathway is achieved either directly through SHP2 binding at the gp130 receptors, or by Gab1 activation. The precise link between Gab1 and the IL-6R complex is not clear and is flagged with ?. Gab1 activation also activates the PI3K (p85 and p110) pathway. Abbreviations; TF, transcription factor; Y, tyrosine; P, phosphate group.

## 1.6 AIMS OF STUDY

It is evident from the previous discussion that the C/EBPs regulate the expression of many important genes required for the inflammatory response. These include, genes encoding multiple cytokines, APR proteins and inflammation-related growth factors and their receptors (see section 1.3.1 for details). Furthermore, certain C/EBP family members, like C/EBPβ but particularly C/EBPδ, are themselves induced by cytokine action, thus providing the potential for maintenance and amplification of cytokine-mediated inflammatory responses.

Of the C/EBPs discussed previously, C/EBPδ in particular, plays a key role in the regulation of the inflammatory response (Section 1.3.1.2-1.3.1.5). Indeed this is further supported by recent evidence presented by Miyoshi and co-workers (2007), where C/EBPδ expression was documented to play a significant role in the regulation of inflammatory gene expression (specifically, IL-6, MCP-1 and COX-2) in a model of glomerulonephritis, an inflammatory disease that affects kidney function. Moreover, given its key role in the regulation of inflammatory gene expression, C/EBPδ expression was also examined in brain tissue of Alzheimer's disease sufferers, since this disease also has an associated inflammatory component to it (Li *et al.* 2004). Significantly increased levels of C/EBPδ were found in brain tissue from Alzheimer's patients, compared to controls. Levels were particularly increased in the vicinity of amyloid-β deposits which were surrounded by reactive astrocytes.

Despite its significance during the inflammatory reaction, the molecular mechanisms by which C/EBPδ is itself induced under such conditions are largely unknown. Previous studies have pointed to an important role of the transcription factor STAT3 in the regulation of C/EBPδ expression by IL-6 (Yamada *et al.* 1997; Cantwell *et al.* 1998). However, investigations in these studies are restricted to the analysis of C/EBPδ gene promoter activation by this cytokine in transfection based assays and have not investigated a role for STAT3 in the regulation of endogenous C/EBPδ gene expression by IL-6. Furthermore, to our knowledge no study has identified the transcription factors regulating C/EBPδ expression in response to other key inflammatory cytokines like IL-1 for example.

Given that C/EBPδ expression is likely to have an impact on inflammatory conditions, like the ones mentioned above, and because the mechanisms underlying its expression are largely unknown, we decided to investigate its expression and the mechanism(s) regulating its expression in response to inflammatory stimuli, such as IL-1 and IL-6.

Numerous studies have reported that a large number of inflammatory mediators, including the cytokines IL-1 and IL-6 strongly induce the expression of C/EBPδ, particularly in hepatocytes, the key cells involved in the production of APPs during the APR (Section 1.3.1.2). Therefore because of their significance in the inflammatory response, we decided to use these cells as a model for our studies. Our initial investigations were aimed at determining the effects of various inflammatory mediators on C/EBPδ expression in these cells, with focus on IL-1. In conjunction, because preliminary results from our laboratory suggested that the expression of C/EBPδ as induced by IL-1 occurred at the level of gene transcription (Dr F.T. Kockar, personal communication), similar to its regulation by IL-6 (Ramji *et al.* 1993a; Yamada *et al.* 1997; Cantwell *et al.* 1998), the initial aims of this project were to:

1) Fully delineate the regulatory elements in the human C/EBPδ gene promoter responsible for IL-1-induced C/EBPδ gene transcription

2) Identify the nuclear factors responsible for the regulation of this response

In conjunction with these aims, we also carried out investigations to determine whether the human C/EBPδ gene was subject to auto-regulation, similar to its rat, mouse and ovine counterparts, given that this area of investigation has gone unreported and also because this mode of gene regulation is likely to play a significant role in modulating C/EBPδ expression during the inflammatory response (Chapter 3).

Further to our initial studies presented in chapter 3, the preliminary aims of this project were revised slightly. More specifically, this was because in contrast to the endogenous gene, the human C/EBPδ gene promoter was not activated by IL-1, in spite of the fact it was shown to be responsive to IL-6 action consistent with previous findings (Yamada *et al.* 1997; Cantwell *et al.* 1998). Similar results were also obtained on analysis of the murine C/EBPδ gene promoter in response to IL-1 and IL-

6 action. Therefore, as a result of this outcome, we decided to change the focus of the project slightly.

Still remaining consistent with the overall objective of the project, we decided to utilise an alternative approach into determining the molecular mechanisms by which C/EBPδ is regulated in response to IL-1 by:

1) Investigating the signal transduction pathways leading to the induction of C/EBPδ expression by IL-1 (Chapter 4-6)

2) Characterising the signalling components that are involved in these pathways (Chapter 7)

Simultaneously, some investigations were also carried out to determine the identity of transcription factor(s) regulating C/EBPδ expression by IL-6 as previous studies (Yamada *et al.* 1997; Cantwell *et al.* 1998) have not extended their investigations to analysing the expression of the human C/EBPδ gene in response to this cytokine (Chapters 3 and 6).

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1 SUPPLIERS

Table 2.0 details materials used during the course of this study and the suppliers from which they were purchased.

**TABLE 2.0 A list of materials and chemicals used in this study with their suppliers.**

| MATERIALS | SUPPLIER |
|---|---|
| Apigenin | Alexis Biochemicals, Nottinghamshire, UK |
| Negative/Scrambled control siRNA | Ambion, Cambridgeshire, UK |
| $\gamma$-$^{32}$P-ATP<br>$\alpha^{32}$P-dCTP<br>ECL Western Blotting Detection Reagent<br>Megaprime DNA Labelling Kit<br>Nick Columns<br>Rainbow Protein Size Markers<br>Random Hexamers ($PdN_6$)<br>X-ray Film | Amersham, GE Healthcare, Buckinghamshire, UK |
| Acrylamide: Bisacrylamide (29:1)<br>Acrylamide: Bisacrylamide (37.5:1) | Anachem, Luton, UK |
| Agarose<br>PCR Grade Magnesium Chloride<br>Taq DNA Polymerase (and 10x Reaction Buffer) | Bioline, London, UK |
| DTT | Boehringer Mannheim, East Sussex, U.K. |
| Bisindolylmaleimide<br>Calphostin C<br>Curcumin<br>LY294002<br>NF-κB Activation Inhibitor<br>PD98059<br>SB202190<br>SB203580<br>SP600125 | Calbiochem, Nottingham, UK |
| DharmaFECT™ | Dharmacon RNA Technologies, USA |
| LB Agar Capsules<br>LB Medium Capsules | DIFCO Laboratories, Surrey, UK |
| Hep3B Cell Line<br>THP-1 Cell Line | European Collection of Animal Cell Cultures (ECACC), Salisbury, UK |
| EDTA<br>Ethanol<br>Hydrochloric Acid<br>Industrial Methylated Spirits<br>Isopropanol<br>Methanol<br>Sodium Chloride<br>Sodium Dodecyl Sulphate<br>Sodium Hydroxide<br>Tris-Base<br>Other General Chemicals | Fisher Scientific, Loughborough, UK |
| Saran Wrap | Genetic Research Instrumentation, Essex, UK |
| Ammonium Persulphate<br>DMEM with GlutaMAX™ | Gibco BRL, Paisley, UK |

| | |
|---|---|
| Fetal Calf Serum<br>2-Mercaptoethanol<br>Trypsin/EDTA | |
| 1ml Cryo-vials<br>96 Well Plates<br>Falcon 15ml and 50ml Polypropylene Tubes | Greiner, Gloucestershire, UK |
| Cell Scrapers<br>Tissue Culture Flasks | Helena Biosciences, Sunderland, UK |
| High Purity Plasmid Maxi Prep System | Marligen Biosciences, High Wycombe, UK |
| PVDF membrane | Millipore Ltd., Gloucestershire, UK |
| DNA Molecular Weight Markers<br>Restriction Endonucleases<br>Anti-mouse HRP Conjugate<br>Anti-p50/p105 NF-κB<br>Anti-p65 NF-κB<br>Anti-rabbit HRP Conjugate<br>Anti-SAPK/JNK<br>Anti-SAPK/JNK pThr183/Tyr185<br>Anti-STAT1<br>Anti-STAT1 pTyr701<br>Anti-STAT3<br>Anti-STAT3 pTyr 705<br>Anti-STAT3 pSer727<br>SAPK/JNK Kinase Assay Kit (Non Radioactive) | New England Biolabs, Hertfordshire, UK |
| Phosphate Buffered Saline Tablets | Oxoid Ltd., Basingstoke, UK |
| Micro BCA Protein Assay Kit | Pierce, Chester, UK |
| dNTPs<br>MMLV Reverse Transcriptase (and MMLV 5x Buffer)<br>Passive Lysis Buffer (5x)<br>RNasin™<br>Wizard SV™ Miniprep Kit | Promega, Southampton, UK |
| SuperFECT™<br>QIAquick™ Gel Extraction Kit<br>RNeasy™ Total RNA Isolation Kit (Mini and Micro) | Qiagen, Crawley, UK |
| Anti-C/EBPδ (M17)X<br>Anti-CK2 α and α'<br>Anti-c-JUN | Santa-Cruz Biotechnology Inc., California, USA |
| Tissue Culture Filters (0.2μm) | Schleicher and Schuell, London, UK |
| DNA Oligonucleotides | Sigma Genosys, Cambridgeshire, UK |
| Anti-β-Actin<br>Ampicillin<br>Aprotonin<br>Benzamidine<br>Bovine Serum Albumin<br>Bromophenol Blue<br>Ethidium Bromide<br>DMSO Molecular Biology Grade<br>Ficoll (Type 400)<br>Glycerol<br>Leupeptin<br>Lipopolysaccharide<br>Molecular Biology Grade Water<br>PEI 25kDa<br>Penicillin/Streptomycin<br>PMA<br>PMSF<br>Sodium Fluoride<br>Sodium Orthovanadate<br>Sodium Pyrophosphate | Sigma, Poole, UK |

| | |
|---|---|
| TBE (x10)<br>TEMED<br>Tissue Culture Grade Water<br>Tissue Culture Grade DMSO<br>Triton X-100<br>Trypan Blue Solution<br>Tween 20<br>Zinc Chloride | |
| Human IFN-γ<br>Human IL-1β<br>Human IL-6<br>Human TGF-β | Totam Biologicals (Tebu Bio), Cambridgeshire, UK |

## 2.2 PREPARATION OF GLASS AND PLASTICWARE

Glass or plastic-ware for the isolation and manipulation of DNA, RNA or protein was autoclaved for 20min at 121°C, at a pressure of 975kPa. Solutions were also autoclaved under the same conditions.

## 2.3 TISSUE CULTURE

All reagents used in tissue culture were pre-warmed to 37°C for at least 20min and subsequently sterilised with 70% (v/v) ethanol prior to use.

### 2.3.1 CELL LINES

The cell lines used for experiments detailed in this study were: the human hepatoma cell line, Hep3B and the human monocytic cell line, THP-1.

#### 2.3.1.1 MAINTENANCE OF CELL LINES IN CULTURE

The Hep3B cells were grown in DMEM with GlutaMAX™ (stabilized L-glutamine) and the THP-1 cell line was grown in RMPI-1640 with GlutaMAX™ cell culture medium. Medium was supplemented with 10% (v/v) heat-inactivated (30min, 56°C) fetal calf serum (HI-FCS), penicillin (100U/ml) and streptomycin (100μg/ml) (hereafter referred to as complete medium). Supplemental reagents were passed through a 0.2μm sterile filter prior to addition to the culture medium. The cells were maintained at 37°C in a humidified incubator with a 5% (v/v) $CO_2$ atmosphere. The cell culture medium was replaced twice a week. It is noteworthy, that no cells beyond passage 10 of growth were used in any experiments.

### 2.3.1.2 SUBCULTURING OF CELL LINES

Confluent (70%) Hep3B cells were washed once with DMEM with GlutaMAX™. The cells were then treated with trypsin/EDTA solution and incubated at 37°C, 5% (v/v) $CO_2$ until the cells were visibly detached from the substratum. Only a minimum volume of trypsin/EDTA adequate to cover the cell monolayer was used for trypsinisation. Once detached, the cells were then resuspended in fresh DMEM complete medium and centrifuged at 800x*g* for 5min. The cell pellet were then resuspended in an appropriate volume of fresh DMEM complete medium and seeded at a ratio of 1:6 (i.e. cells from one tissue culture flask were subcultured into six new flasks of the same size).

Confluent THP-1 cells (70%) were split at a ratio of 1:4, by diluting the cells into fresh RPMI-1640 complete medium.

### 2.3.1.3 PRESERVING AND STORING OF CELL LINES

Only cells of an early passage (between 2-4) were used for long term storage. Cell suspensions (prepared as described above) were pelleted by centrifugation at 800x*g* for 5min. Following this, the cell pellets were resuspended in an appropriate volume of HI-FCS containing 10% (v/v) DMSO (Hep3B) or containing 10% (v/v) sterile glycerol (THP-1). These cell suspensions were dispensed as 1ml aliquots into sterile cryo-vials and insulated with polystyrene. The cells were then frozen at -80°C overnight. Following the overnight freeze, the cells were placed into liquid nitrogen for long-term storage.

### 2.3.1.4 THAWING FROZEN CELLS

For thawing cells, cryo-vials were removed from liquid nitrogen storage and incubated at 37°C until their contents had thawed completely. The content of each vial was added to 15ml of HI-FCS and centrifuged at 800x*g* for 5min. Following centrifugation, the HI-FCS was aspirated from the cell pellet, which was then resuspended in an appropriate volume of DMEM or RMPI-1640 complete medium depending on the cell line. The cells were then seeded into tissue culture flasks and incubated at 37°C [5% (v/v) $CO_2$].

### 2.3.1.5 TREATMENT OF CELLS WITH CYTOKINES

In the case of Hep3B cells prior to cytokine treatment, the medium was aspirated from the cells and replaced with an appropriate volume of fresh DMEM complete medium. Cytokines were added directly to the culture medium, at various concentrations depending on the nature of the experiment. Routinely, IL-6 was added at 1000U/ml, IL-1β at 100U/ml and IFN-γ at 100U/ml. Treated cells were incubated at 37°C [5% (v/v) $CO_2$] and harvested at indicated time points for experimental analysis.

### 2.3.1.6 TREATMENT OF CELL LINES WITH PHARMACOLOGICAL INHIBITORS

Pharmacological inhibitors were prepared according to the manufacturer's instructions (Alexis Biochemicals, Calbiochem). The majority of pharmacological agents were dissolved in DMSO, unless otherwise stated.

Prior to treatment of Hep3B cells, the medium was aspirated from the cells and replaced with an appropriate volume of fresh DMEM complete medium and the cells returned to the incubator [37°C, 5% (v/v) $CO_2$] for 30min. For THP-1 monocytes, approximately 24h prior to treatment, confluent cells were diluted with RPMI-1640 complete medium, seeded into two new flasks and incubated [37°C, 5% (v/v) $CO_2$] overnight. On the day of treatment, the contents of both flasks was combined and centrifuged at 800x*g* for 5min, the medium aspirated, and the resultant cell pellet resuspended in an appropriate volume of RPMI-1640 complete medium. The cells were then plated into new flasks at a ratio of 1:2 and incubated for a further 4h [37°C, 5% (v/v) $CO_2$], at which point they were ready for inhibitor treatment.

Inhibitors were added directly to the culture medium, at various concentrations depending on the nature of the experiment. As a control, the cells were also treated with an appropriate volume of vehicle that was used to dissolve the inhibitor, typically DMSO. In all experiments the cells were incubated [37°C, 5% (v/v) $CO_2$] with the inhibitors for 1h (to ensure their uptake and action), after which they were treated with an appropriate cytokine [or PMA (0.16μM) in the case of THP-1 cells] and incubated further. Following this, the cells were harvested at indicated time points for experimental analysis.

#### 2.3.1.7 TRYPAN BLUE EXCLUSION ASSAY

Trypan blue solution [0.4% (w/v)] was added to the cell culture medium at a ratio of 1:100 (v/v) and incubated at 37°C (5-10min). The medium was then aspirated from the cells and the cells were washed once with phosphate-buffered saline (PBS). The number of cells taking up the blue stain was estimated by counting those in a representative area using phase contrast microscopy. The proportion of dead cells was calculated from this.

## 2.4 DNA TRANSFECTIONS

### 2.4.1 POLYETHYLENIMINE TRANSFECTION (PEI)

Approximately 48h prior to transfection, Hep3B cells were split 1:6 (Section 2.3.1.2) and seeded equally into 6-well plates, so that on the day of transfection the cells were between 40-60% confluent. Prior to transfection, the medium was changed to fresh complete medium (2ml/well of plate). Plasmid DNA (2.5-12.5μg) was diluted to a volume of 10μl in 5% (w/v) glucose. Polyethylenimine (PEI) solution (5.625mg/ml, pH 7.2) (1.5μl) was added to the DNA, mixed vigorously by pipetting and the resulting complex immediately suspended in 1ml of DMEM complete medium, after which it was added dropwise to cells. The cells were then incubated overnight [37°C, 5% (v/v) $CO_2$] after which they were washed twice in DMEM with GlutaMAX™ and the medium replaced with fresh DMEM complete medium. At this stage, if necessary the mediator in question was added to the cells and incubated further for the requisite time period.

### 2.4.2 PREPARATION OF CELL EXTRACTS FOR THE DETERMINATION OF REPORTER GENE ACTIVITY

The medium was aspirated from the cells and replaced with PBS. The PBS was then aspirated from the cells and 200μl of 1x passive lysis buffer (Promega) was added directly to the cell monolayer and left to incubate for 15min at room temperature. The cells were then scraped into the buffer, transferred into a microcentrifuge tube, vortexed for 45s and centrifuged at 12,000x*g* for 2min. The supernatant was transferred into a fresh tube and was either stored at -80°C or used immediately for the measurements of luciferase activity.

### 2.4.3 MEASUREMENT OF LUCIFERASE ACTIVITY

The luciferase activity of samples was determined as described by the manufacturer (Promega). Briefly, 20µl of cell extracts (Section 2.4.2) were mixed, at room temperature, with 100µl of luciferase assay buffer (Promega) in a luminometer tube. The light output for each sample was determined using a luminometer (TD-20/20 Turner Designs, California, USA). The luminometer was set at a 45% sensitivity value, with a 2s delay period and a 20s integrate period. All measurements were taken in duplicate and the background values were subtracted from the sample values. Also note that the total protein concentration of each sample was determined (Section 2.7.4). Protein concentrations were then used to analyse the data generated from transfection studies. That is, the luciferase counts were normalised to the protein concentration (µg/µl) in each sample.

### 2.4.4 PLASMIDS

During this study, several DNA constructs were used to analyse promoter activity and to investigate various signalling pathways. Many of these constructs were kind gifts from various laboratories, the details of which are discussed briefly below. Where applicable, an appropriate reference is also provided. Plasmid maps for all expression and reporter vectors can be found in Appendix I.

#### *2.4.4.1 pHuC/EBPδ[1.6kb]-Luc*

A 1.6kb region of the human *C/EBPδ* promoter upstream of the transcriptional start site was cloned into the luciferase reporter vector pLUC (Cleutjens *et al.* 1993). This plasmid was obtained from Dr J Trapman (Erasmus University, Netherlands).

#### *2.4.4.2 pHuC/EBPδ[0.2kb]-Luc*

A 0.2kb region of the human *C/EBPδ* promoter upstream of the transcriptional start site was cloned into the pGL2-Basic luciferase reporter vector. This plasmid was created by Dr F.T. Kockar (Balikesir University, Turkey).

#### *2.4.4.3 pMoC/EBPδ[2.2kb]-Luc*

A 2.2kb region of the murine *C/EBPδ* promoter (-2146 to +73) site was cloned into the pGL2-Basic luciferase reporter vector (O'Rourke *et al.* 1999a; Hutt *et al.* 2000).

#### *2.4.4.4 pNFκB-Luc*

This plasmid was obtained from Dr D. Leake (University of Reading, U.K) and is based on the luciferase reporter vector pTAL-Luc (Clontech) containing an insert of four NF-κB consensus enhancer elements.

#### *2.4.4.5 SAPKα-VPF*

This expression plasmid encodes a SAPK/JNK protein, in which the normally conserved phosphorylation motif of Thr-Pro-Tyr is altered to Val-Pro-Phe, producing an inactive, dominant negative SAPK/JNK protein. This plasmid was a kind gift from E. Nishida (Kyoto University, Japan) (Moriguchi *et al.* 1995).

#### *2.4.4.6 D/N c-JUN/TAM67*

This expression plasmid encodes a dominant-negative mutant of c-Jun that dimerises with endogenous c-Jun and c-Fos. Thus, this mutant is a potent inhibitor of AP-1 mediated *trans*-activation. The mechanism of action of this dominant negative mutant has been discussed by Brown *et al.* (1994).

#### *2.4.4.7 D/N CK2α-K68A*

This expression plasmid encodes a kinase inactive mutant of the CK2α catalytic subunit in which Lys68 has been substituted for Ala in the ATP-binding domain of this subunit. This plasmid was a generous gift from Drs E.M. Chamburg and C Cochet (INSERM, Grenoble, France) (Lebrin *et al.* 2001).

#### *2.4.4.8 IκB Super-Repressor*

This expression plasmid contains a cDNA insert that encodes the human IκBα protein, which acts to inhibit NF-κB signalling when over-expressed. This plasmid was a kind gift from Dr D. Krappman (Berlin, Germany) (Krappmann *et al.* 1996).

#### *2.4.4.9 MSVβ*

This expression plasmid contains a cDNA insert that encodes the wild-type murine C/EBPβ protein. This plasmid was a kind gift from Prof. S.L. McKnight (Carnegie Institution of Washington, Baltimore, U.S.A) (Cao *et al.* 1991).

#### *2.4.4.10 MSVδ*

This expression plasmid contains a cDNA insert that encodes the wild-type murine C/EBPδ protein. This plasmid was a kind gift from Prof. S.L. McKnight (Carnegie Institution of Washington, Baltimore, U.S.A) (Cao *et al.* 1991).

#### *2.4.4.11 pCS2xα*

This expression plasmid contains a cDNA insert that encodes the wild-type *Xenopus laevis* C/EBPα protein. This plasmid was created by Dr S. Kousteni (Cardiff University, U.K) (Kockar *et al.* 2001).

## 2.5 SMALL INTERFERING RNA (SIRNA) TRANSFECTIONS

All siRNA transfections were carried out using validated siRNAs against target mRNAs obtained from Qiagen and Invitrogen, the details of which are presented in Tables 2.1 and 2.2 respectively. Qiagen retains the right to withhold the sequences of its validated siRNAs and instead provides customers with an 'SI' reference number that identifies every siRNA. As every SI reference number is unique, this can be used to identify siRNAs required for publication purposes (Dr K. Brennan, Qiagen, personal communication). Therefore in Table 2.1, for siRNAs ordered from Qiagen no sequence is provided, in place the SI reference number for each siRNA is specified. Invitrogen provides its customers with two validated siRNA duplexes for each target mRNA, the sequences of which are presented in Table 2.2. It is noteworthy that in all experiments presented in thesis, involving the use of these siRNAs, the results have been verified with the use of both the duplexes presented.

siRNA stock solutions were prepared according to the manufacturer's instructions (Qiagen and Invitrogen) from lyophilised siRNAs.

**TABLE 2.1 Details of validated siRNAs used in this study as ordered from Qiagen.**

| TARGET TRANSCRIPT(S) | NCBI ACCESSION NUMBER(S) OF TARGET(S) | GENE SYMBOL | SI REFERENCE NUMBER/CATALOGUE NUMBER† |
|---|---|---|---|
| CK2α' | NM_001896 | CSNK2A2 | SI00605409 |
| c-JUN | NM_002228 | JUN | SI00300580 |
| JNK1 | NM_002750<br>NM_139046<br>NM_139047<br>NM_139049 | MAPK8 | SI02758637<br>1022515† |
| JNK2 | NM_002752<br>NM_139068<br>NM_139069<br>NM_139070 | MAPK9 | SI02222920<br>1022632† |
| TAK1 | NM_003188<br>NM_145331<br>NM_145332<br>NM_145333 | MAP3K7 | SI02758756 |
| p65 NF-κB | NM_021975 | RELA | SI02663094 |
| p50 NF-κB | NM_003998 | NFKB1 | SI02654932 |

† Where SI reference numbers were not available, the unique catalogue number identifying the specified siRNA is provided.

**TABLE 2.2 Details of validated siRNAs used in this study as ordered from Invitrogen.**

| TARGET TRANSCRIPT(S) | GENE SYMBOL | NCBI PRIMARY ACCESSION NUMBER | siRNA TARGET LOCATION BASED ON ACCESSION NUMBER | TARGET SEQUENCE (5' → 3') |
|---|---|---|---|---|
| CK2α | CSNK2A1 | NM_001895.3 | 650<br>761 | Duplex 1 5'-ACCAGACGUUAACAGACUAUGAUAU-3'<br>Duplex 2 5'-UCAUGAUUGAUCAUGAGCACAGAAA-3' |
| p38 MAPK | MAPK14 | NM_139012 | 758<br>691 | Duplex 1 5'-CCAAAUUCUCCGAGGUCUAAAGUAU-3'<br>Duplex 2 5'-GGGCAGAUCUGAACAACAUUGUGAA-3' |
| STAT1 | STAT1 | NM_007315.2 | 608<br>882 | Duplex 1 5'-GCAAGCGUAAUCUUCAGGAUAAUUU-3'<br>Duplex 2 5'-GCAGAACAGAGAACACGAGACCAAU-3' |
| STAT3 | STAT3 | NM_139276 | 453<br>501 | Duplex 1 5'-CCUGCAAGAGUCGAAUGUUCUCUAU-3'<br>Duplex 2 5'-GCAGUUUCUUCAGAGCAGGUAUCUU-3' |

### 2.5.1 siRNA TRANSFECTIONS USING DHARMAFECT™

Approximately 48h prior to transfection, Hep3B cells were split 1:6 (Section 2.3.1.2) and seeded equally into 24-well or 6-well culture plates depending on the experiment. Following an overnight incubation [37°C, 5% (v/v) $CO_2$], the medium was changed to DMEM complete medium with no antibiotics and the cells were left to incubate [37°C, 5% (v/v) $CO_2$] for a further 24h. Having reached approximately 40% confluence, Hep3B cells were ready to transfect.

The following procedure was carried out for the transfection of cells in one $2cm^2$ cell culture dish. For multiple transfections, the volumes detailed in the protocol below were multiplied by the number of desired transfections. For co-transfections, involving the use of two siRNAs together and for transfections involving different cell culture formats the appropriate volumes of reagents required for transfections are shown in Table 2.3.

On the day of transfection, the medium was aspirated from the cells and replaced with 1ml of DMEM complete medium, containing no antibiotics. Essentially, the transfection protocol detailed below is as described by the manufacturer, with minor modifications (Dharmacon RNA Technologies). A 2.5μM siRNA solution [siRNA was diluted in an appropriate volume of RNase-free water or siRNA suspension buffer (Qiagen)] was prepared. In a sterile tube (Tube 1 in Table 2.3), 2.5μl of 2.5μM siRNA solution was diluted with 47.5μl of HI-FCS-free and antibiotic-free DMEM medium. In a separate tube (Tube 2 in Table 2.3), 0.5μl of DharmaFECT™ reagent (Dharmacon RNA Technologies) was diluted in 49.5μl of HI-FCS-free and antibiotic-free DMEM medium. The content of each tube was mixed thoroughly by pipetting, before being left to incubate at room temperature for 5min. Subsequently, the content of each tube was combined, mixed and this 'transfection-mix' was incubated for a further 20min at room temperature. The resultant transfection-mix was added (100μl, dropwise) to the cells in the $2cm^2$ culture dish and the cells were returned to incubate [37°C, 5% (v/v) $CO_2$] for approximately 65h. By observing this protocol, the approximate concentration of siRNA in a $2cm^2$ culture dish (containing 1ml of medium) is 6.25nM. Following the incubation period cells were harvested for RNA (Section 2.6.2) or protein extraction (Section 2.7.1). Alternatively, where appropriate, the medium was aspirated from the cells and replaced with DMEM complete medium

without antibiotics. The cells were then treated with the appropriate mediator, incubated further for the requisite time period, before being harvested for RNA (Section 2.6.2) or protein extraction (Section 2.7.1).

**TABLE 2.3 Volumes of reagents required for various siRNA transfections in different cell culture formats.**

| REAGENT | $2CM^2$ CULTURE DISH | $2CM^2$ CULTURE DISH (CO-TRANSFECTION) | $9.5CM^2$ CULTURE DISH | $9.5CM^2$ CULTURE DISH (CO-TRANSFECTION) |
|---|---|---|---|---|
| **DMEM complete medium (minus antibiotics) on cells (ml)** | 1 | 1 | 2 | 2 |
| **siRNA at 2.5µM (µl) TUBE 1** | 2.5 | 2.5 (siRNA 1)<br>+<br>2.5 (siRNA 2) | 5 | 5 (siRNA 1)<br>+<br>5 (siRNA 2) |
| **HI-FCS- and antibiotic-free DMEM medium (µl) TUBE 1** | 47.5 | 45 | 45 | 40 |
| **DharmaFECT™ (µl) TUBE 2** | 0.5 | 0.5 | 1 | 1 |
| **HI-FCS- and antibiotic-free DMEM medium (µl) TUBE 2** | 49.5 | 49.5 | 49 | 49 |
| **Volume of transfection-mix applied to cells (µl)** | 100 | 100 | 100 | 100 |
| **Final siRNA concentration (nM)** | 6.25 | 12.5 | 6.25 | 12.5 |

siRNA 1 and siRNA 2 denote the different siRNAs used in combination in the co-transfection.

## 2.6 RNA/DNA RELATED TECHNIQUES

### 2.6.1 TOTAL RNA ISOLATION USING RNEASY™ MINI KIT (QIAGEN)

Total RNA was isolated from cells using the RNeasy™ Total RNA Isolation kit (Qiagen) according to the instructions supplied by the manufacturer. Briefly, the medium was aspirated from the cells and the cells were then washed once in PBS.

The cells were then scraped into the PBS, transferred to a 15ml polypropylene tube and centrifuged at 1000x*g* for 5min. The resulting supernatant was discarded and the cell pellet was thoroughly resuspended in 350-600µl of buffer RLT (containing 10µl/ml β-mercaptoethanol) supplied in the kit. This lysate was then homogenised by passing it through a 0.9mm needle attached to a 1ml syringe (5 times). At this stage, the lysate was either stored at -80°C for use at a later date, or immediately used for RNA extraction. The integrity of the RNA was determined by resolving 1µg on a 1% (w/v) agarose gel (see Section 2.6.4.1 for details). The purity and concentration of the RNA was assessed by measuring $O.D._{260}$ and $O.D._{280}$ using a U-1800 Hitachi spectrophotometer.

### 2.6.2 TOTAL RNA ISOLATION USING RNEASY™ MICRO KIT (QIAGEN)

For small scale preparations of total RNA the RNeasy™ Micro Total RNA Isolation Kit (Qiagen) was used according to the manufacturer's instructions. Briefly, Hep3B cells were washed once with PBS, which was then aspirated from the growing cells. At this stage, 350µl of Buffer RLT was added directly to the cell monolayer (growing in a $2cm^2$ cell culture dish). The resultant lysate was collected and passed through a Qiagen QI shredder column for homogenisation. At this stage, the total RNA isolation procedure was carried out exactly as described by the manufacturer (Qiagen) or the lysate was stored at -80°C for use at a later date.

### 2.6.3 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR can be separated into two steps:

1. Synthesis of cDNA from RNA using reverse transcriptase (RT).
2. Polymerase chain reaction (PCR), with cDNA from step 1 using gene-specific primers to amplify a product of interest.

#### 2.6.3.1 CDNA SYNTHESIS (RT)

cDNA was synthesised from 1µg of total cellular RNA (isolated as described in Section 2.6.1 and Section 2.6.2). Briefly, to 1µg of total RNA was added, 1µl of (200pmol, $PdN_6$) random hexameric primers and molecular biology grade water to achieve final volume of 13.5µl. Samples were then incubated for 5min at 70°C and then quenched on ice. Subsequently, 4µl of 5× Moloney murine leukaemia virus

(MMLV) reverse transcriptase buffer (Promega), 1µl of dNTP mix (10mM each of dATP, dGTP, dTTP and dCTP), 0.5µl of 50U/µl recombinant RNase inhibitor (RNasin®) and 1µl of MMLV reverse transcriptase (200U/µl) was added to each reaction. Samples were then incubated for 1h at 37°C and the reaction was terminated by incubation at 94°C for 2min. The synthesised cDNA was diluted 5-fold by the addition of 80µl nuclease-free $H_2O$. cDNA prepared in this way was stored at -20°C or used for PCR analysis (Section 2.6.3.2).

### 2.6.3.2 POLYMERASE CHAIN REACTION (PCR)

PCR conditions were specific for each primer pair used to amplify various products. Table 2.4 documents the PCR primer sequences, the expected PCR product size for each primer pair and where applicable, the references from which primer sequences were obtained. The reaction conditions required for the amplification of the various PCR products analysed during this study are specified in Table 2.5. It is noteworthy, that PCR conditions and cycles of amplification were optimised for each primer set so that products were generated in the exponential phase of amplification. That is, there was a direct correlation between the amount of product and the amount of the original cDNA template used. Temperature cycling was carried out on either a Techne Thermal Cycler or a Peltier Thermal Cycler (PTC-200).

**TABLE 2.4 Sequences of forward and reverse primers used to PCR-amplify specified products and their sizes in base pairs (bp).**

| GENE (REFERENCE) | FORWARD PRIMER (5'-3') | REVERSE PRIMER (5'-3') | PCR PRODUCT SIZE (BP) |
|---|---|---|---|
| Hu β-actin (Sabatakos *et al.* 1998b) | ATGATATCGCCGCGCTCG | CGCTCGGTGAGGATCTTCA | 580 |
| Hu β-2-microglobulin | TTTCTGGCCTGGAGGCTATC | CATGTCTCGATCCCACTTAACT | 314 |
| Hu C/EBPδ | GCGCGAGCGCAACAACATC | CCAGGTCCCGCGTGAGCT | 143 |
| Hu CK2α | TGAGCTACTTGTAGACTATCA | CTGAAGAAATCCCTGACATCA | 473 |
| Hu CK2α' | GAGCTGGGGTAATCAAGATG | TAGAATTCTGCCAGACCCCA | 453 |
| Hu GAPDH (Sabatakos *et al.* 1998b) | CCCTTCATTGACCTCAACTACATGG | AGTCTTCTGGGTGGCAGTGATGG | 455 |
| Hu JNK1 MAPK | ACCTATAGGCTCAGGAG | CTCCATCTGAATCACT | 277 |
| Hu JNK2 MAPK (primer set 1) | ATAGTGTGCAAGTGGCAG | GCATCCATTAATTCCA | 307 |
| Hu JNK2 MAPK (primer set 2) (Dadoune *et al.* 2005) | GATCAGCCTTCAGATGCAGCAGTAAGTAG | CAAAGTGCTAGATGGGCAAGTCCAAG | 524 |
| Hu LPL (Irvine *et al.* 2005) | GAGATTTCTCTGTATGGCACC | CTGCAAATGAGACACTTTCTC | 276 |
| Hu p38 MAPK | GTGCCCGAGCGTTACCAGAACC | CTGTAAGCTTCTGACATTTC | 313 |
| Hu p50 NF-κB | CAACTATGTGGGACCAGCAA | CATAGATGGCGTCTGATACC | 461 |

| | | | |
|---|---|---|---|
| **Hu p65 NF-κB (RelA)** | ATGGCTTCTATGAGGCTGAG | GCAGGTACTGGAATTCCATG | 579 |
| **Hu 28S rRNA (Singh and Ramji 2006)** | TGAACTATGCTTGGGCAGGG | AGCGCCATCCATTTTCAGGG | 513 |
| **Hu STAT1** | AATCCAGATGTCTATGATCAT | AACTATAGTGAACCAGTTCT | 505 |
| **Hu STAT3** | AAGAATCACGCCTTCTACAGA | TTGACCAGCAACCTGACTTTA | 730 |
| **Hu TAK1 (Klatt *et al.* 2006)** | CCGTGTGAACCATCCTAATA | GGGCCACCAATCTCATCAAA | 459 |

**TABLE 2.5 Reaction conditions required for the amplification of the various PCR products analysed during this study.**

| GENE | Forward primer (100µM) µl | Reverse primer (100µM) µl | dNTPs (10mM) µl | $MgCl_2$ (50mM) µl | PCR buffer (x10) µl | DMSO µl | cDNA µl | Taq polymerase (5U/µl) µl | Initial melting | Annealing | Extension | Melting | Final long extension | Number of cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hu β-actin | 0.5 | 0.5 | 1 | 1.5 | 5 | 2.5 | 10 | 0.5 | 95°C 5min | 57°C 1min | 72°C 2min | 93°C 0.5min | 72°C 10min | 16 |
| Hu β-2-microglobulin | 0.5 | 0.5 | 1 | 1 | 5 | - | 5 | 0.25 | 95°C 5min | 60°C 1min | 72°C 2min | 95°C 0.5min | 72°C 8min | 19 |
| Hu C/EBPδ | 0.5* | 0.5* | 1 | 1 | 5 | 2.5 | 10 | 0.5 | 94°C 2min | 60°C 2min | 72°C 2min | 94°C 2min | 72°C 5min | 25-29 |
| Hu CK2α | 0.5 | 0.5 | 1 | 2 | 5 | 2.5 | 10 | 0.25 | 95°C 2min | 61°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 26 |
| Hu CK2α' | 0.5 | 0.5 | 1 | 1.5 | 5 | 2.5 | 10 | 0.25 | 96°C 5min | 60.8°C 1min | 72°C 1mim | 96°C 0.5min | 72°C 10min | 27 |
| Hu GAPDH | 0.5 | 0.5 | 0.5 | 2 | 5 | - | 10 | 0.5 | 96°C 5min | 60°C 1min | 72°C 2min | 93°C 0.5min | 72°C 10min | 17 |
| Hu JNK1 MAPK | 1 | 1 | 1 | 1 | 5 | 2.5 | 10 | 0.5 | 95°C 5min | 55°C 1min | 72 °C 1min | 95 °C 0.5min | 72 °C 10min | 30 |
| Hu JNK2 MAPK (primer set 1) | 1 | 1 | 1 | 1 | 5 | 2.5 | 10 | 0.5 | 95°C 2min | 51°C 1min | 72°C 1min | 95°C 0.5min | 72°C 5min | 30 |
| Hu JNK2 MAPK (primer set 2) | 1 | 1 | 1 | 1.5 | 5 | 2.5 | 10 | 0.5 | 94°C 2min | 71°C 0.5min | 72°C 1min | 94°C 0.5min | 72°C 10min | 27 |
| Hu LPL | 0.5 | 0.5 | 1 | 2 | 5 | - | 10 | 0.25 | 96°C 5min | 55°C 1min | 72°C 2min | 93°C 0.5min | 72°C 10min | 24 |
| Hu p38 MAPK | 0.5 | 0.5 | 0.5 | 1.5 | 5 | - | 10 | 0.25 | 95°C 2min | 55°C 1min | 72°C 1min | 95°C 1min | 72°C 5min | 22 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hu p50 NF-κB** | 0.5 | 0.5 | 1 | 1.5 | 5 | 2.5 | 10 | 0.25 | 95°C 2min | 62°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 25 |
| **Hu p65 NF-κB (RelA)** | 0.5 | 0.5 | 1 | 1 | 5 | 2.5 | 10 | 0.25 | 95°C 2min | 62°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 26 |
| **Hu 28S rRNA** | 1 | 1 | 1 | 0.5 | 5 | - | 5 | 0.25 | 95°C 5min | 62°C 1min | 72°C 2min | 93°C 0.5min | 72°C 10min | 10 |
| **Hu STAT1** | 0.5 | 0.5 | 1 | 1.5 | 5 | 2.5 | 10 | 0.5 | 95°C 2min | 58°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 28 |
| **Hu STAT3** | 0.5 | 0.5 | 1 | 1.5 | 5 | 2.5 | 10 | 0.5 | 95°C 2min | 58°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 25 |
| **Hu TAK1** | 0.5 | 0.5 | 1 | 2 | 5 | 2.5 | 10 | 0.25 | 95°C 2min | 59°C 1min | 72°C 1min | 95°C 0.5min | 72°C 10min | 25 |

*C/EBPδ PCR primers were prepared at 33.3μM, not 100μM.
All PCRs were prepared in a 50μl reaction volume achieved with the addition of the appropriate volume of molecular biology grade water.

Abbreviations: Mo, murine; Hu, human.

### 2.6.4 AGAROSE GEL ELECTROPHORESIS OF RNA/DNA

For the electrophoresis of DNA and total RNA, a 5x loading dye solution was prepared [0.5x TBE, 40% (v/v) glycerol, 0.25% (w/v) bromophenol blue]. The 5x loading dye was autoclaved prior to use (Section 2.2). For electrophoresis, a 10x TBE [0.89M Tris borate (pH 8.3), 0.02M EDTA (Sigma)] stock buffer was used to prepare a 1x TBE buffer, which was made up using ultra-violet (UV) -light treated double-distilled water ($ddH_20$).

#### 2.6.4.1 RESOLVING RNA ON AGAROSE GELS

Routinely, 1µg of RNA was resolved on 1% (w/v) agarose gels to assess its integrity. The gels were made using 1xTBE containing ethidium bromide (0.5µg/ml). RNA samples were prepared with sterile 5x loading dye and made up to a final volume of 20µl with RNase free water (Qiagen), before loading. Electrophoresis was carried out using a Fisherbrand Horizonal Gel Unit at 100V for 30min, in 1xTBE buffer. RNA was visualised under UV light using a Syngene Gel Documentation System.

#### 2.6.4.2 RESOLVING DNA ON AGAROSE GELS

To resolve DNA, gels were prepared between 0.8-2% (w/v) agarose, depending on the size of the DNA requiring size fractionation. Typically, PCR products were resolved on 1.5% (w/v) agarose gels and the fragment size determined by comparison to standard DNA molecular markers (see Appendix II). Plasmid DNA (approximately 500ng) was size-fractionated on 0.8% (w/v) agarose gels and again compared against standard DNA molecular markers (see Appendix II). The gels were made using 1xTBE containing ethidium bromide (0.5µg/ml). DNA samples were prepared using 5x DNA loading dye, prior to gel loading. DNA electrophoresis was carried out on a Fisherbrand Horizontal Gel Unit, at 100V for 1h, in 1xTBE buffer. DNA was visualised under UV light using a Syngene Gel Documentation System.

### 2.6.5 EXTRACTION OF DNA FROM AGAROSE GELS

DNA bands were excised from ethidium bromide stained agarose gels and purified using the QIAquick™ gel extraction kit (Qiagen), according to the manufacturer's

instructions. The efficiency of purification was assessed by analysing an aliquot of the purified DNA on a 1.5% (w/v) agarose gel by electrophoresis (Section 2.6.4.2).

### 2.6.6 BACTERIAL STRAINS AND VECTORS

All bacterial culture media (LB-agar and LB-liquid media) was prepared according to the manufacturer's instructions (DIFCO Laboratories). Table 2.6 specifies the genotypes of the *Escherichia coli (E. coli)* strains used during the course of this study.

**TABLE 2.6 Genotypes of Escherichia coli (*E. coli)* used**

| STRAIN | GENOTYPE | REFERENCE |
|---|---|---|
| JM109 | *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*$^q$ *lacZDM15*] | (Yanisch-Perron *et al.* 1985) |
| DH5α | *supE44 ΔlacU169 (ϕ80 lacZΔM15) hsd17 recA1 endA1 gyrA96 thi-1 relA1* | (Hanahan 1983) |

#### 2.6.6.1 PREPARATION OF COMPETENT CELLS

For the preparation of competent cells, a modified version of the method described by Mandel and Higa (1970) was used. LB-medium (5ml, pre-heated to 37°C) was inoculated with a single bacterial colony of *E. coli* strain DH5α or JM109 and incubated for 12-18h with shaking (300rpm) at 37°C. Subsequently, 0.1ml of this overnight culture was used to inoculate 9.9ml of fresh LB-medium that was then incubated with shaking (300rpm) at 37°C until an $O.D._{550}$ of between 0.5-0.6 was observed. The cells were then pelleted by centrifugation at 3000x*g* for 5min at 4°C, resuspended in half their original volume (5ml) of ice-cold 50mM $CaCl_2$, and kept on ice for 25min. The cells were then pelleted again by centrifugation as above, and resuspended in 1/10 of their original volume (1ml) of ice-cold 50mM $CaCl_2$. Competent cells were kept on ice until required for transformation or mixed with an equal volume of 40% (v/v) sterile glycerol and dispensed into 200µl aliquots in pre-chilled microcentrifuge tubes and stored at -80°C.

### 2.6.6.2 TRANSFORMATION OF COMPETENT CELLS

Competent cells were thawed on ice for 30min prior to transformation. Plasmid DNA (10ng) was added to 200µl of competent cells, which were subsequently kept on ice for 40-60min. The cells were then heat-shocked at 42°C for 90s and placed back on ice for a further 2min. LB-medium (800µl) was added to the cells, which were then incubated at 37°C with shaking (300rpm) for 1h. For the selection of ampicillin resistant bacterial colonies, the following method was used. The cells (200µl) were spread on LB-agar plates containing 100µg/ml ampicillin. The remaining cells (800µl) were pelleted (3000x*g*, 3min), the supernatant discarded, resuspended gently in 100µl of fresh LB-medium and plated on agar plates containing 100µg/ml ampicillin. Plates were incubated overnight at 37°C, and analysed for ampicillin resistant colonies.

### 2.6.6.3 SMALL-SCALE PREPARATION OF PLASMID DNA (MINI-PREP)

LB-medium (10ml) containing ampicillin (100µg/ml) was inoculated with a single colony of transformed *E. coli* and incubated for 16h at 37°C with constant shaking at 300rpm. Preparation of plasmid DNA from the bacterial culture was carried out using Wizard SV™ Miniprep Kit according to the manufacturer's instructions (Promega).

### 2.6.6.4 LARGE-SCALE PREPARATION OF PLASMID DNA (MAXI-PREP)

LB-medium (500ml) containing ampicillin (100µg/ml) was inoculated with 1ml of a bacterial culture (from Section 2.6.6.3) derived from a single colony and grown in antibiotic selective media for 16h at 37°C with shaking (300rpm). Plasmid DNA was subsequently prepared using a High Purity Plasmid Purification Maxiprep kit (Marligen Biosciences) according to the manufacturer's instructions (Marligen Biosciences). The plasmid DNA was diluted in sterile molecular biology grade water following purification. The concentration and purity of the DNA was determined by measuring $O.D._{260}$ and $O.D._{280}$ using a U-1800 Hitachi spectrophotometer.

### 2.6.7 RESTRICTION ENDONUCLEASE DIGESTION OF RECOMBINANT PLASMID DNA

Typically, restriction endonuclease reactions were performed with 100-500ng of DNA in 20µl reaction volume. Restriction endonuclease digestion reactions were carried

out in the presence of a two-fold excess of the appropriate restriction endonuclease(s). Single and double enzyme digestions were typically performed at 37°C (unless otherwise stated by the enzyme supplier) for 2h in the recommended buffers [with or without BSA (100μg/ml) depending on the enzyme] provided by the enzyme supplier (New England Biolabs). If required, restriction endonucleases were heat-inactivated at the end of the digestion to terminate the reaction. Restriction digests were analysed using agarose gel electrophoresis (Section 2.6.4.2).

### 2.6.8 AUTOMATED DNA SEQUENCING

DNA was sequenced by LARK using an automated fluorescent dye termination method of sequencing. For further information see http://www.lark.com.

## 2.7 PROTEIN ANALYSIS

Micro-centrifugation steps described in this section were carried out in a Biofuge pico micro-centrifuge (Heraeus).

**TABLE 2.7 lists the stock solutions required for protein analysis used during the course of this study.**

| SOLUTION | COMPOSITION |
|---|---|
| Phosphatase-free whole cell extraction buffer | 10mM Tris-HCl (pH 7.05), 50mM NaCl, 50mM NaF, 1% (v/v) Triton X-100, 30mM $Na_4P_2O_7$ (sodium pyrophosphate), 5μM $ZnCl_2$, 100μM $Na_3VO_4$ (sodium orthovanadate), 1mM DTT, 2.8μg/ml aprotinin, 2.5μg/ml each of leupeptin and pepstatin, 0.5mM benzamidine, 0.5mM PMSF |
| Nuclei extraction buffer A | 10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF, 1μg/ml pepstatin A, 10μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml type I-S soybean trypsin inhibitor |
| Nuclear extraction buffer C | 25% (v/v) glycerol, 20mM HEPES (pH 7.9), 420mM NaCl, 1.5mM $MgCl_2$, 0.5mM PMSF in isopropanol, 10μg/ml type I-S soybean trypsin inhibitor, 0.2mM EDTA, 0.5mM DTT, 1μg/ml pepstatin A, 10μg/ml aprotinin, 10μg/ml leupeptin |
| Laemmli sample buffer | 0.125M Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β-2-mercaptoethanol |
| Bromophenol blue solution | 0.05% (w/v) bromophenol blue in $H_20$ |
| SDS-PAGE lower gel buffer | 1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS |
| SDS-PAGE upper gel buffer | 1M Tris-HCl (pH 6.8), 10% (w/v) SDS |
| SDS-PAGE gel loading buffer | 50mM Tris-HCl (pH 6.8), 100mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol |
| SDS-PAGE running buffer | 25mM Tris, 250mM glycine, 0.1% (w/v) SDS |
| Western blot transfer buffer | 25mM Tris, 192mM glycine, 20% (v/v) methanol |
| 10x Tris-buffered saline (TBS) | 10mM Tris-HCl, 20mM NaCl, pH 7.4 |

### 2.7.1 PREPARATION OF PROTEIN EXTRACTS USING LAEMMLI SAMPLE BUFFER

Hep3B cells growing in a 9.5cm$^2$ dish were harvested for protein analysis using Laemmli sample buffer (Table 2.7). The medium was aspirated and the cells were washed with 1ml of PBS. Once the PBS was removed by aspiration, freshly prepared Laemmli sample buffer (75μl) was added directly to the cells and these cells were scraped into this buffer. The extracts were then collected and micro-centrifuged (12,000rpm, 5min). At this point, the extracts were either frozen (-80°C) for use on a later date or subjected to SDS-PAGE and subsequently, western blotting (Sections 2.7.7 and 2.7.8).

### 2.7.2 PREPARATION OF PHOSPHATASE-FREE WHOLE CELL PROTEIN EXTRACTS

Phosphatase-free whole cell extracts were prepared as described by Hipskind *et al.* (1994). Hep3B cells were scraped into the medium in which they were growing, transferred to a 50ml polypropylene tube (Falcon) and centrifuged at 1000x*g* for 5min. The cell pellet was washed (x2) with ice-cold PBS containing 10mM NaF and 100μM sodium orthovanadate (1ml), and micro-centrifuged (13,000rpm, 1min) between washes. The supernatant was discarded and the cell pellet resuspended in freshly prepared phosphatase-free whole cell extraction buffer (100-200μl) (see Table 2.7) by vortexing (45s) and lysed by vigorous pipetting. Following centrifugation (10,000x*g* for 10min at 4°C), the supernatant, containing the total cellular protein was removed and stored at -80°C. The concentration of total cellular protein was determined using the Micro BCA protein assay reagents (Pierce) (Section 2.7.4).

### 2.7.3 PREPARATION OF NUCLEAR PROTEIN EXTRACTS

Nuclear extracts were prepared according to the protocol described by Ramji *et al.* (1993a) with minor modifications. Hep3B cells were pelleted by centrifugation (1000x*g*, 5min) and washed three times by resuspension in ice-cold PBS and micro-centrifugation (10,000rpm, 1min). After the final washing step, the supernatant was discarded and the pellet resuspended in ice-cold buffer A (50μl) (Table 2.7) and incubated on ice (15min). The cells were lysed by drawing 5 times through a Hamilton syringe and subjected to micro-centrifugation (10,000rpm, 20s) at 4°C. The resulting nuclear pellet was gently resuspended in ice-cold buffer C (60μl) (Table 2.7) and incubated on ice (30min). After micro-centrifugation at 4°C (10,000rpm, 5min) the supernatant was removed and stored at -80°C. The concentration of nuclear protein extract was determined using the Micro BCA protein assay reagents (Pierce) (Section 2.7.4).

### 2.7.4 DETERMINATION OF PROTEIN CONCENTRATION

The concentration of protein extracts was determined using the Micro BCA Protein Assay Reagent Kit (Pierce) in accordance with the manufacturer's instruction. A standard curve was produced for each assay using bovine serum albumin (BSA) solution at concentrations of 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml in

100µl final volume. Each sample, for which the protein concentration was to be determined, was diluted between 1/15-1/200 with PBS. For each dilution, samples (100µl) were aliquoted in duplicate and 100µl of protein assay reagent was subsequently added. After gentle agitation, the plate was incubated at 37°C for 2h to allow colour change to develop. The absorbance of each sample was read at 595nm using a Dynex Technologies MRX microplate reader. The protein concentration of each sample was then calculated from the standard curve.

### 2.7.5 SAPK/JNK KINASE ACTIVITY ASSAY

The SAPK/JNK kinase assays were carried out using the SAPK/JNK non-radioactive kinase assay kit (Cell Signalling Technology) with minor modifications to the protocol provided by the manufacturer essentially as described by Greenow (2004). Phosphatase-free whole cell extracts were prepared as described in Section 2.7.2. To 250µg of whole cell extract, 20µl of c-Jun fusion protein beads were added and the sample was left to incubate overnight at 4°C, with gentle rocking. Following this incubation period, the sample was micro-centrifuged at 13,000rpm for 1min. The resultant pellet was washed twice in 300µl of phosphatase-free whole cell extraction buffer minus the Triton-X-100 (Table 2.7), with micro-centrifugation at 13,000rpm for 1min between washes. The pellet was then washed (x2) with 1xJNK kinase buffer [25mM Tris-HCl (pH 7.5), 5mM β-glycerophosphate, 2mM DTT, 0.1mM $Na_3VO_4$, 10mM $MgCl_2$] with micro-centrifugation at 13,000rpm for 1min between washes. Following the final wash, the pellet was resuspended in 50µl of 1xJNK kinase buffer supplemented with 100µM ATP. The kinase mix was incubated at 30°C for 30min. The kinase reaction was terminated by the addition of 25µl of SDS-PAGE gel loading buffer (Table 2.7). The sample was then boiled for 5min and loaded immediately onto a 10% (w/v) SDS-PAGE gel. SDS-PAGE and western blotting were carried out as described in Sections 2.7.7 and 2.7.8 respectively. An antibody specific to this kit [anti-phospho-c-Jun (Ser63)] was used for the immunodetection of proteins (Section 2.7.9, see Table 2.9 also).

### 2.7.6 CASEIN KINASE 2 (CK2) ASSSAY

This method was derived from the protocol used by Sung *et al.* (2001) and Lodie *et al.* (1997) with minor modifications. Phosphatase-free whole cell extracts were

prepared as described in section 2.7.2 and the anti-CK2α subunit antibody was used to immunoprecipitate CK2α from 150μg of extracts. Immunoprecipitation was carried out using Protein A/G agarose beads (Santa Cruz Biotechnology). Whole cell extracts were left to incubate overnight at 4°C, with gentle rocking with the anti-CK2α antibody (2μg/ml). The resulting protein-antibody complex was then 'captured' with the addition of 20μl of Protein A/G agarose with gentle rocking at 4°C for 2h. The immunoprecipitates were recovered by micro-centrifugation (13,000rpm, 2min) and the resulting pellet was washed (200μl) twice in phosphatase-free whole cell extraction buffer minus the Triton-X-100. The pellet was resuspended in 25μl of 1x kinase buffer [100mM Tris-HCl (pH 8.0), 100mM NaCl, 20mM $MgCl_2$, 50mM KCl, 100μM $Na_3V0_4$, 5mg/ml β-casein] to which 1μCi [$\gamma$-$^{32}$P]-ATP was added. The kinase reaction was incubated for 15min at 37°C and terminated by the addition of 10μl of SDS-PAGE reducing gel loading buffer (Table 2.7). Samples were immediately subjected to SDS-PAGE (Section 2.7.7) using 15% (w/v) acrylamide gel. Following the electrophoresis, the gel was fixed for 20min in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. After the gel had been fixed, it was briefly washed with $ddH_2O$, transferred to Whatmann 3MM paper and dried under vacuum using a Gel Dryer (Model 583, Bio-Rad) at 80°C (1h). The dried gel was exposed to Kodak X-Ray film in a light proof cassette (GRI) at –80°C for varying exposure periods (12-72h). X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert).

### 2.7.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed under reducing conditions following the method of (Laemmli 1970). Typically, separating gels and stacking gels, containing 10% (w/v) and 5% (w/v) acrylamide respectively were used. Gels were prepared from stock solutions (see Table 2.7) as shown below in Table 2.8.

**TABLE 2.8 Composition of separating and stacking gels used for SDS-PAGE**

| GEL COMPONENT | 7.5% (W/V) SEPARATING GEL | 10% (W/V) SEPARATING GEL | 12.5% (W/V) SEPARATING GEL | 15% (W/V) SEPARATING GEL | 5% (W/V) STACKING GEL |
|---|---|---|---|---|---|
| UPPER BUFFER | - | - | - | - | 2.5ml |
| LOWER BUFFER | 2.5ml | 2.5ml | 2.5ml | 2.5ml | - |
| ACRYLAMIDE:BISACRYLAMIDE (37.5:1) | 1.875ml | 2.5ml | 3.125ml | 3.75ml | 1.25ml |
| DD$H_2O$ | 5.625ml | 5ml | 4.26ml | 3.75ml | 6.25ml |
| 10% (W/V) APS | 100µl | 100µl | 100µl | 100µl | 100µl |
| TEMED | 10µl | 10µl | 10µl | 10µl | 10µl |

Electrophoresis was carried out using the Mini-PROTEAN II slab electrophoresis cell from Bio-Rad Laboratories with the gel apparatus being assembled as described by the manufacturer (Bio-Rad Laboratories).

The separating gel was poured to within 3cm of the upper edge of the inner glass plate, isopropanol was layered on top of the gel solution to exclude any air bubbles and the gel was allowed to polymerise for 30-40min. Once the gel had set, the isopropanol was washed off with dd$H_2O$ and the upper surface dried with Whatman 3MM paper. The stacking gel was then poured on top and the well-forming comb inserted. Following polymerisation of the stacking gel, the comb was removed and the wells were washed out with dd$H_2O$. The gels were then placed in the electrophoresis tank and the upper and lower chambers were filled with 1× running buffer containing 0.1% (w/v) SDS (Table 2.7).

Protein samples were prepared for SDS-PAGE with either phosphatase-free whole cell extraction buffer or Laemmli sample buffer (Table 2.7). Prior to SDS-PAGE, phosphatase-free whole cell extracts (typically between 10-80µg) were mixed with an appropriate volume of SDS-PAGE gel loading buffer (Table 2.7). Where samples were prepared with Laemmli sample buffer, extracts were mixed with an appropriate volume of bromophenol blue solution (Table 2.7). All samples were then heated to 100°C for 5min, after which they were quenched on ice. At this stage, samples were loaded immediately onto the gel. Rainbow protein size markers (10µl) (GE Healthcare) were loaded into the first lane of each gel (see Appendix II). The gel was

then subjected to electrophoresis at a constant voltage of 200V for 45-50min. The gels were then used for western blotting (see below).

### 2.7.8 WESTERN BLOTTING

This technique was adapted from Burnette (1981). Electrophoretic transfer of proteins was carried out using a Bio-Rad Trans Blot Electrophoretic transfer cell (Bio-Rad Laboratories). The transfer of protein to PVDF membranes (Millipore) was carried out as described by the manufacturer. Briefly, the gel was removed from the glass plates and the stacking gel was cut away. The gel was then equilibrated by incubation in transfer buffer (see Table 2.7) for 15min at room temperature. Whatman 3MM paper, PVDF membrane (pre-wetted in methanol for 15s), both cut to the same size as the gel and the sponge pads of the transfer apparatus were also pre-wetted in transfer buffer. The PVDF membrane was then placed on the gel and sandwiched between the Whatman 3MM paper and the sponge pads before being placed into the blotting cassette. The cassette was then subjected to electro-blotting in a tank containing transfer buffer at 4°C at a constant voltage of 15V for 12-18h or 150V for 1h.

### 2.7.9 IMMUNODECTECTION OF PROTEINS

Blotted PVDF membranes were probed immunochemically as described below. The membrane was incubated in 20ml of blocking solution [1× TBS containing 5-10% (w/v) non-fat milk powder and 0.1% (v/v) Tween-20] for 1h. Excess blocking solution was removed at this point and the membrane washed three times (15min washes) in wash solution [1× TBS containing 0.1% (v/v) Tween-20]. The membrane was then incubated with primary antibody which was diluted in 1× TBS containing 5% (w/v) skimmed milk powder or 5% (w/v) BSA and 0.1% Tween-20 for 1-1.5h at room temperature or overnight at 4°C (see Table 2.9 for details). The membrane was then washed as described above and immersed in the appropriate secondary antibody (10-20ml) [horseradish peroxidase-conjugated, anti-rabbit IgG, anti-mouse IgG or anti-goat IgG diluted 1:2000-1:10,000 in 1× TBS containing 5-10% (w/v) skimmed milk powder and 0.1% (v/v) Tween-20] and incubated for 1h at room temperature. Following another set of washes, detection of membrane bound protein was carried

out as described in the instructions supplied with the Enhanced Chemi-Luminescent (ECL) detection kit (GE Healthcare).

**TABLE 2.9 Primary antibodies used for the immunodetection of proteins.**

| PRIMARY ANTIBODY | DILUTION IN 1XTBS 0.1% (V/V) TWEEN 20 | % (W/V) NON-FAT MILK OR BSA | INCUBATION PERIOD (H) (RT OR 4°C) | SIZE (KDA) |
|---|---|---|---|---|
| anti-C/EBPδ | 1:2000 | 5 non-fat milk | 1.5 (RT) | 32 |
| anti-β-actin | 1:10,000 | 5 non-fat milk | 1 (RT) or 12-18 (4°C) | 42 |
| anti-phospho-SAPK/JNK (Thr183/Tyr185) | 1:1000 | 5 BSA | 12-18 (4°C) | 54/46 |
| anti-SAPK/JNK | 1:1000 | 5 BSA | 12-18 (4°C) | 54/46 |
| anti-phospho-c-Jun (Ser63) | 1:1000 | 5 BSA | 12-18 (4°C) | 35/37 |
| anti-c-Jun | 1:1000 | 5 BSA | 12-18 (4°C) | 42 |
| anti-CK2α | 1:1000 | 5 non-fat milk | 1 (RT) | 42 |
| anti-CK2α' | 1:1000 | 5 non-fat milk | 1 (RT) | 40 |
| anti- NF-κB p105/p50 | 1:1000 | 5 BSA | 12-18 (4°C) | active form 50 precursor 120 |
| anti-NF-κB p65 | 1:1000 | 5 BSA | 12-18 (4°C) | 75 |
| anti-TAK1 | 1:1000 | 5 BSA | 12-18 (4°C) | 80-82 |
| anti-STAT1 | 1:1000 | 5 BSA | 1 (RT) | 84/91 |
| anti-phospho-STAT1 (Tyr701) | 1:1000 | 5 BSA | 12-18 (4°C) | 84/91 |
| anti-STAT3 | 1:1000 | 5 non-fat milk | 12-18 (4°C) | 79/86 |

| | | | | |
|---|---|---|---|---|
| anti-phospho-STAT3 (Tyr705) | 1:1000 | 5 BSA | 12-18 (4°C) | 79/86 |
| anti-phospho-STAT3 (Ser727) | 1:1000 | 5 BSA | 12-18 (4°C) | 86 |

RT denotes room temperature.

### 2.7.10 DETECTION OF CHEMILUMINESCENT SIGNAL

Membranes were placed in contact with Kodak X-ray film in a Genetic Research Instrumentation (GRI) Hi-Speed-X light proof cassette, for varying periods of time (10s-10min), depending on the strength of the signal. The exposed film was then developed in a Gevamatic 60 automatic developer (Agfa-Gevaert).

## 2.8 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

This technique was carried out essentially as described by Ramji *et al.* (1993a) with minor modifications.

### 2.8.1 PREPARATION OF RADIOLABELLED OLIGONUCLEOTIDE PROBE DNA

The preparation of radiolabelled oligonucleotide probes was divided into two steps.

1) Annealing of oligonucleotides
2) Radiolabelling of double-stranded oligonucleotides with $\alpha$-$^{32}$P-dCTP

#### 2.8.1.1 ANNEALING OF OLIGONUCLEOTIDES

The sequences of the oligonucleotides used for EMSA analysis are shown in Table 2.10. Forward and reverse sequences were designed to leave 5' overhangs containing at least one G residue following annealing to allow for complementary binding of [$\alpha$-$^{32}$P]-dCTP during radiolabelling.

Forward and reverse oligonucleotides (0.5nmol of each) were incubated together at 100°C (10min) in the presence of a medium salt buffer (1x NEB Buffer 3) in a final volume of 100µl. The mixture was then allowed to cool to room temperature. Double stranded oligonucleotides prepared in this way were either radiolabelled immediately or stored at -20°C.

**TABLE 2.10 Sequences of double-stranded oligonucleotides used for EMSA analysis.**

| PROBE | ANNEALED OLIGONUCLEOTIDE |
|---|---|
| NF-κB consensus | 5' GG**A GTT GAG GGG ACT TTC CA**A **GGC** 3'<br>3' **T CAA CTC CCC TGA AAG GGT CCG** G 5' |
| AP-1 consensus | 5' CGC TTG **ATG AGT CAG** 3'<br>3' **TAC TCA GTC** GGC CTT 5' |

### 2.8.1.2 RADIOLABELLING OF DOUBLE-STRANDED OLIGONUCLEOTIDES

Radiolabelling was carried out using the reagents supplied in the Megaprime™ Labelling Kit (GE Healthcare) according the manufacturer's protocol. Annealed double-stranded oligonucleotides (60ng) were incubated with 1x Labelling buffer, [α-$^{32}$P]-dCTP (3µl) and Klenow polymerase (2U) in a final volume of 50µl at 37°C (30min). Separation of the radiolabelled probe from unincorporated nucleotides was carried out using a Sephadex G50 nick column. The column was equilibrated with 1× TE [10mM Tris-HCl (pH 7.5), 1mM EDTA] buffer (9ml) and the reaction mix (50µl) loaded onto the column and eluted in 1× TE buffer as a 400µl fraction which was then discarded. The column was then further eluted with 1× TE (400µl) and this second fraction, collected and stored at –20°C.

### 2.8.2 DNA-PROTEIN BINDING REACTIONS

The following stock solutions were used in this procedure:

- 10x Binding Buffer: 340mM KCl, 50mM $MgCl_2$, 1mM DTT.
- Dilution Buffer: 40mM KCl, 0.1mM EDTA.

The binding of the radiolabelled probe to phosphatase-free whole cell or nuclear extracts was carried out as described below. For this, 5µg of phosphatase-free whole cell extracts or 5µg of nuclear extracts were mixed with dilution buffer to a final volume of 26µl. Then, 2-5µl of 10x binding buffer and 3-6µl of poly-(dI-dC) (1µg/µl) were added to the reaction mixture and this was incubated on ice for 10min. Subsequently, 3µl of the radiolabelled double-stranded oligonucleotide was added and the mixture was left at room temperature for 20min to allow binding to occur.

Following the addition of 12µl of 20% (w/v) Ficoll, the mixture was subjected to electrophoresis (Section 2.8.4).

### 2.8.3 ANTIBODY SUPERSHIFT/INTERFERENCE AND COMPETITION BINDING STUDIES

For supershift/interference experiments, antibody (0.5µg) was added to the protein (whole cell or nuclear) binding reaction and incubated on ice for 20min, prior to the addition of the radiolabelled probe. In competition binding studies, prior to the addition of the radiolabelled probe, the binding reaction mixture was incubated with a 250 molar excess of unlabelled competitor oligonucleotides (10min on ice).

### 2.8.4 ELECTROPHORESIS OF DNA-PROTEIN COMPLEXES

**TABLE 2.11 Composition of non-denaturing polyacrylamide gels for EMSA analysis.**

| COMPONENT | 4% (W/V) ACRYLAMIDE | 6% (W/V) ACRYLAMIDE |
|---|---|---|
| ACRYLAMIDE: BISACRYLAMIDE (29:1) | 5ml | 7.5ml |
| 10X TBE | 2.5ml | 2.5ml |
| DD$H_2$0 | 42.5ml | 40ml |
| 10% APS | 500µl | 500µl |
| TEMED | 50µl | 50µl |

DNA-protein complexes were resolved by electrophoresis on 4-6% (w/v) non-denaturing polyacrylamide gels (Table 2.11). Electrophoresis was carried out for 3-4h at 150V or 12-18h at 35V (both at 4°C) using vertical gel apparatus (20x20cm, Scotlab) with 0.5× TBE as running buffer. Following electrophoresis, the gel was transferred to Whatmann 3MM paper and dried under vacuum using a Gel Dryer (Model 583, Bio-Rad) at 80°C (1h). The dried gel was exposed to Kodak X-Ray film in a light proof cassette (GRI) at –80°C for varying exposure periods (12-72h). X-ray films were developed using a Gevamatic 60 automatic developer (Agfa-Gevaert).

## 2.9 DENSITOMETRIC ANALYSIS OF DATA

For analysis, the intensity of bands from agarose gel images, immunoblots and EMSAs were analysed using GeneTools™ (Syngene) software as described by Harvey *et al.* (2007). Also see section 3.2.1 for a detailed account on the densitometric analysis of data generated by RT-PCR.

## 2.10 STATISTICAL ANALYSIS OF DATA

To assess data for statistical significance a standard student's T-test was carried out, the details of which are in Appendix III. Where $p<0.05$, this was concluded as statistically significant.

# CHAPTER THREE: AN INVESTIGATION INTO THE FACTORS REGULATING C/EBPδ EXPRESSION IN HEPATOCYTES

## 3.1 INTRODUCTION

The transcription factor C/EBPδ is an important regulator of gene expression during the inflammatory response. Aberrant inflammation contributes to the pathophysiology of diseases such as asthma (Borger *et al.* 2002), atherosclerosis (Kelkenberg *et al.* 2002; Hansson 2005) and glomerulonephritis (Miyoshi *et al.* 2007). Studying the cytokine-mediated regulation of C/EBPδ expression could potentially aid advances in the search for therapeutic targets against such diseases.

As discussed in section 1.3.1, the initial phase of inflammation in the liver is referred to as the APR and is characterised by changes in the levels of several serum APPs, synthesised primarily by hepatocytes (Baumann and Gauldie 1994; Cantwell *et al.* 1998). The expression of liver acute phase genes is triggered by several inflammatory mediators, including the cytokines IL-6, IL-1 and TNF-α (reviewed in Mackiewicz 1997; Poli 1998).

Several lines of evidence indicate C/EBPδ is a key transcription factor for the regulation of liver APR genes. For example, the expression of C/EBPδ is regulated by a number of inflammatory mediators, including IL-6, IL-1 and IFN-γ in hepatocytes (Juan *et al.* 1993; Ramji *et al.* 1993a; Cantwell *et al.* 1998; Poli 1998). Furthermore, in a number of experimental animal models of the APR, C/EBPδ is one of the major transcription factors whose activity/expression is induced in response to inflammatory stimuli (Alam *et al.* 1992; Ray and Ray 1994a; Magalini *et al.* 1995; Dinic *et al.* 2004). C/EBPδ is also reported to functionally interact with a number of APP gene promoters including, complement C3 (Juan *et al.* 1993), hemopexin (Poli and Cortese 1989), haptoglobin (Oliviero and Cortese 1989; Milosavljevic *et al.* 2003), CRP (Majello *et al.* 1990), rat α2-macroglobulin (Milosavljevic *et al.* 2002), rabbit α-1-acid glycoprotein (Ray and Ray 1994b) and more recently PAI-1 (Dong *et al.* 2005).

### 3.1.1 EXPERIMENTAL STRATEGY

It was decided that the work presented in this thesis would be carried out in hepatocytes as these cells are known to be responsive to a range of inflammatory mediators, express C/EBPδ as well as other C/EBP family members and are principally involved in the production of serum APPs during the APR.

The human hepatoma cell line, Hep3B has been used extensively as a model to study the effects of cytokines on hepatic acute phase gene expression (Oliviero and Cortese 1989; Juan *et al.* 1993; Ramji *et al.* 1993a; Zhang *et al.* 1995; Cantwell *et al.* 1998; Foka *et al.* 2003). Hep3B cells are fully adherent hepatocytes first established in culture by Aden *et al.* (1979). These cells share many of their liver specific phenotypes with normal hepatocytes. Specifically, Hep3B cells have been characterised with respect to their ability to produce liver specific markers such as hepatitis B surface antigen, albumin and α-fetoprotein, as well as a battery of other liver specific genes (Knowles *et al.* 1980). In addition, these cells have also been shown to produce cytokine-mediated responses similar to those observed in humans during inflammatory states (Hiron *et al.* 1992). The use of such liver cell lines has several advantages over the use of primary hepatocytes and *in vivo* models for study. Firstly, there are obvious limitations concerning the availability of primary human hepatocytes for research. Also, primary cells cannot be used for the long term culture in the laboratory and in comparison, cell lines generally provide a more homogeneous system for study allowing for the extraction of greater yields of RNA and protein. Conducting studies in cell lines also avoids having to address the ethical issues that surround the use of animal models in scientific research.

Several studies conducted in our laboratory and by others have successfully used the Hep3B cell line in investigations involving the regulation of C/EBPs during the inflammatory response (Ramji *et al.* 1993a; Ramji *et al.* 1994; Poli 1998 and references therein; Davies *et al.* 2000; Foka *et al.* 2001; Ramji and Foka 2002 and references therein; Foka *et al.* 2003). Although some studies into the regulation of C/EBPδ expression by cytokines have been fruitful, particularly in relation to IL-6 action (Yamada *et al.* 1997; Cantwell *et al.* 1998), there is limited information available regarding its regulation by other cytokines including IL-1.

Given that there is good evidence for an important role for C/EBPδ during the APR, we initially decided to investigate the effect of various inflammatory mediators on the expression of this transcription factor. In light of the findings of the work presented in this chapter, we then pursued a line of investigation that would help elucidate the signalling pathways by which selected cytokines regulate the expression of C/EBPδ (Chapters 4-7).

### 3.1.1.1 SPECIFIC AIMS

Previously published data shows that C/EBPδ expression is induced by IL-1, IL-6 and IFN-γ in Hep3B cells (Juan *et al.* 1993; Ramji *et al.* 1993a; Cantwell *et al.* 1998). In addition, studies have also demonstrated that in response to other inflammatory stimuli including LPS and turpentine oil, C/EBPδ is up-regulated in animal models of the APR (Alam *et al.* 1992; Ray and Ray 1994a). Therefore, we initially wanted to investigate the effects of such mediators on the expression of C/EBPδ in Hep3B cells in an effort to initially confirm and then expand on the data presented in these published reports. From the mediators tested, we decided to focus on the effect of IL-1 action on C/EBPδ expression.

Because initial studies strongly suggested that this response was regulated at the transcriptional level, further studies were aimed to analysing the action of IL-1 on C/EBPδ promoter activation as an initial step towards functional dissection of the promoter. IL-6-mediated activation of the rat and mouse C/EBPδ promoter has previously been reported (Yamada *et al.* 1997; Cantwell *et al.* 1998). Therefore we also included this cytokine in our experiments for comparative purposes and so that we could assess whether the human C/EBPδ gene promoter was regulated in a similar manner. In conjunction, experiments were also carried out to determine whether the human C/EBPδ gene was subject to auto-regulation. We decided to pursue this line of investigation because previously published reports, including those published by our laboratory, have shown that the rat, mouse and ovine C/EBPδ genes are regulated through auto-regulatory mechanisms (Yamada *et al.* 1998; O'Rourke *et al.* 1999a; Davies *et al.* 2000; Tanabe *et al.* 2000). However, to our knowledge these findings have not yet been extended to the analysis of the human C/EBPδ gene promoter. In addition, these studies have also suggested the existence of species-specific differences in the mechanisms by which the C/EBPδ gene is auto-regulated. For example, the 5' regions of the mouse and ovine C/EBPδ genes are necessary for auto-activation (O'Rourke *et al.* 1999a; Davies *et al.* 2000), whereas the rat gene is auto-regulated through sequences present at the 3' end of the gene (Yamada *et al.* 1998). Given that these mechanisms of gene regulation may play a role in controlling the expression of C/EBPδ during the inflammatory response (Ramji and Foka 2002 and references therein) and the existence of species-specific mechanisms of auto-regulation it was of interest to pursue studies into this novel area.

Figure 3.1 summarises our overall experimental approach that was designed to achieve the outlined experimental objectives.

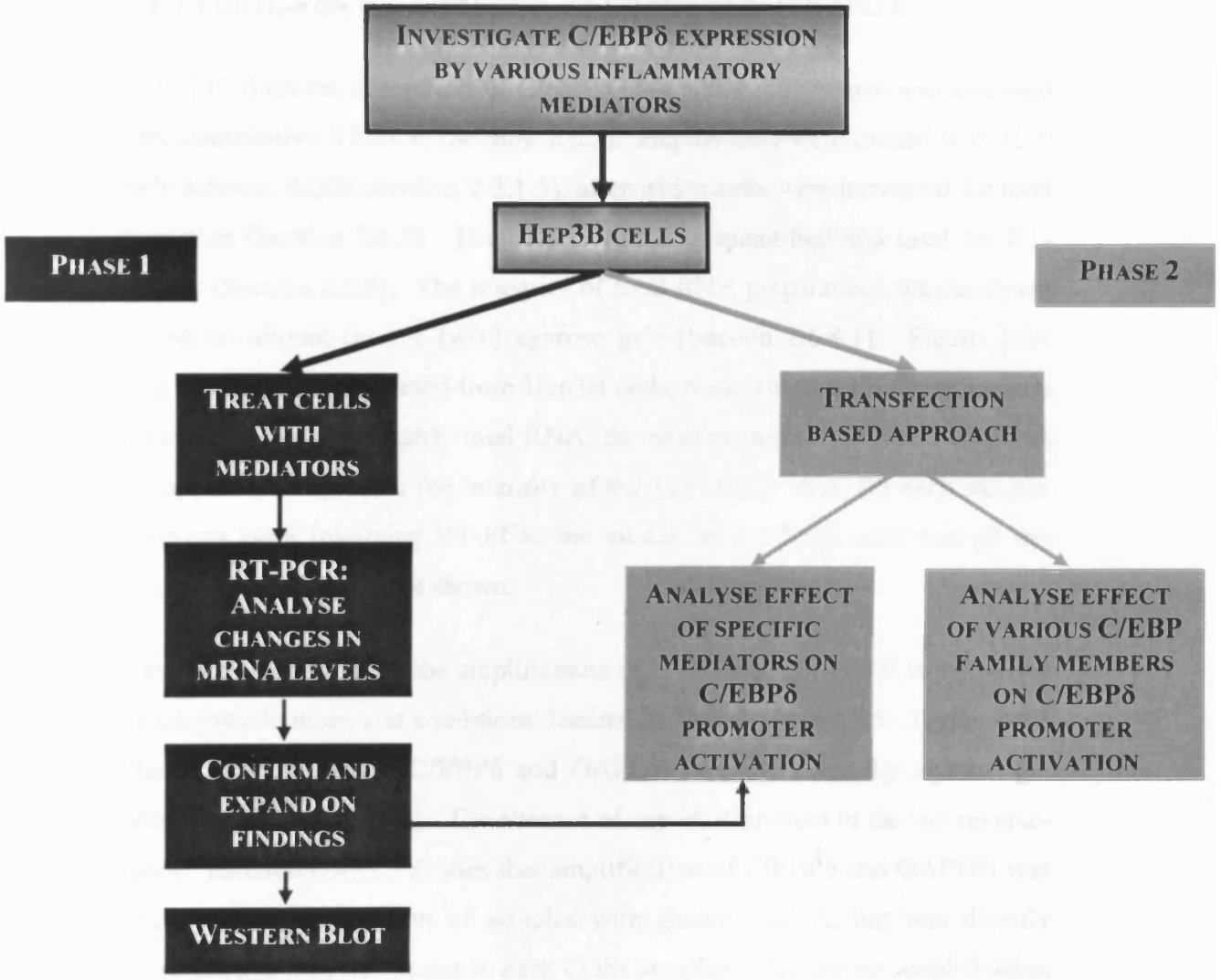



**FIGURE 3.1 Experimental strategy.** The aim of Phase 1 of the experimental strategy was to establish which inflammatory mediators affect C/EBPδ expression in Hep3B cells. The Phase 2 experimental strategy was designed to expand on the findings from Phase 1 to the analysis of the C/EBPδ gene promoter and to initiate investigations into auto-activation of the C/EBPδ gene.

## 3.2 INVESTIGATIONS INTO THE EFFECT OF INFLAMMATORY MEDIATORS ON C/EBPδ EXPRESSION

### 3.2.1 EFFECT OF IL-6 ON THE EXPRESSION OF C/EBPδ IN HEP3B CELLS

The effect of IL-6 on the expression of C/EBPδ over a 24h time course was analysed using semi-quantitative RT-PCR (Section 2.6.3). Hep3B cells were treated with IL-6 for periods between 0-24h (Section 2.3.1.5), after which cells were harvested for total RNA extraction (Section 2.6.1). Total RNA was then quantified and used for RT-PCR analysis (Section 2.6.3). The integrity of total RNA preparations was analysed by resolving an aliquot on 1% (w/v) agarose gels (Section 2.6.4.1). Figure 3.2A shows 1μg of total RNA extracted from Hep3B cells, resolved on a 1% (w/v) agarose gel. As expected for good quality total RNA, the relative intensity of the 28S rRNA band was approximately twice the intensity of the 18S rRNA band, for each sample. For subsequent work involving RT-PCR, the quality of the RNA used was of this standard although the data is not shown.

Polymerase chain reactions for the amplification of C/EBPδ and GAPDH were carried out using oligonucleotides and conditions detailed in Tables 2.4 and 2.5. Figure 3.2B shows the PCR products for C/EBPδ and GAPDH as fractionated by agarose gel electrophoresis (Section 2.6.4.2). The absence of any PCR product in the 'no reverse-transcriptase' reaction (-RT) indicates that amplification of C/EBPδ and GAPDH was not derived from contamination of samples with genomic DNA, but was directly related to respective mRNA species in each of the samples. The correct amplification of C/EBPδ and GAPDH was determined by comparing the PCR product size to standard DNA molecular weight markers (Appendix II). In addition, the PCR amplification product for C/EBPδ was analysed by DNA sequence analysis (Section 2.6.8) and its identify confirmed.

The relative intensity of PCR products for C/EBPδ and GAPDH at each time-point were determined using Syngene GeneTools software and used for densitometric analysis. The software assigns numerical values to each of the PCR products under analysis, relating directly to the intensity of the PCR product. Densitometric analysis of the data was determined by relating the intensity of the C/EBPδ PCR product at each time-point to the intensity of the PCR product for the housekeeping gene,

GAPDH at the equivalent time-point. The data was normalised to the 0h control and expressed as a fold change relative to this (Figure 3.2C). The results indicate that IL-6 induces expression of C/EBPδ (maximally at 3h) and that this high level of expression is maintained up to 24h following stimulation of cells with IL-6.

Ramji *et al.* (1993a) and Cantwell *et al.* (1998) have previously demonstrated that IL-6 induces expression of C/EBPδ mRNA in Hep3B cells for up to 24h following treatment by northern blotting. We also report similar results using semi-quantitative RT-PCR. Importantly, these results establish that our laboratory has a valid *in vitro* cellular system for examining the effects of cytokines on C/EBPδ expression and that semi-quantitative RT-PCR is a suitable method used to determine expression levels of C/EBPδ in Hep3B cells.

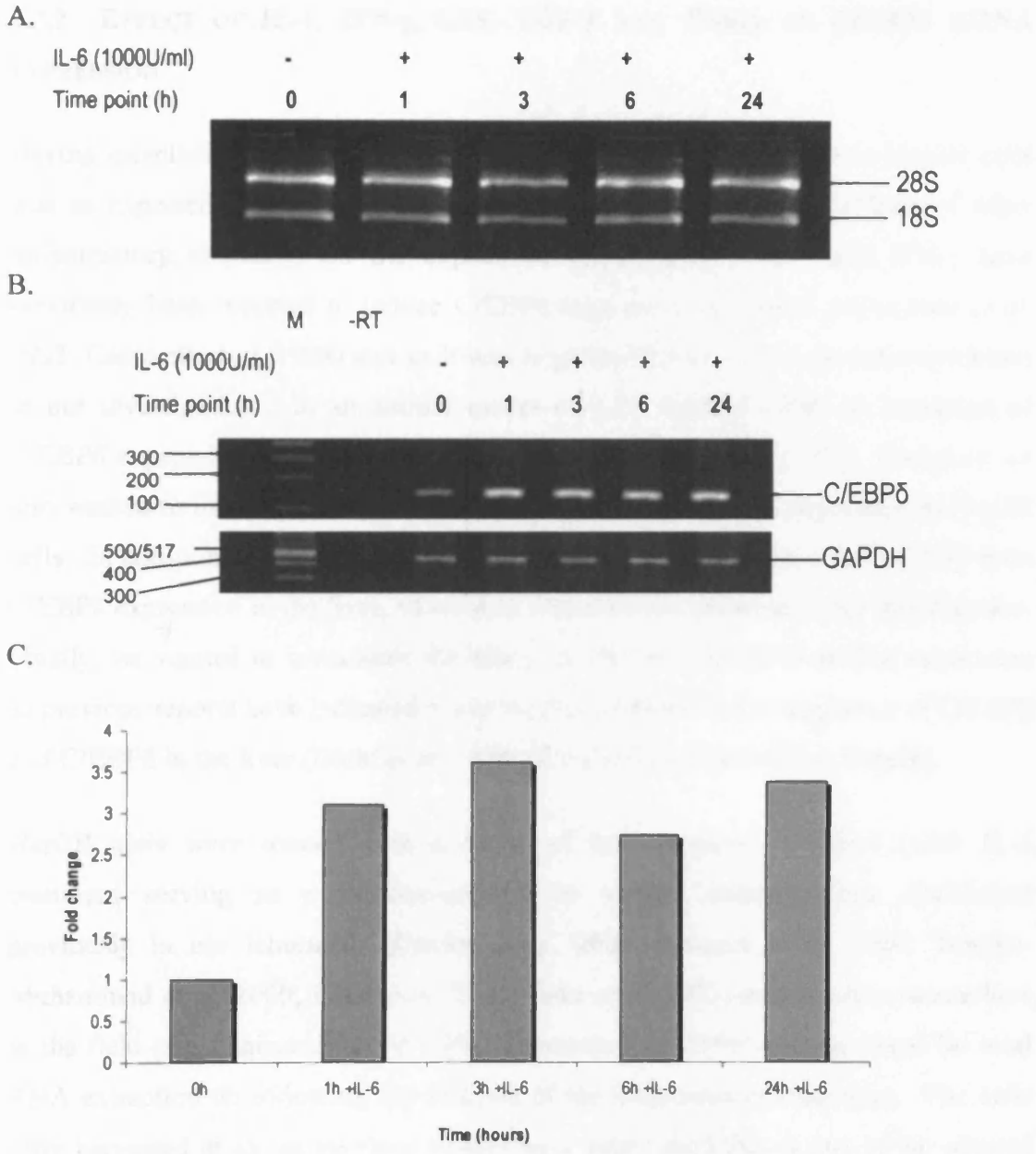



**FIGURE 3.2. Time dependent effect of IL-6 on C/EBPδ mRNA expression in Hep3B cells over a period of 24h.** Hep3B cells were treated with IL-6 and harvested for total RNA extraction at each of the indicated time-points. 0h denotes untreated cells harvested at the beginning of the 24h time-course. Total RNA was extracted, its integrity examined (A) and used for RT-PCR analysis (Section 2.6.3). The PCR amplification products for C/EBPδ and GAPDH were resolved on 1.5% (w/v) agarose gels by electrophoresis (B). The size of each PCR-amplified product was determined by comparing it to standard DNA markers, denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). The sizes of some of the markers are shown on the left of the images in Panel B. –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using 0h RNA). Panel C displays the ratios of C/EBPδ:GAPDH, normalised to the 0h control as determined by densitometric analysis. These data are representative of two independent experimental series.

### 3.2.2 EFFECT OF IL-1, IFN-γ, LPS, TGF-β AND TNF-α ON C/EBPδ MRNA EXPRESSION

Having established the effect of IL-6 on C/EBPδ mRNA expression in Hep3B cells was as expected (Figure 3.2), we next decided to investigate the effects of other inflammatory mediators on the expression of this gene. IL-1 and IFN-γ have previously been reported to induce C/EBPδ expression in Hep3B cells (Juan *et al.* 1993; Cantwell *et al.* 1998) and so it was important for us to include these cytokines in our investigation. In an animal model of LPS induced APR, an induction of C/EBPδ expression in the liver has been reported (Alam *et al.* 1992). Therefore we also wanted to investigate the effect of this mediator on C/EBPδ expression in Hep3B cells. In addition, to our knowledge, no study has determined the effect of TGF-β on C/EBPδ expression in the liver, so we also included this cytokine in our investigation. Finally, we wanted to investigate the effect of TNF-α on C/EBPδ mRNA expression as previous reports have indicated a role for this cytokine in the regulation of C/EBPβ and C/EBPδ in the liver (Diehl *et al.* 1995; Diehl 1998 and references therein).

Hep3B cells were treated with a range of inflammatory mediators (with IL-6 treatment serving as a positive-control) at various concentrations established previously in our laboratory (Davies *et al.* 2000; Granger *et al.* 2000; Tengku-Muhammad *et al.* 2000; Foka *et al.* 2001; Foka *et al.* 2003) and by other researchers in the field (Angchaisuksiri *et al.* 1996; Fransson *et al.* 1999) and harvested for total RNA extraction 6h following the addition of the inflammatory mediators. The cells were harvested at 6h, as previous studies have established this as one of the optimal time-points for maximum induction of C/EBP mRNA/protein in a range of cellular systems (Ramji *et al.* 1993a; Cardinaux *et al.* 2000; Granger *et al.* 2000). Having verified the integrity of total RNA preparations (data not shown), this RNA was used for RT-PCR analysis (Figure 3.3A and B). The data generated from these experimental series was analysed as described in Section 3.2.1 [with amplification of a housekeeping gene (e.g. β-actin or β-2-microglobulin) serving as a control], normalised to the untreated control (UN) and expressed numerically in Figure 3.3C and D.

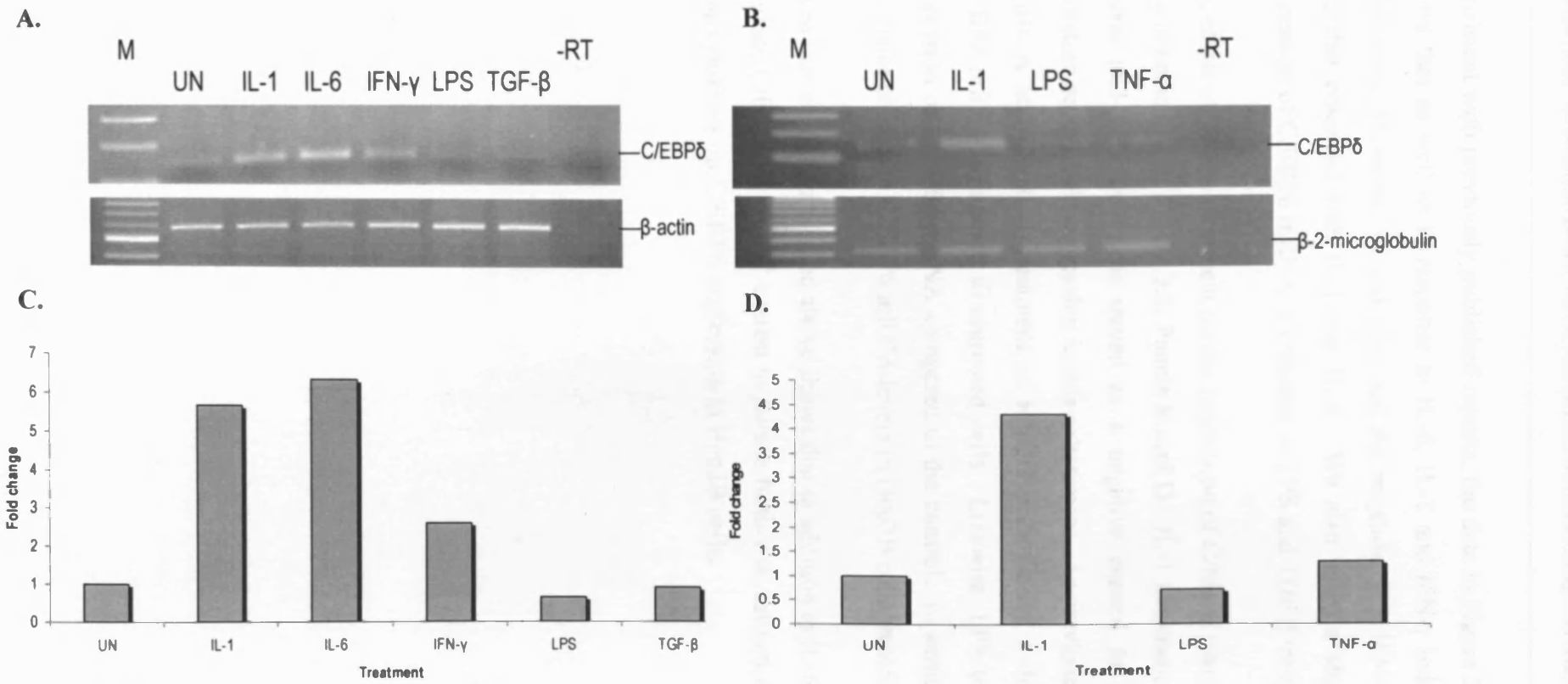



**FIGURE 3.3. Effects of various inflammatory mediators on C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were treated with inflammatory mediators at various concentrations (IL-1, IL-6, IFN-γ and TNF-α were all added to a final concentration of 1000U/ml; TGF-β was added to a final concentration of 0.04μg/ml and LPS was added to achieve a final concentration of 1μg/ml). Total RNA was extracted from cells at 6h following the addition of the inflammatory mediator and used for RT-PCR analysis (Section 2.6.3). The PCR amplification products for C/EBPδ, β-actin and β-2-microglobulin were resolved on 1.5% (w/v) agarose gels by electrophoresis. The data is separated into two panels, (A and B) with β-actin serving as a control in the experiment displayed in Panel A and β-2-microglobulin serving as a control in the experiment displayed in Panel B. The size of each PCR-amplified product was determined by comparing it to standard DNA markers, denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using untreated RNA). Panels C and D display the mean ratios of C/EBPδ:β-actin and C/EBPδ:β-2-microglobulin respectively, normalised to the untreated (UN) control as determined by densitometric analysis. These data are representative of two independent experimental series.

Consistent with previously published reports, the data in Figure 3.3 (Panels A and C) shows that as well as in response to IL-6, IL-1 and IFN-γ induce C/EBPδ mRNA expression. However, we did note that the response with IFN-γ was less profound than that observed with IL-1 and IL-6. We also noted a slight reduction in the expression of C/EBPδ mRNA in response to LPS and TGF-β treatment.

The effect of TNF-α treatment on the expression of C/EBPδ was also examined. This data is presented in Figure 3.3, Panels B and D. IL-1 treatment served as a positive control and LPS treatment served as a negative control for the effect of these mediators on C/EBPδ expression in this experiment. As previously noted (Figure 3.3, Panels A and C), IL-1 treatment of Hep3B cells caused a dramatic induction of C/EBPδ mRNA compared to untreated cells. Likewise, LPS treatment reduced the expression of C/EBPδ mRNA compared to the control. In addition, we did not note any major change in C/EBPδ mRNA levels in Hep3B cells treated with TNF-α.

Given that the data presented above shows that in addition to IL-6, both IL-1 and IFN-γ induce C/EBPδ, it was of interest to pursue further investigations into the effects of these cytokines on C/EBPδ expression in Hep3B cells.

### 3.2.3 EFFECT OF DIFFERENT IL-1 CONCENTRATIONS ON C/EBPδ MRNA EXPRESSION

To confirm the induction of C/EBPδ mRNA expression by IL-1 and to determine an optimal concentration of this cytokine required to produce the response for future experiments, Hep3B cells were treated with three different concentrations of IL-1. The cells were then harvested for semi-quantitative RT-PCR analysis. Figure 3.4 shows the effect of various concentrations of IL-1 on C/EBPδ mRNA expression in Hep3B cells. These results indicate that the expression of C/EBPδ is induced maximally at a concentration of 100U/ml of IL-1.

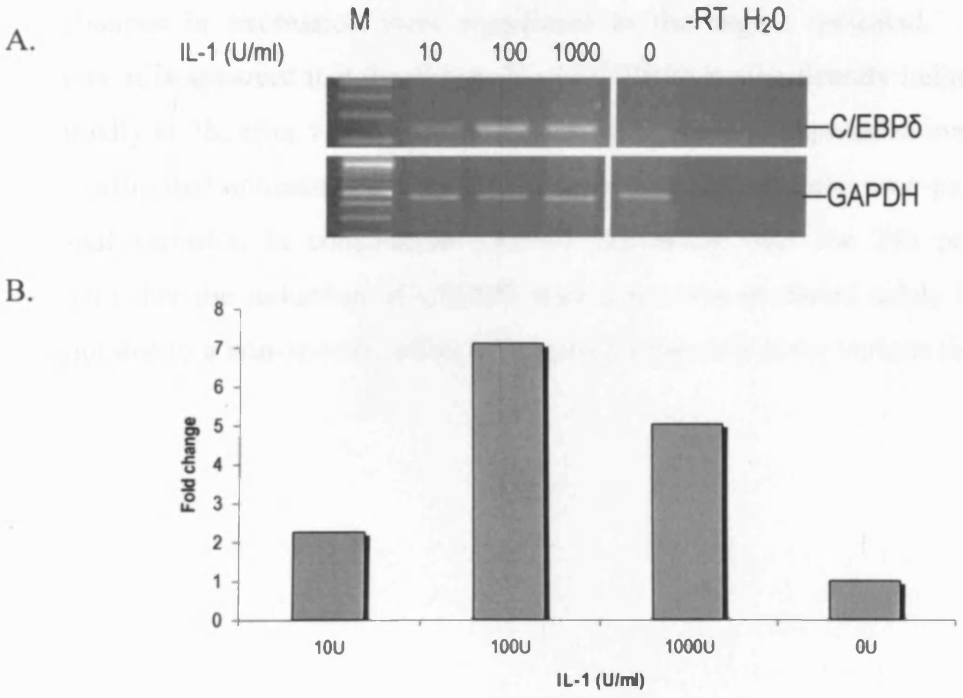
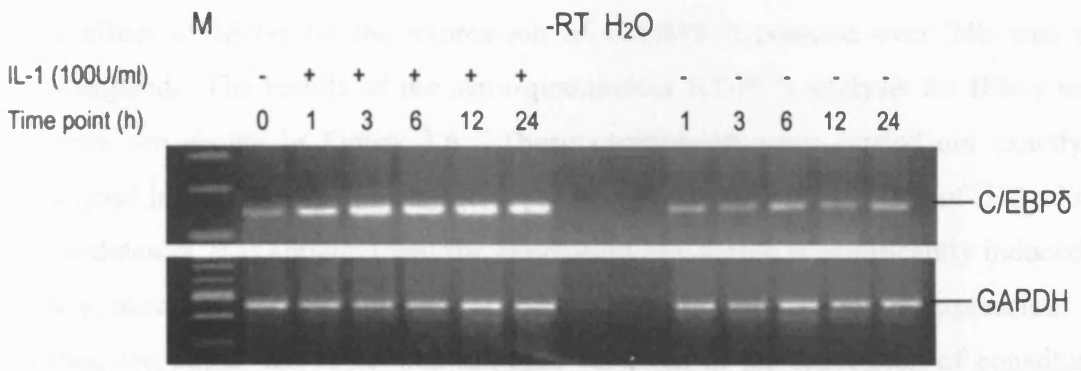



**FIGURE 3.4. Concentration-dependent effects of IL-1 on C/EBPδ mRNA expression in Hep3B cells**. Hep3B cells were treated with indicated concentrations of IL-1 and harvested for analysis 6h following treatments. Total RNA was subjected to RT-PCR, with the amplification of GAPDH serving as a control and PCR products were resolved by agarose gel electrophoresis as described in Figure 3.3. M denotes the 100bp DNA ladder used to determine the size of the PCR products. –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using 0U RNA). $H_2O$ denotes a PCR in which cDNA was replaced with an equal volume of water. Panel B displays the ratios of C/EBPδ:GAPDH, normalised to the untreated (0U) control as determined by densitometric analysis.

### 3.2.4 EFFECT OF IL-1 ON C/EBPδ MRNA EXPRESSION OVER A 24H TIME COURSE

Having established an appropriate concentration of IL-1 that produces maximal induction of C/EBPδ expression, it was next necessary to examine the effects of this cytokine on C/EBPδ induction over a period of 24h. This experiment was important because it was necessary to determine an optimal time-point for maximal C/EBPδ expression (as mediated by IL-1) for future experiments. The results of the semi-quantitative RT-PCR analysis for this experiment are shown in Figures 3.5. Hep3B cells were either treated with IL-1 (100U/ml) or left untreated and harvested for RT-PCR analysis at indicated time-points within a period of 24h (see figure legend for details). A standard statistical t-test (Appendix III) was carried out for this data and the changes in expression were significant to the degree indicated. From these datasets, it is apparent that the expression of C/EBPδ is significantly induced by IL-1, maximally at 3h, after which there is a gradual decrease in expression over 24h. It is noteworthy that untreated cells harvested at each of the indicated time-points showed minimal variation in constitutive C/EBPδ expression over the 24h period. This indicates that the induction of C/EBPδ expression was mediated solely by IL-1 and was not due to a non-specific effect of harvesting the cells at the various time-points.

A.




B.

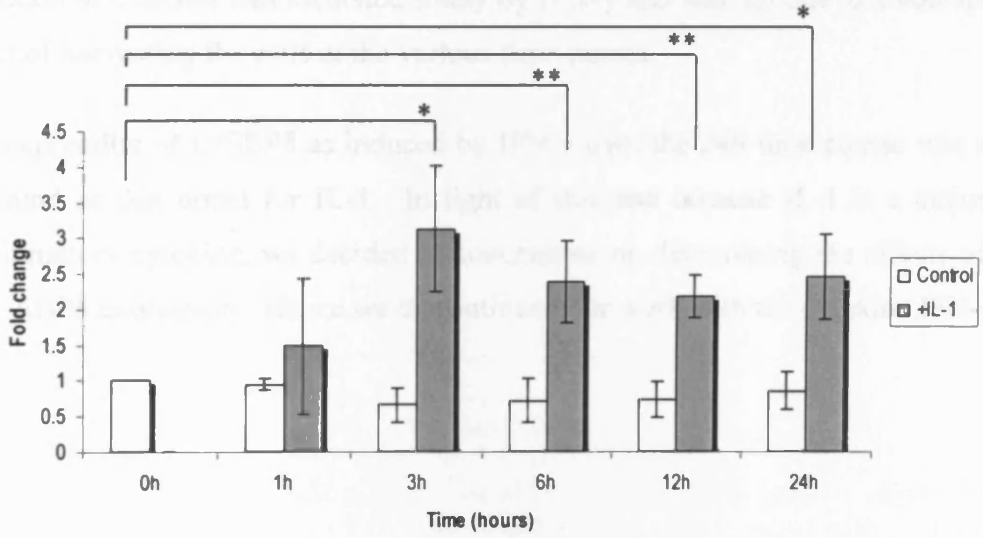



**FIGURE 3.5. Time dependent effect of IL-1 on C/EBPδ mRNA expression in Hep3B cells over a 24h time course.** Hep3B cells were either treated with IL-1 or left untreated and harvested for total RNA extraction at each of the indicated time-points. Total RNA was subjected to RT-PCR analysis and agarose gel electrophoresis as described previously. M denotes the 100bp DNA ladder used to determine the size of the PCR products. –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using 0h RNA). $H_2O$ denotes a PCR in which cDNA was replaced with an equal volume of water. Panel B displays the ratios of C/EBPδ:GAPDH, normalised to the 0h control as determined by densitometric analysis. The data shown is the mean ratio ± standard deviations (±SD) at each time-point from three independent experimental series. *P<0.05, **P<0.01.

### 3.2.5 EFFECT OF IFN-γ ON C/EBPδ MRNA EXPRESSION OVER A 24H TIME COURSE

The effect of IFN-γ on the expression of C/EBPδ expression over 24h was also investigated. The results of the semi-quantitative RT-PCR analysis for IFN-γ time-courses are shown in Figure 3.6. These experiments were carried out exactly as described in Section 3.2.4, where IFN-γ was added to the cells in place of IL-1. From these datasets, it is apparent that the expression of C/EBPδ is significantly induced by IFN-γ, maximally at 3h, after which there was a gradual decrease in expression. As before, we noted that there was minimal variation in the expression of constitutive C/EBPδ expression at indicated time points in untreated samples. Therefore, the induction of C/EBPδ was mediated solely by IFN-γ and was not due to a non-specific effect of harvesting the cells at the various time-points.

The expression of C/EBPδ as induced by IFN-γ over the 24h time course was not as profound as that noted for IL-1. In light of this and because IL-1 is a major pro-inflammatory cytokine, we decided to concentrate on determining the effects of IL-1 on C/EBPδ expression. Hence we discontinued our work with the cytokine IFN-γ.

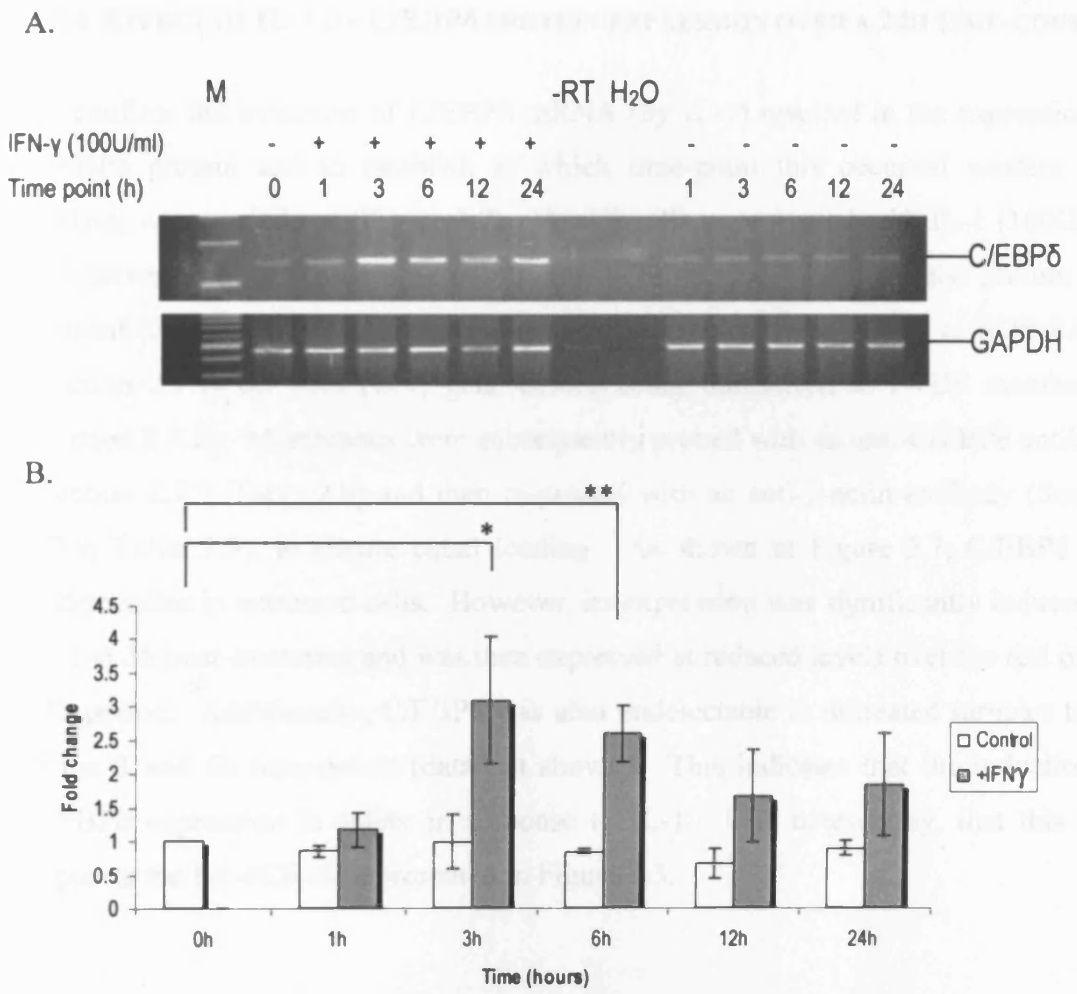



**FIGURE 3.6. Time-dependent effect of IFN-γ on C/EBPδ mRNA expression in Hep3B cells over a period of 24h**. Hep3B cells were either treated with IFN-γ or left untreated and harvested for total RNA extraction at each of the indicated time-points. Total RNA was subjected to RT-PCR analysis and agarose gel electrophoresis as described previously. M denotes the 100bp DNA ladder used to determine the size of the PCR products. –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using 0h RNA). $H_2O$ denotes a PCR in which cDNA was replaced with an equal volume of water. Panel B displays the ratios of C/EBPδ:GAPDH, normalised to the 0h control as determined by densitometric analysis. The data shown is the mean ratio ±SD at each time-point from three independent experimental series. *P<0.05, **P<0.01.

### 3.2.6 EFFECT OF IL-1 ON C/EBPδ PROTEIN EXPRESSION OVER A 24H TIME-COURSE

To confirm the induction of C/EBPδ mRNA (by IL-1) resulted in the expression of C/EBPδ protein and to establish at which time-point this occurred western blot analysis was carried out (Figure 3.7). Hep3B cells were treated with IL-1 (100U/ml) and harvested at indicated time points over a 24h period. Total cellular protein was isolated (Section 2.7.2) and 60μg of protein extracts were subjected to SDS-PAGE (Section 2.7.7) on 10% (w/v) gels, before being transferred to PVDF membranes (Section 2.7.8). Membranes were subsequently probed with an anti-C/EBPδ antibody (Section 2.7.9, Table 2.9) and then re-probed with an anti-β-actin antibody (Section 2.7.9, Table 2.9), to ensure equal loading. As shown in Figure 3.7, C/EBPδ was undetectable in untreated cells. However, its expression was significantly induced by IL-1 at 3h post-treatment and was then expressed at reduced levels over the rest of the 24h period. Additionally, C/EBPδ was also undetectable in untreated samples taken at the 3 and 6h time-points (data not shown). This indicates that the induction of C/EBPδ expression is solely in response to IL-1. It is noteworthy, that this data supports the RT-PCR data presented in Figure 3.5.

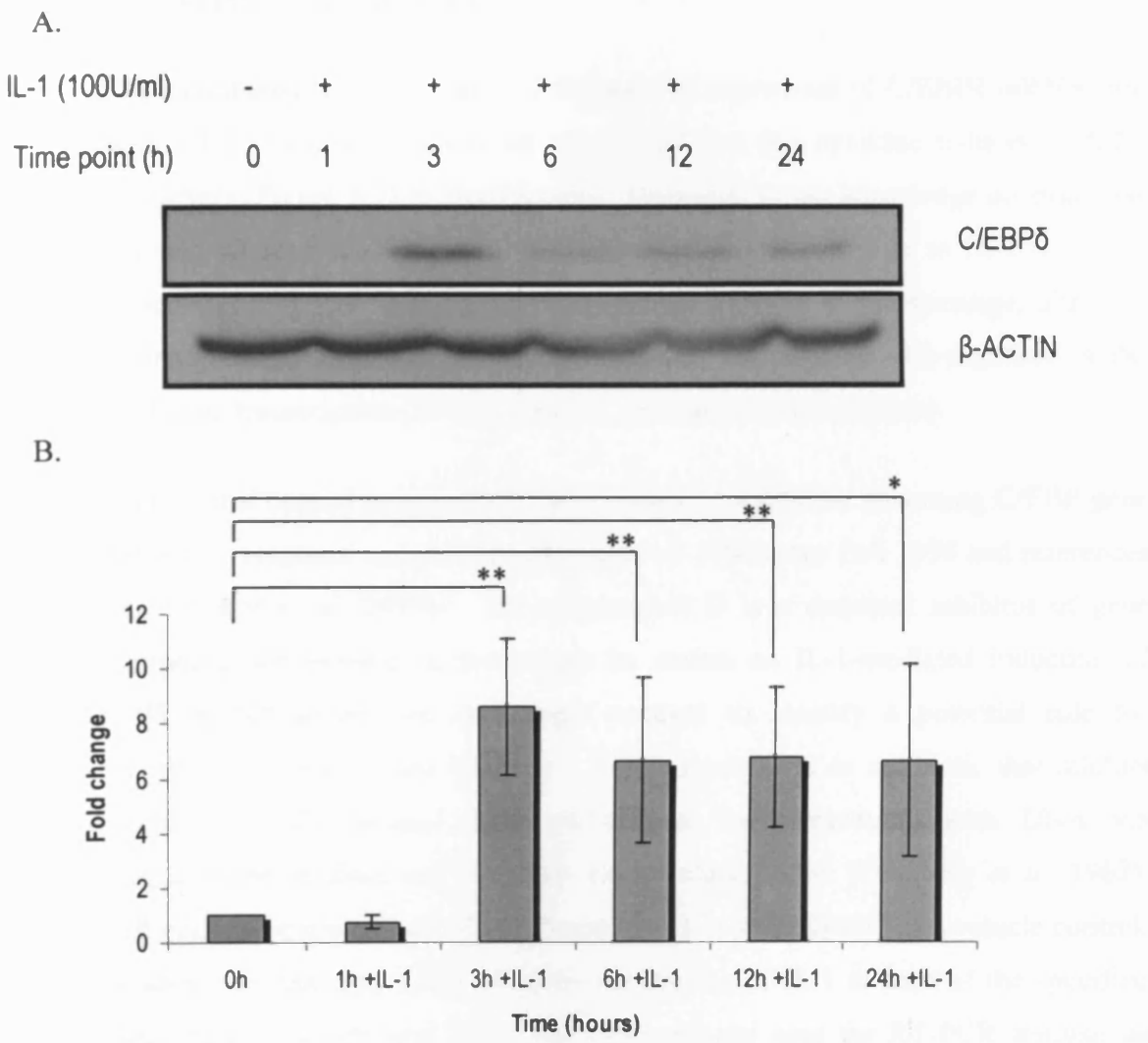



**FIGURE 3.7. Time dependent effect of IL-1 on C/EBPδ protein expression in Hep3B cells over a period of 24h**. Hep3B cells were either treated with IL-1 or left untreated and harvested for protein extraction at each of the indicated time-points. Western blot analysis was carried out using antibodies specific for C/EBPδ and β-actin (A). Panel B displays the ratios of C/EBPδ:β-actin, normalised to the 0h control as determined by densitometric analysis. The data shown is the mean ratio ± SD from three independent experimental series. *P<0.05, **P<0.01

### 3.2.7 EFFECT OF ACTINOMYCIN D ON THE IL-1-MEDIATED INCREASE OF C/EBPδ MRNA LEVELS IN HEP3B CELLS

Our data consistently shows that IL-1 induces the expression of C/EBPδ mRNA (see Figures 3.3-3.5) and in addition we also show that this cytokine induces C/EBPδ protein levels (Figure 3.7) in Hep3B cells. However, to our knowledge no study has determined whether the induction of C/EBPδ mRNA in response to IL-1 is due to increased transcription of the gene or increased stability of the message, although preliminary results from our laboratory suggested the response was regulated at the level of gene transcription (Dr F.T. Kockar, personal communication).

Transcriptional control is one of the main modes of regulation governing C/EBP gene expression in response to cytokines (Ramji *et al.* 1993a; see Poli 1998 and references therein; Niehof *et al.* 2001b). As actinomycin D is a chemical inhibitor of gene transcription, we decided to investigate its action on IL-1-mediated induction of C/EBPδ mRNA expression as a rapid method to identify a potential role for transcriptional control in the response. Actinomycin D is an antibiotic that inhibits the action of DNA-primed RNA polymerase by complexing with DNA via deoxyguanosine residues and therefore blocks transcription (Goldberg *et al.* 1962). Hep3B cells were pre-treated with actinomycin D or with DMSO as a vehicle control. They were then cultured in the presence or absence of IL-1 at each of the specified time-points after which total RNA was extracted and used for RT-PCR analysis as described previously. As shown in Figure 3.8, the presence of actinomycin D prevented the increase in C/EBPδ mRNA by IL-1 at each of the indicated time-points. In contrast, the induction of C/EBPδ in response to IL-1 was evident in cells treated with DMSO alone, harvested at the 3h time-point as a control. Together, these results suggest that increased transcriptional activation of the C/EBPδ gene is necessary to produce the induction in mRNA levels by IL-1.

To firmly determine whether transcriptional activation of the human C/EBPδ gene is the main mechanism by which IL-1 mediates its induction, other experiments would have to be carried out. Monitoring the decay of C/EBPδ mRNA over a time, in Hep3B cells pre-treated with actinomycin D, would be useful to determine the half-life of the message. Also, nuclear run-on transcription assays conducted with extracts

from IL-1 treated and untreated Hep3B cells would help definitively establish whether the C/EBPδ gene was indeed transcribed in response to IL-1.

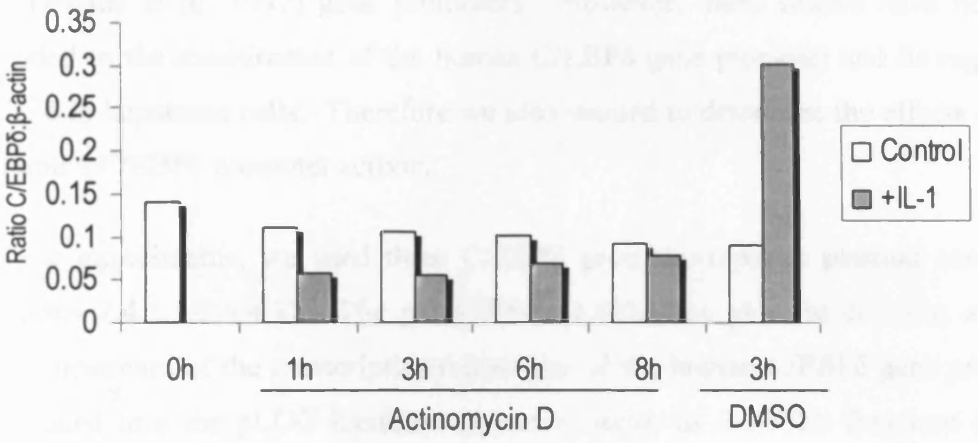



**FIGURE 3.8 Effect of actinomycin D on the induction of C/EBPδ mRNA levels by IL-1.** Hep3B cells were pretreated with actinomycin D (5μg/ml) or with DMSO as a vehicle control for 1h (Cavin *et al.* 2003). They were then either treated with IL-1 (100U/ml) or left untreated (Control) and harvested for RT-PCR analysis at each of the indicated time-points. The PCR products for C/EBPδ and β-actin were resolved by agarose gel electrophoresis as previously described. The graph shows the ratios of C/EBPδ:β-actin at each of the specified time-points as determined by densitometric analysis. Results are representative of two independent experiments.

## 3.3 INVESTIGATIONS INTO THE REGULATION OF C/EBPδ PROMOTER ACTIVITY

### 3.3.1 EFFECT OF CYTOKINES ON C/EBPδ PROMOTER ACTIVATION

Our data suggests that IL-1 induces the expression of C/EBPδ at the level of gene transcription (Figure 3.8). As a result, we decided to initiate investigations into studying the effects of this cytokine on C/EBPδ promoter activation. It was of particular interest for us to pursue this line of investigation because to our knowledge no study has reported the effects of this cytokine on C/EBPδ promoter activity.

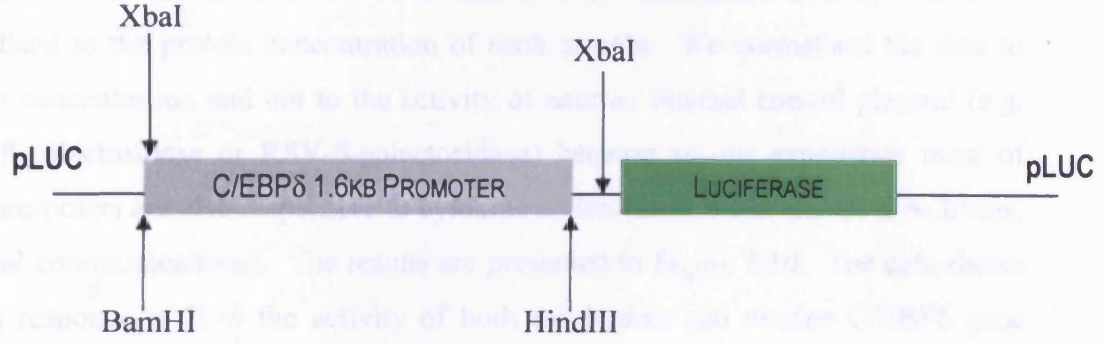
It has also been reported that IL-6-mediated expression of the C/EBPδ is a transcriptional response (see section 1.3.1.3), as demonstrated by several studies

(Ramji *et al.* 1993a; Yamada *et al.* 1997; Cantwell *et al.* 1998; Davies *et al.* 2000). These studies (Yamada *et al.* 1997; Cantwell *et al.* 1998) show that C/EBPδ gene transcription is regulated at least in part, by key proximal promoter regions, containing STAT3 and Sp1 *cis*-acting elements. Mutation of these sites abrogates IL-6-mediated C/EBPδ gene promoter activation from mouse (Cantwell *et al.* 1998) and rat (Yamada *et al.* 1997) gene promoters. However, these studies have not been extended to the examination of the human C/EBPδ gene promoter and its regulation by IL-6 in hepatoma cells. Therefore we also wanted to determine the effects of IL-6 on human C/EBPδ promoter activity.

For our experiments, we used three C/EBPδ promoter-reporter plasmid constructs (Sections 2.4.4.1-2.4.4.3). The pHuC/EBPδ[1.6kb]-Luc plasmid contains a 1.6kb region upstream of the transcriptional start site of the human C/EBPδ gene promoter sub-cloned into the pLUC luciferase reporter vector as a 1.75kb fragment (Figure 3.9). The pHuC/EBPδ[0.2kb]-Luc plasmid contains a 0.2kb region upstream of the transcriptional start site of the human C/EBPδ gene promoter cloned into the pGL2-Basic luciferase reporter vector (Appendix I). Both these constructs were considered to be suitable models to study the activity of the human C/EBPδ gene promoter in order to provide data directly relevant to the regulation of gene expression in Hep3B cells. Finally, the pMoC/EBPδ[2.2kb]-Luc plasmid which contains a 2.2kb (-2146 to +73) region of the murine C/EBPδ gene promoter cloned into the pGL2-Basic luciferase reporter vector was also used. This reporter plasmid was used in our experiments alongside the human C/EBPδ promoter-reporter constructs described above for comparative purposes, since the murine C/EBPδ gene promoter has previously been extensively characterised (Cantwell *et al.* 1998; O'Rourke *et al.* 1999a). All the plasmids were confirmed by sequencing (Section 2.6.8) or digestion with restriction enzymes (Section 2.6.7). Both the pMoC/EBPδ[2.2kb]-Luc and pHuC/EBPδ[0.2kb]-Luc plasmid inserts were sequenced using the GL1 and GL2 universal primers (Appendix IV). The pHuC/EBPδ[1.6kb]-Luc plasmid insert was confirmed by restriction endonuclease digestion (Figure 3.9) and by partial DNA sequence analysis. Two internal primers (Appendix IV) were designed to confirm the sequence of the proximal region of this promoter fragment. The entire sequence of this insert has previously been determined by Dr F.T. Kockar (personal communication). For the preparation of plasmid constructs for transfection based

assays, plasmid DNA was used to transform the bacterial *E. coli* strain DH5α. Plasmid DNA was then extracted from mini cultures grown from a single bacterial colony by the Miniprep method (Section 2.6.6.3). The same bacterial colony was also used to prepare glycerol stocks and for further extraction of plasmid DNA by the Maxiprep method (Section 2.6.6.4). All plasmid constructs that were used are described briefly in section 2.4.4.

A.




B.

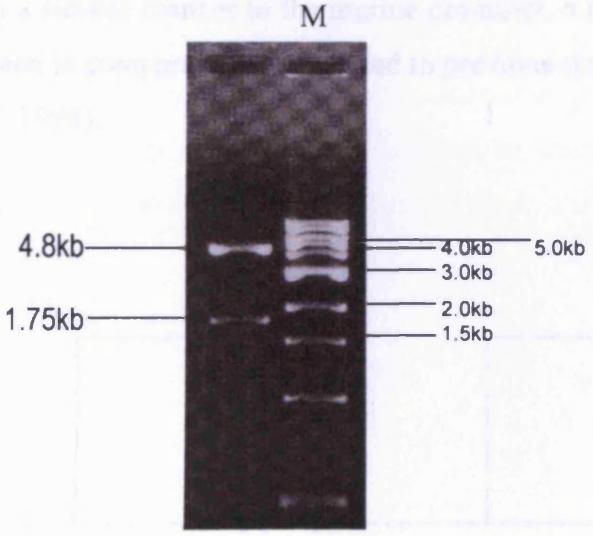



**FIGURE 3.9. A schematic representation and an analytical digest of the pHuC/EBPδ[1.6kb]-Luc plasmid.** The 1.6kb human C/EBPδ gene promoter was sub-cloned into the pLUC luciferase reporter vector as a 1.75kb BamHI-HindIII fragment. The promoter insert is also flanked by two XbaI sites (A). Panel B shows the results of the analytical digest of the pHuC/EBPδ[1.6kb]-Luc plasmid as digested with the XbaI restriction endonuclease. The digested plasmid was size fractionated on a 1% (w/v) agarose gel. The sizes of the digested DNA fragments were determined by comparing them to standard DNA markers, denoted as 'M' (New England Biolabs, 1kb DNA ladder. See Appendix II). The size of some of these DNA markers is indicated on the right. The expected size fragments of 4.8kb (plasmid) and 1.75kb (cloned promoter insert) were obtained as shown on the left of the agarose gel image.

### 3.3.1.1 EFFECT OF IL-6 ON C/EBPδ PROMOTER ACTIVATION

Hep3B cells were transfected with either the pHuC/EBPδ[1.6kb]-Luc plasmid or the pMoC/EBPδ[2.2kb]-Luc plasmid (Section 2.4.1). Transfected cells were treated with either IL-6 or left untreated for 30h [previously identified as the optimal time-point by Dr F.T. Kockar (personal communication)]. The cells were then harvested and extracts prepared for luciferase assay and the protein concentration for each sample was determined (Sections 2.4.2, 2.4.3 and 2.7.4). Luciferase activity was then normalised to the protein concentration of each sample. We normalised the data to protein concentration and not to the activity of another internal control plasmid (e.g. CMV-β-galactosidase or RSV-β-galactosidase) because in our experience most of these promoters are also responsive to cytokine action (Dr P. Foka and Dr S.A. Irvine, personal communications). The results are presented in Figure 3.10. The data shows that in response to IL-6 the activity of both the human and murine C/EBPδ gene promoters is significantly induced, indicating that the human C/EBPδ gene promoter is regulated in a similar manner to the murine promoter, a novel finding. The degree of induction seen is comparable to that noted in previous studies (Yamada *et al.* 1997; Cantwell *et al.* 1998).

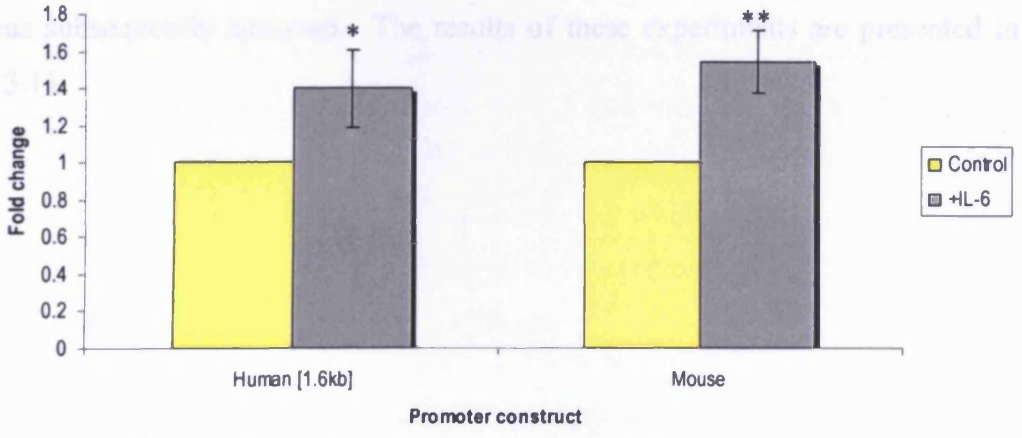



**FIGURE 3.10 Analysis of the effect of IL-6 treatment on the activity of the human and mouse C/EBPδ promoters in Hep3B cells.** Hep3B cells were transfected with either the pHuC/EBPδ[1.6kb]-Luc (5μg) or the pMoC/EBPδ[2.2kb]-Luc (5μg) plasmids (Human [1.6kb] and Mouse respectively) as described in section 2.4.1. Transfected cells were either treated with IL-6 (1000U/ml) or left untreated (Control) for 30h. Cell extracts were then prepared for luciferase reporter activity assay. Luciferase activity was normalised to the protein concentration of each sample and is presented as a fold change in response to IL-6 relative to the untreated control (assigned as 1). Results are presented as mean ±SD from three independent experiments, where all treatments were carried out in triplicate in each independent experimental series. *P<0.05, **P<0.01.

### 3.3.1.2 EFFECT OF IL-1 ON C/EBPδ PROMOTER ACTIVATION

We next determined the effect of IL-1 on C/EBPδ promoter activity. As before both the human (pHuC/EBPδ[1.6kb]-Luc ) and murine (pMoC/EBPδ[2.2kb]-Luc) C/EBPδ promoter-reporter plasmids were used in these experiments. In addition we also decided to assess the effect of IL-1 on promoter activity of the pHuC/EBPδ[0.2kb]-Luc plasmid (Section 2.4.4.2), containing the shorter fragment of the human C/EBPδ gene promoter. This is because large gene promoter fragments can potentially contain silencer elements that could mask the effects of extracellular mediators on promoter activity in transient transfection assays, as previously shown (Cantwell *et al.* 1998). To verify that our transfection system was able to produce a response to IL-1 action at the level of promoter activity, we decided to assess the effect of IL-1 on NF-κB activation. The IL-1-mediated activation of NF-κB is a well documented phenomenon (see O'Neill 2000; O'Neill 2002 and references therein). For this, we used the plasmid pNFκB-Luc (Section 2.4.4.4). This reporter plasmid contains four NF-κB consensus enhancer elements fused upstream of the firefly luciferase gene. Hep3B cells were transfected with each of the plasmids described above exactly as detailed in the figure legend of Figure 3.10, except the cells were treated with IL-1 in place of IL-6. The activity of the various luciferase reporter-constructs in response to IL-1 was subsequently analysed. The results of these experiments are presented in Figure 3.11.

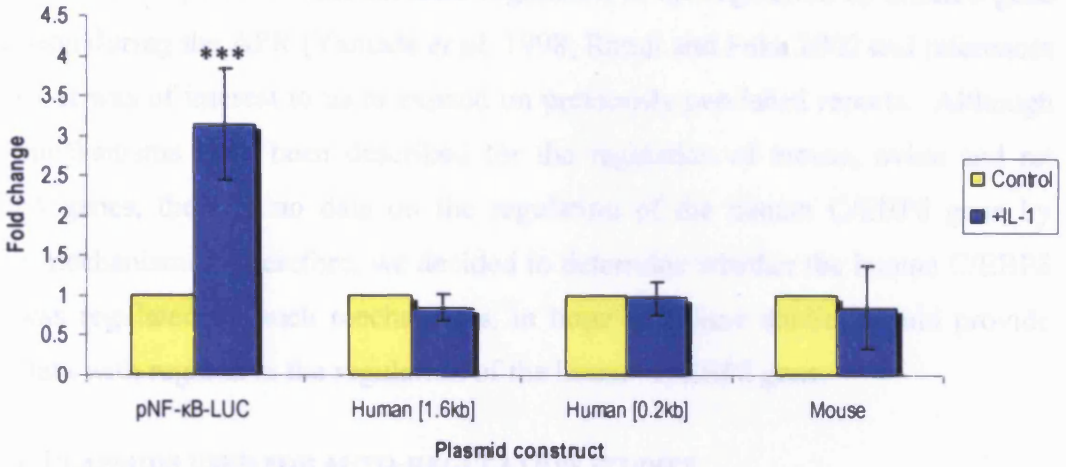



**FIGURE 3.11 Analysis of the effect of IL-1 treatment on the activity of the human and mouse C/EBPδ promoters in Hep3B cells.** Hep3B cells were transfected with the pNFκB-Luc plasmid (5μg) as a control, or each of the three specified C/EBPδ gene promoter-reporter plasmid constructs (5μg) as described in Section 2.4.1. Transfected cells were either treated with IL-1 (100U/ml) or left untreated (Control) for 30h. Cell extracts were then prepared for luciferase reporter activity assay. Luciferase activity was normalised to the protein concentration of each sample and is presented as a fold change in response to IL-1 relative to the untreated control (assigned as 1). Results are presented as mean ±SD from three independent experiments, where all treatments were carried out in triplicate in each independent experimental series. ***P<0.001.

As expected, the data presented in Figure 3.11 shows that IL-1 significantly activates pNFκB-Luc plasmid in Hep3B cells, with a ~3.2 fold induction in reporter activity observed in response to this cytokine. In addition, we did not observe an increase in C/EBPδ promoter activity in response to IL-1 in any of the three promoter-reporter constructs used in our experiments. Some potential explanations for these results are discussed in detail in the section 3.4 of this chapter.

### 3.3.2 INVESTIGATIONS INTO C/EBPδ PROMOTER ACTIVATION THROUGH AUTO-REGULATORY MECHANISMS

A number of studies have shown that the expression of the C/EBPδ gene is regulated through auto-regulatory mechanisms in a variety of species, although species-specific differences have been reported (Yamada *et al.* 1998; O'Rourke *et al.* 1999a; Davies *et al.* 2000; Tanabe *et al.* 2000).

As there is also a potential role for auto-regulation in the regulation of C/EBPδ gene expression during the APR (Yamada *et al.* 1998; Ramji and Foka 2002 and references therein), it was of interest to us to expand on previously published reports. Although these mechanisms have been described for the regulation of mouse, ovine and rat C/EBPδ genes, there is no data on the regulation of the human C/EBPδ gene by similar mechanisms. Therefore, we decided to determine whether the human C/EBPδ gene was regulated by such mechanisms, in hope that these studies would provide novel data with regards to the regulation of the human C/EBPδ gene.

#### 3.3.2.1 PLASMIDS USED FOR AUTO-REGULATION STUDIES

For our experiments, we used the three promoter-reporter constructs detailed above and three expression plasmids present in our laboratory (Section 2.4.4). The MSVβ and –δ expression plasmids contain full length cDNA encoding wild-type murine C/EBPβ and –δ respectively. The pCS2xα expression plasmid contains a cDNA encoding wild-type *Xenopus laevis* C/EBPα protein. Given that the C/EBP proteins are both highly conserved at the level of sequence and function (see Ramji and Foka 2002 and references therein) it was considered acceptable to use expression plasmids specifying C/EBP proteins from different species in our experiments as previously demonstrated (Foka *et al.* 2001). All plasmids were confirmed by restriction endonuclease digestion (carried out by an undergraduate project student Anoushka Takooree). Table 3.0 details the restriction enzymes used to confirm the identity of the plasmids and the size of the cDNA insert excised by digestion. Due to the unavailability of the empty plasmid vectors used in the MSV-β and –δ constructs, pcDNA3 was transfected into cells as a control, as previously described (O'Rourke *et al.* 1999a). The empty plasmid vector pCS2x, was used as a control plasmid for experiments involving the expression plasmid pCS2xα.

**TABLE 3.0 Restriction endonuclease digestion of expression plasmid constructs.**

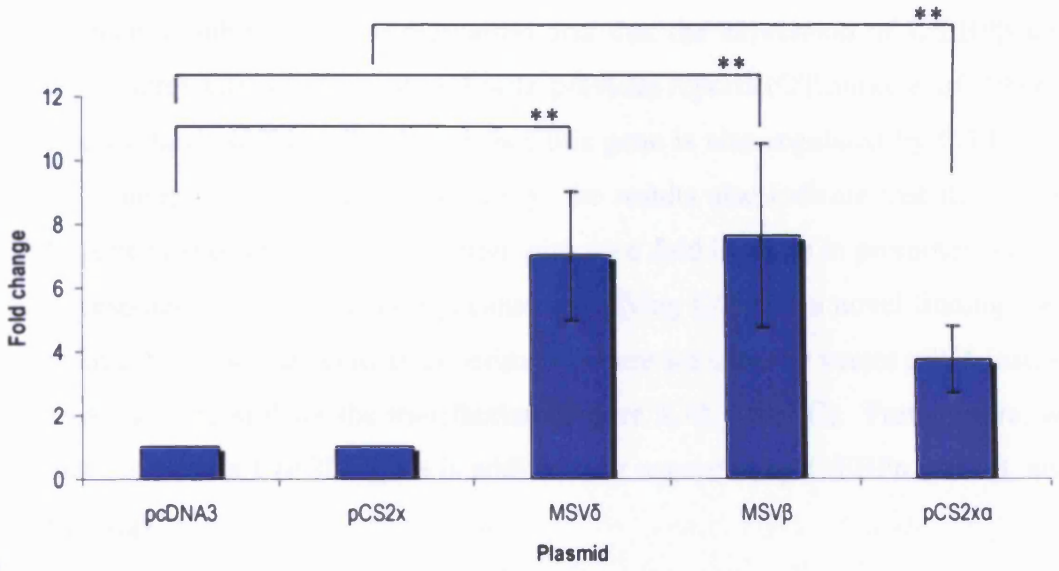
| PLASMID | RESTRICTION ENDONUCLEASE(S) | cDNA INSERT (kB) |
|---|---|---|
| MSVβ | EcoR1, BamH1 | 1.5 |
| MSVδ | EcoR1, BamH1 | 1 |
| pCS2xα | EcoR1 | 1.7 |

### 3.3.2.2 EFFECT OF C/EBPα, -β AND –δ PROTEINS ON THE ACTIVITY OF THE MURINE AND HUMAN C/EBPδ GENE PROMOTERS

As previous studies have indicated that the murine C/EBPδ gene is subject to auto-regulation (O'Rourke *et al.* 1999a), we decided to include the murine C/EBPδ gene promoter-reporter construct in our experiments for comparative purposes. Hep3B cells were co-transfected with either the pMoC/EBPδ[2.2kb]-Luc promoter construct or the pHuC/EBPδ[1.6kb]-Luc promoter construct and expression plasmids specifying either C/EBPδ (MSVδ), C/EBPβ (MSVβ) or C/EBPα (pCS2xα) protein. As a control, cells were also co-transfected with either the pMoC/EBPδ[2.2kb]-Luc promoter construct or the pHuC/EBPδ[1.6kb]-Luc promoter construct and the empty expression vectors pcDNA3 or pCS2x appropriately. As an additional control, Hep3B cells were also co-transfected with the pHuC/EBPδ[1.6kb]-Luc promoter construct and either MSVδ or MSVβ and an alternative empty expression vector, pSG5. Transfections were carried out as described previously and results are presented in Figure 3.12.

**FIGURE 3.12. Effect of C/EBPδ, C/EBPβ and C/EBPα proteins on the activity of the murine (pMoC/EBPδ[2.2kb]-Luc) and human (pHuC/EBPδ[1.6kb]-Luc) C/EBPδ gene promoters in Hep3B cells.** Hep3B cells were co-transfected with either the pMoC/EBPδ[2.2kb]-Luc promoter construct (**A**) or the pHuC/EBPδ[1.6kb]-Luc promoter construct (**B**) and expression plasmids specifying either C/EBPδ (MSVδ), C/EBPβ (MSVβ) or C/EBPα (pCS2xα) proteins. As a control, cells were also co-transfected with either the pMoC/EBPδ[2.2kb]-Luc promoter construct or the pHuC/EBPδ[1.6kb]-Luc promoter construct and the vectors pcDNA3 or pCS2x appropriately. For the experiment displayed in Panel **C**, Hep3B cells were co-transfected with pHuC/EBPδ[1.6kb]-Luc and MSVδ or MSVβ plasmids. As a control, pHuC/EBPδ[1.6kb]-Luc was also co-transfected with another control expression plasmid pSG5. All transfections were carried out as specified in Section 2.4.1 using 5μg of each of the specified plasmids and cells were harvested 36h post transfection for luciferase reporter assays. In all experiments, luciferase activity was normalised to the protein concentration of each sample. In Panels **A** and **B** this is presented as a fold change relative to the pcDNA3 or pCS2x transfected controls appropriately (assigned as 1). Results are presented as mean ±SD from four independent experiments, where all treatments were carried out in triplicate in each independent experimental series. **P<0.01, ***P<0.001. In Panel **C**, luciferase activity is expressed as the mean ratio of triplicate samples. Error bars show the ±SD between triplicate samples.

A.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12
10
8
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4
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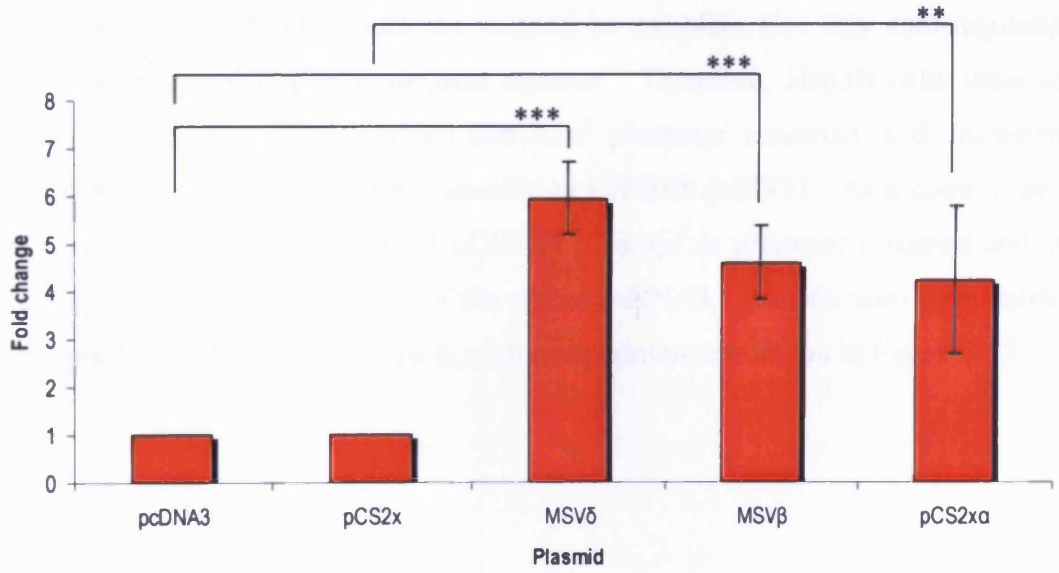
pCS2x
MSVδ
MSVβ
pCS2xα
Plasmid

B.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8
7
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pCS2x
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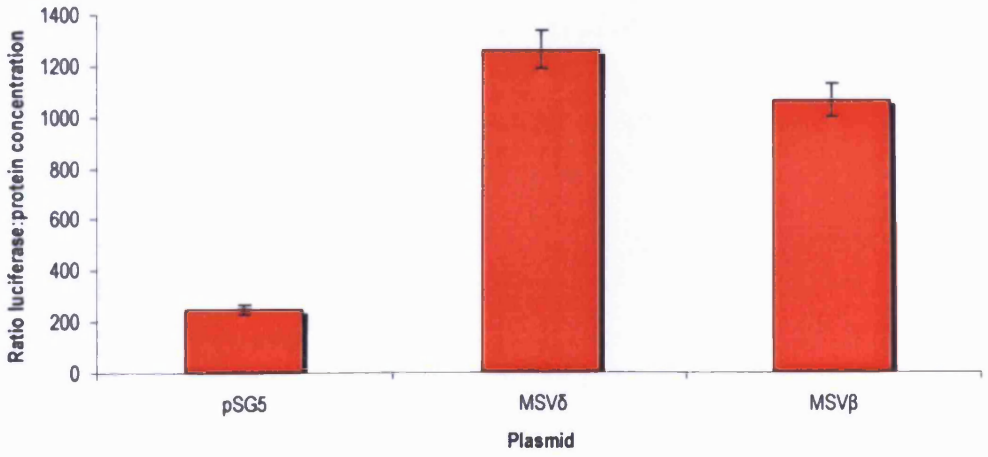
pCS2xα
Plasmid

C.


1400
1200
1000
800
600
400
200
0
Ratio luciferase:protein concentration
pSG5
MSVδ
MSVβ
Plasmid

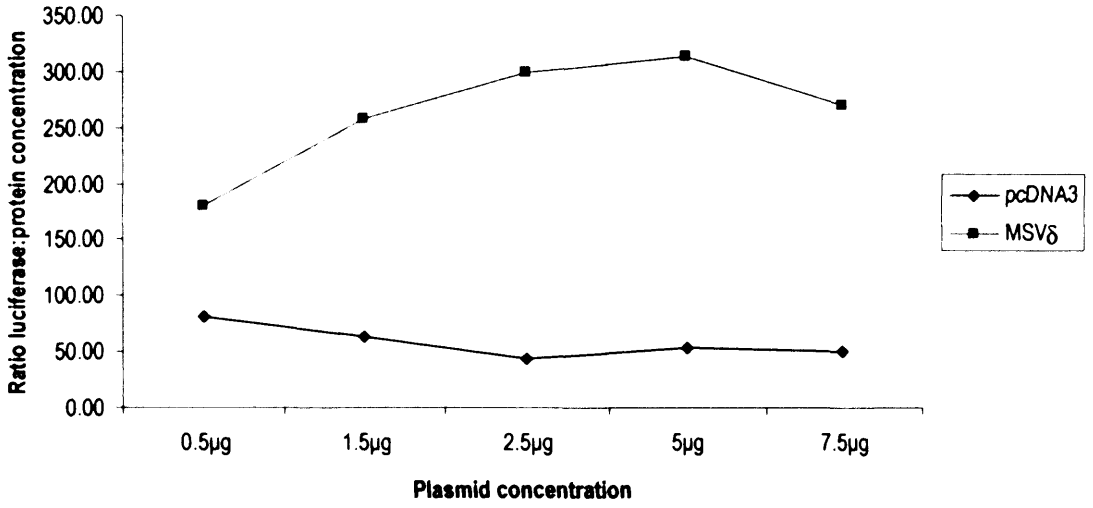



**FIGURE 3.13. Effect of increasing amounts of the expression plasmid MSVδ, on the activity of the human C/EBPδ promoter (pHuC/EBPδ[1.6kb]-Luc).** Hep3B cells were co-transfected with the pHuC/EBPδ[1.6kb]-Luc promoter construct (5μg) and increasing amounts of the expression plasmid specifying for C/EBPδ (MSVδ). As a control, cells were also co-transfected with the pHuC/EBPδ[1.6kb]-Luc promoter construct (5μg) and the increasing corresponding amounts of the vector pcDNA3. Transfections were carried out as specified in Figure 3.12. Luciferase activity was normalised to the protein concentration of each sample and is expressed as the mean ratio of duplicate samples. This experiment was carried out in conjunction with an undergraduate project student, Anoushka Takooree.

Consistent with the data in Figure 3.12, the results of Figure 3.13 show that the human C/EBPδ gene is subject to auto-regulation. In addition, we show that this activation occurs in a concentration dependent manner. That is, there is an increase in promoter activation in response to increasing amounts of the expression plasmid MSVδ, with no noted increase in control cells transfected with pcDNA3. We did note a slight decrease in promoter activation with 7.5μg of the expression plasmid, possibly due to transcriptional squelching (Ptashne 1988; Natesan *et al.* 1997).

To begin to isolate the DNA sequence elements that may be responsible for this auto-regulatory effect, we wanted to investigate whether the plasmid construct containing the minimal human C/EBPδ gene promoter (pHuC/EBPδ[0.2kb]-Luc) was also responsive to the expression plasmids specifying C/EBPδ, -β and –α. Therefore, Hep3B cells were co-transfected exactly as described in Figure 3.12 with the pHuC/EBPδ[0.2kb]-Luc reporter construct. The results of these experiments are presented in Figure 3.14.

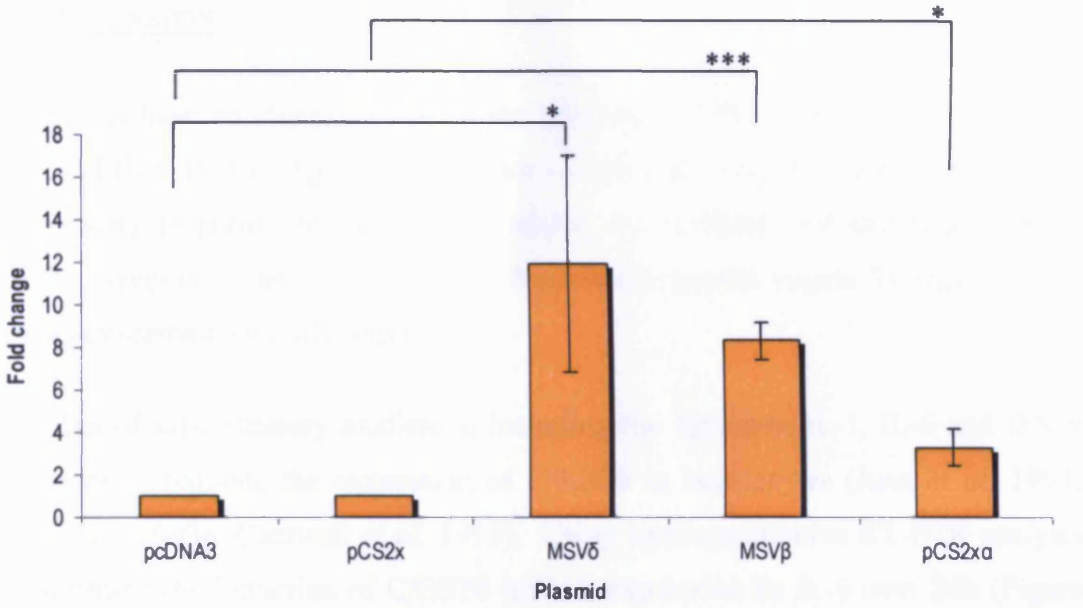



**FIGURE 3.14. Effect of C/EBPδ, C/EBPβ and C/EBPα proteins on the activity of the human C/EBPδ (pHuC/EBPδ[0.2kb]-Luc) minimal promoter in Hep3B cells.** Hep3B cells were co-transfected with 5μg of the pHuC/EBPδ[0.2kb]-Luc promoter construct and 5μg of the expression plasmids specifying either C/EBPδ (MSVδ), C/EBPβ (MSVβ) or C/EBPα (pCS2xα) protein. As a control, cells were also co-transfected with 5μg of the pHuC/EBPδ[0.2kb]-Luc promoter construct and 5μg of the vectors pcDNA3 or pCS2x appropriately. Transfections were carried out exactly as specified in Figure 3.12. Results are presented as mean ±SD from three independent experiments, where all treatments were carried out in triplicate in each independent experimental series. *P<0.05, ***P<0.001.

From the data presented in Figure 3.14, it is apparent that the pHuC/EBPδ[0.2kb]-Luc promoter construct is also activated in response to expression plasmids specifying for C/EBPδ, -β and –α. These results indicate that the sequence elements responsible for mediating the auto-regulatory effects of the human C/EBPδ gene are located within the proximal promoter region of the human C/EBPδ gene.

## 3.4 DISCUSSION

Hepatocytes have an important role in the regulation of the APR. The transcription factor C/EBPδ is an important regulator of hepatic gene expression during the inflammatory response (Section 3.1). Studying the cytokine-mediated regulation of C/EBPδ expression may help develop potential therapeutic targets for treatment of diseases exacerbated by inflammation.

A number of inflammatory mediators, including the cytokines IL-1, IL-6 and IFN-γ are known to regulate the expression of C/EBPδ in hepatocytes (Juan *et al.* 1993; Ramji *et al.* 1993a; Cantwell *et al.* 1998). Using semi-quantitative RT-PCR analysis we confirmed the induction of C/EBPδ mRNA expression by IL-6 over 24h (Figure 3.2). The observation that C/EBPδ expression is maintained over 24h suggests that further signalling events are induced to sustain its expression, possibly through auto-regulation (see Ramji and Foka 2002 and references therein). We also showed the induction of C/EBPδ expression by IL-1 and IFN-γ in Hep3B cells (Figure 3.3) consistent with data presented in the aforementioned publications. In addition, we noted that the induction of C/EBPδ mRNA in response to IFN-γ compared to the induction by IL-6 and IL-1 was notably less. These data are consistent with the results presented by Cantwell *et al.* (1998). Data presented in this paper show that both IL-6 and IFN-γ induce C/EBPδ mRNA expression and that the observed induction with IFN-γ is less profound that that noted for IL-6 (as determined by northern blotting) in Hep3B cells.

Previous studies have reported that hepatic C/EBPδ expression is induced in animal models of the APR, stimulated with LPS (Alam *et al.* 1992). However, we did not note any increase in C/EBPδ expression in hepatocytes stimulated with this mediator (Figures 3.3). Data presented by Hetherington *et al.* (1999) indicates that Hep3B cells express the human LPS receptor, CD14 at very low levels compared to macrophages for example. This observation may potentially explain why we did not note an increase in C/EBPδ expression in response to LPS in our experiments. It is therefore likely that the induction in C/EBPδ expression noted in liver extracts of LPS-treated animals is not triggered by LPS stimulation of hepatocytes directly but is secondary to macrophage activation by this mediator. Intravenous administration of LPS induces an inflammatory response primarily through macrophage activation in treated

animals. Activated macrophages release a broad spectrum of cytokines including IL-1 and TNF-α. The release of these cytokines also triggers the production of other cytokines such as IL-6 at the site of inflammation. These cytokines can act both locally and distally and can trigger APP release from the liver (Baumann and Gauldie 1994). Therefore, it is possible that this wave of cytokine release is responsible for triggering the expression of hepatic C/EBPδ observed in LPS-stimulated animal models of the APR. Alternatively, the increase in C/EBPδ expression observed in liver extracts may be due to an induction of C/EBPδ expression in Kupffer cells (liver macrophages) as C/EBPδ is also induced in LPS-treated macrophages (Tengku-Muhammad *et al.* 2000).

We also did not note any increase in C/EBPδ expression in response to the cytokine TNF-α (Figure 3.3). This is consistent with the findings of Yin *et al.* (1996). This study showed that rather than inducing the expression (determined at the mRNA and protein level) of C/EBPβ and –δ, TNF-α promoted rapid nuclear accumulation of C/EBPβ and –δ, (between 5-180min after treatment) in conditionally transformed rat hepatocytes (cultured at the permissive temperature of 33°C). Therefore, it is plausible that whilst cytokines such as IL-1 and IL-6 induce the expression of hepatic C/EBPδ (Juan *et al.* 1993; Ramji *et al.* 1993a), the effect of TNF-α may be to promote nuclear localisation of the newly synthesised C/EBPδ protein inside the cell, rather than induce its expression.

We also determined that the expression of C/EBPδ mRNA as induced by IL-1 and IFN-γ was maximal at 3h post treatment and that the levels of C/EBPδ remained induced although at reduced levels over 24h (Figures 3.5 and 3.6). These are novel data, as to our knowledge no study has determined the effects of these cytokines on C/EBPδ mRNA expression over an extensive time-course in Hep3B cells. We additionally showed that C/EBPδ protein expression was induced at the same time-point after IL-1 treatment and that C/EBPδ was undetectable in untreated cells (Figure 3.7). Data presented by Juan *et al.* (1993) report similar findings in nuclear extracts prepared from Hep3B cells treated with IL-1.

The IL-6-mediated expression of C/EBPδ is reported to be regulated at the level of gene transcription (Ramji *et al.* 1993a; Yamada *et al.* 1997; Cantwell *et al.* 1998; Davies *et al.* 2000). Studies have reported a ~2 fold increase in C/EBPδ promoter

activation in response to this cytokine (Yamada *et al.* 1997; Cantwell *et al.* 1998; Davies *et al.* 2000) from various species. However, similar investigations into of the effect of IL-6 on human C/EBPδ gene promoter activity have not been published. This prompted us to initiate investigations into this area. We showed that IL-6 did significantly induce the activation of the human C/EBPδ gene promoter (Figure 3.10) and confirmed the effect of this cytokine on the murine gene promoter (Cantwell *et al.* 1998). Consistent with our results a recent study reported similar findings (Sanford and DeWille 2005). That is, the human C/EBPδ gene promoter is indeed activated in response to IL-6 (although in a different cell line, LNCaP) and the degree of induction is comparable to that observed in our transfection system. Previous reports have implicated STAT3 and Sp1 transcription factors in conferring IL-6-mediated activation of the C/EBPδ gene promoters (Yamada *et al.* 1997; Cantwell *et al.* 1998) and Sanford and DeWille (2005) confirm that these transcription factors are required for the IL-6-mediated activation of the human C/EBPδ gene promoter.

Despite showing that the IL-1-mediated induction of C/EBPδ mRNA may be a transcriptional response (Figure 3.8) we were unable to note an increase in C/EBPδ promoter activation in response to this cytokine in any of the three promoter-reporter constructs used (Figure 3.11). We ruled out the possibility that this was a result of our transfection system because we showed the activation of NF-κB by IL-1 at the promoter level. It is possible that we did not observe promoter activation in response to IL-1 in our transfection system due to a potential requirement for a specific chromatin context that is absent in our transfected plasmids but present in the endogenous C/EBPδ gene *in situ*. Additionally, it may be that the promoter elements necessary to confer IL-1-mediated promoter activation are simply absent from the promoter reporter constructs we used. Another potential explanation is that the 5' end of the C/EBPδ gene is not responsible for mediating the IL-1-mediated induction of C/EBPδ gene transcription. Indeed, the rat C/EBPδ gene is auto-regulated through sequences present at the 3' end of the gene (Yamada *et al.* 1998). Moreover, despite the fact that studies have shown that IL-1 readily induces C/EBPδ mRNA and protein levels (Juan *et al.* 1993; Magalini *et al.* 1995; Hungness *et al.* 2002b) in a number of different systems, there are no published reports examining the effect of IL-1 on C/EBPδ promoter activity.

Studies have reported that the C/EBPδ gene promoter is activated through auto-regulation (O'Rourke *et al.* 1999a; Davies *et al.* 2000). Auto-regulation of the mouse C/EBPδ gene may occur through two C/EBP binding sites identified in the distal 5' promoter region (O'Rourke *et al.* 1999a). We confirmed that the murine C/EBPδ gene is auto-regulated with a ~7 fold increase in promoter activity observed when this promoter reporter construct was co-transfected with an expression plasmid specifying for C/EBPδ, consistent with previous findings (Figure 3.12, Panel A). We also showed that this promoter is activated in the presence of an expression plasmid specifying for C/EBPβ (Figure 3.12, Panel A), again consistent with previous findings (O'Rourke *et al.* 1999a). In addition, we have also demonstrated that the murine promoter is activated by C/EBPα (~4 fold, Figure 3.12, Panel A), in contrast to the findings of O'Rourke *et al.* (1999a). Whilst this study showed that both C/EBPδ and –β activated the murine C/EBPδ gene promoter in HC11 mouse mammary epithelial cells. C/EBPα did not. The difference may be attributed to the use of different cell lines between this study and that of O'Rourke *et al.* (1999a).

We also demonstrate, for the first time, that the 5' flanking region of the human C/EBPδ gene is likely to mediate auto-regulation of this gene (Figures 3.12-3.14). We have identified the regulatory elements that are responsible for this effect to within ~200bp of the transcriptional start site of this gene (Figure 3.14). This promoter region is also activated by C/EBPα and –β, demonstrating that other C/EBP family members can regulate the expression of the human C/EBPδ gene also (Figure 3.14). Analysis of this proximal promoter region of the C/EBPδ gene for putative C/EBP transcription factor binding sites [TESS (Transcription Element Search Software) at www.cbil.upenn.edu/cgi-bin/tess/tess. See Appendix V for search results] did not identify any putative C/EBP binding motifs in this region of the human C/EBPδ gene promoter. The absence of any consensus C/EBP sites in this promoter region suggests that like the ovine C/EBPδ gene (Davies *et al.* 2000) and the human C/EBPα gene (Timchenko *et al.* 1995), the human C/EBPδ gene may be subject to indirect auto-regulation. Our search did however reveal putative binding sites for potential transcription factors that could mediate this auto-regulatory effect. The human C/EBPα gene is subject to indirect auto-regulation through the binding of Upstream Stimulating Factor protein (USF) to the C/EBPα promoter (Timchenko *et al.* 1995). Similarly the ovine C/EBPδ is subject to indirect auto-regulation as no

C/EBP binding sites were identified in the promoter region of this gene. However, in common with the human C/EBPα gene promoter, putative USF sites were also identified in the promoter region of the ovine C/EBPδ gene (Davies *et al.* 2000). Additionally, C/EBPδ itself has been reported to bind with high affinity to cAMP response elements (CRE) and activate gene transcription through binding at these sites (Inoue *et al.* 1995). Therefore, it is possible that C/EBPδ may auto-regulate its own promoter by binding at CRE sites. Putative USF and CRE sites were identified in the proximal promoter region of the human C/EBPδ gene (Figure 3.15).

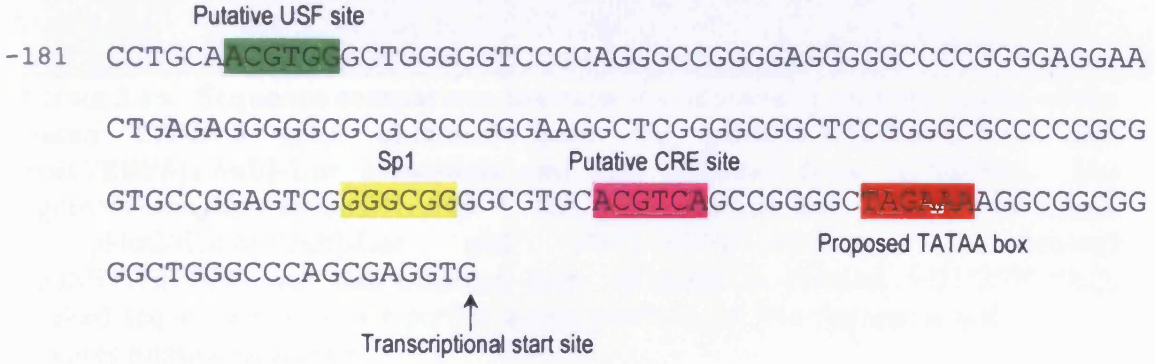



**FIGURE 3.15. Putative *cis*-acting elements in the proximal promoter region of the human C/EBPδ gene promoter as sequenced from the pHuC/EBPδ[0.2kb]-Luc promoter reporter plasmid construct.** Transcription factor binding sites were identified using TESS (Transcription Element Search Software) at www.cbil.upenn.edu/cgi-bin/tess/tess. Putative sites are blocked in colour, the reported TATAA box and functional Sp1 site (Sanford and DeWille 2005) are also shown for reference purposes. The transcriptional start site is indicated with the vertical arrow.

Although our proximal promoter sequence of the human C/EBPδ gene is identical to that reported by Cleutjens *et al.* (1993), we nevertheless decided to compare our sequence against those submitted to GenBank (National Center for Biotechnology Information at www.ncbi.nlm.nih.gov). With the completion of the Human Genome Project (www.ornl.gov/sci/techresources/Human_Genome/home/shtml), it was likely that this sequence had been updated. We compared our sequence of the proximal promoter region of the human C/EBPδ gene against the RP11-137L15 clone containing the human chromosome 8 sequence (GenBank accession number AC023991). The results of this comparison are presented in Figure 3.16.

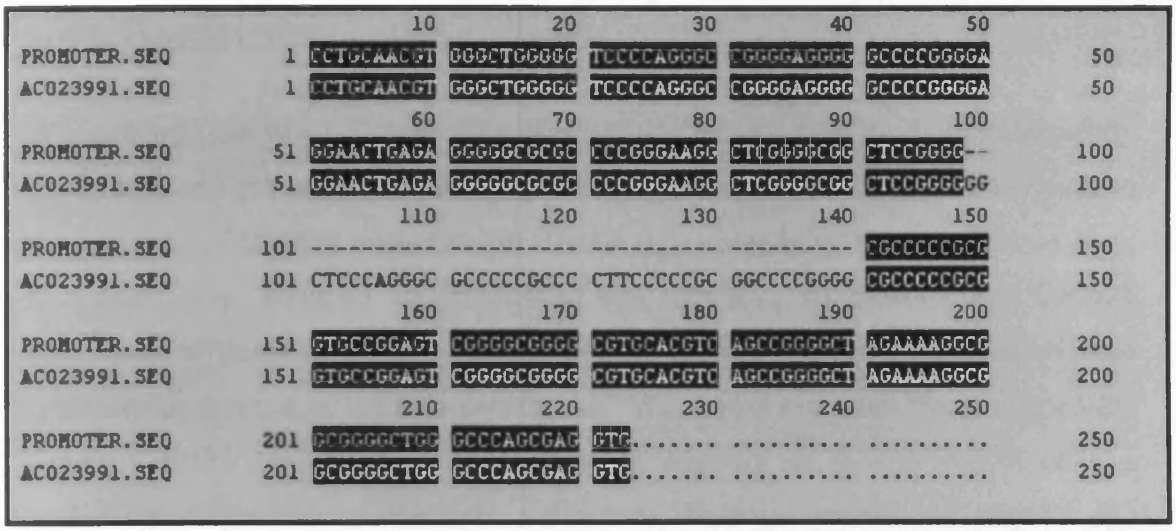



**FIGURE 3.16. Sequence comparison between the proximal promoter region of the human C/EBPδ gene obtained from the pHuC/EBPδ[0.2kb]-Luc and pHuC/EBPδ[1.6kb]-Luc constructs and that obtained from AC023991.** The alignment was done using DNASIS™. Sequence obtained from the promoter inserts in pHuC/EBPδ[0.2kb]-Luc and pHuC/EBPδ[1.6kb]-Luc is denoted PROMOTER.SEQ and that obtained from GenBank is denoted AC023991.SEQ. Blocked sequence indicates a perfect match between the two sequences and ---------- indicates missing sequence.

It became apparent to us that both our human C/EBPδ promoter constructs (pHuC/EBPδ[1.6kb]-Luc and pHuC/EBPδ[0.2kb]) were lacking a 42bp sequence that was present in the AC023991 sequence and therefore may be present in the human wild-type C/EBPδ gene promoter. The precise reasons for the noted difference between the two sequences remain unclear but could possibly be due to allelic variations or cloning artefacts.

The fact that both our human C/EBPδ promoter reporter constructs (pHuC/EBPδ[1.6kb]-Luc and pHuC/EBPδ[0.2kb]-Luc) are lacking this 42bp sequence at the proximal end of the C/EBPδ gene promoter (Figure 3.16), may explain why we did not observe promoter activation in response to IL-1. However, we believe this is unlikely as the murine C/EBPδ gene promoter is also not responsive to IL-1 action (Figure 3.11). Nevertheless, we presently cannot rule out the involvement of this deleted sequence in the regulation of human C/EBPδ gene transcription. Further experiments will be required to ascertain the role of the sequence elements present in this deleted sequence in the regulation of C/EBPδ expression. However, we can rule out the involvement of this sequence in mediating

the auto-regulatory effects observed above (Figures 3.12-3.14) since it is absent in both our human C/EBPδ promoter constructs.

In summary, the work presented in this chapter shows that we have successfully reproduced and extended the findings published from previous studies with regards to the induction of C/EBPδ expression in Hep3B cells as mediated by the cytokines IL-6, IL-1 and IFN-γ. Because we established that both IL-1 and IL-6 induced C/EBPδ much more efficiently than IFN-γ, we decided to focus on studying the action of these cytokines in relation to C/EBPδ expression. We have shown that the activity of the human C/EBPδ gene promoter is induced in response to IL-6 in Hep3B cells, a previously unreported observation. However, because we did not observe any increase in C/EBPδ promoter activity in response to IL-1 we decided not to pursue this area of investigation further. Therefore, we decided to focus on determining the signalling pathways by which IL-1 and to a lesser extent IL-6, regulate the expression of C/EBPδ, this being the focus of the remainder of this thesis. Finally, we have also shown for the first time that like the rat, mouse and ovine C/EBPδ genes, the human gene is also subject to auto-regulation although through a yet unidentified indirect mechanism. We have additionally isolated the putative regulatory elements responsible for this effect to within ~200bp of the transcriptional start site of this gene.

# CHAPTER FOUR: AN INVESTIGATION INTO THE EFFECTS OF PHARMACOLOGICAL INHIBITORS ON IL-1-MEDIATED EXPRESSION OF C/EBPδ

## 4.1 INTRODUCTION

As previously described (Section 1.3.1), C/EBPδ plays an important role in regulating the inflammatory response and therefore may contribute towards the pathophysiology of inflammatory diseases. Indeed a role for C/EBPs has been described in models of atherosclerosis (Kelkenberg *et al.* 2002), asthma (Borger *et al.* 2002) and recently C/EBPδ action has been described in a model for glomerulonephritis (Miyoshi *et al.* 2007) and in Alzheimer's disease (Li *et al.* 2004). Understanding the signalling pathways by which inflammatory cytokines regulate C/EBPδ expression/action may help to expand our knowledge on the molecular mechanisms underlying such diseases and may also help contribute towards the development of therapeutic drugs against such conditions.

Little work had been published identifying the signalling pathways by which IL-1 regulates the expression of C/EBPδ. We therefore decided to make this the main focus of our studies. IL-1 has been reported to activate a variety of signalling pathways that are discussed in brief below.

***Nuclear Factor-κ B (NF-κB)-*** The activation of NF-κB by IL-1 is a well documented phenomenon (see Martin and Wesche 2002; Dunne and O'Neill 2003 and references therein). Many genes involved in the inflammatory response contain NF-κB *cis*-acting elements in their promoters, including C/EBPδ (Akira and Kishimoto 1997; Yamada *et al.* 1997; Delhalle *et al.* 2004). NF-κB, is a dimer composed of various subunits that are members of the Rel family of proteins [p105/50, p100/52, p65 (RelA), RelB and c-Rel]. In resting cells, NF-κB proteins reside in the cytoplasm where they are sequestered by a family of inhibitors of κB (IκB) proteins. Stimulation of cells with a variety of inflammatory agents including cytokines (IL-1 and TNF-α) and LPS triggers the phosphorylation of the IκB and targets it for subsequent ubiquitinylation and degradation by the 26S proteasome. Degradation of IκB allows translocation of activated NF-κB (typically the p50:p65 heterodimers) from the cytoplasm to the nucleus where it can induce the transcription of target genes. Phosphorylation of IκB is dependent on the activity of IκB kinase (IKK). The IKK complex is composed of three subunits, two of which are catalytic (IKKα and IKKβ) and one regulatory (IKKγ/NEMO). IKKβ regulates the phosphorylation and

subsequent degradation of IκB proteins in response to IL-1 and TNF-α. IKKα is however responsible for the phosphorylation-mediated processing of the NF-κB precursor p100 into the mature p52 subunit in certain cell types such as B cells (see Ghosh and Karin 2002; Delhalle *et al.* 2004; Chen *et al.* 2006b for reviews).

***Mitogen-Activated Protein Kinases (MAPK)-*** MAPK signalling pathways play an important role in regulating eukaryotic gene expression (see Yang *et al.* 2003) and have been shown to regulate a variety of cellular processes including growth, differentiation, inflammation and apoptosis (Woodgett 2003). There are three sub-groups of the MAPK family of proteins namely the ERK, JNK and the p38 MAPKs. The ERK module is mainly activated by mitogenic stimuli such as growth factors and hormones while JNK and p38 modules are activated by cytokines and other stress stimuli (Lewis *et al.* 1998; Kyriakis and Avruch 2001). MAPKs can regulate transcription through the phosphorylation of a range of transcription factors and co-regulator proteins or by regulating mRNA stability (Dunne and O'Neill 2003; Yang *et al.* 2003). IL-1 has been shown to activate all three MAPK modules (ERK1/2, JNK and p38) and many of the upstream kinases involved in MAPK signalling including TAK1 (see O'Neill 2000; Dunne and O'Neill 2003 and references therein). A previously published study indicates a potential role for the ERK MAPK in the regulation of C/EBPδ by IL-1 in human enterocytes (Hungness *et al.* 2002b).

***Casein kinase 2 (CK2)-*** CK2 is a ubiquitous, constitutively active serine/threonine protein kinase, typically found in tetrameric complexes consisting of two catalytic (α and/or α') subunits and two regulatory β subunits (Litchfield 2003). However, a third α subunit, designated α'' has also been described (Shi *et al.* 2001). CK2 has been isolated in a variety of cellular compartments and is known to catalyse the phosphorylation of a broad range of substrates (Meggio and Pinna 2003). IL-1 stimulates CK2, which has been reported to phosphorylate directly the p65 subunit of NF-κB (Bird *et al.* 1997). CK2 has also been reported to directly phosphorylate C/EBPδ at least *in vitro* (Osada *et al.* 1996).

***Phosphatidylinositol-3 kinase (PI3K)-*** PI3K modulates several physiological processes including metabolism, cell growth, proliferation and cell migration (Wymann *et al.* 2003). PI3K consists of a p110 catalytic subunit and a p85 regulatory subunit. Following ligand-mediated activation, the SH2 (Src Homology 2) domain of

p85 interacts with a tyrosine-phosphorylated motif in associated receptors. For the type 1 IL-1 receptor (IL-1R), tyrosine$^{476}$ was identified as a putative phosphorylation site and mutation of this residue abrogated PI3K activation (Marmiroli *et al.* 1998). Nuclear translocation of PI3K has also been reported, stimulated in response to IL-1 (Bavelloni *et al.* 1999). Furthermore, the downstream target of PI3K, Akt/PKB (Protein Kinase B) has been implicated in IL-1-mediated activation of NF-κB (Cenni *et al.* 2003).

***Protein Kinase C (PKC)-*** PKC is a family of protein kinases consisting of ~10 isozymes. These are divided into three subfamilies: conventional (or classical), novel, and atypical based on their structure, second messenger and co-factor requirements. PKCs regulate a diverse range of biological responses including cellular growth, immune responses, memory and learning (see Newton 1995; Dempsey *et al.* 2000 for reviews). A plethora of studies propose an involvement of PKC isoforms in IL-1 signalling frequently by employing pharmacological inhibitors (see Schutze *et al.* 1994; Martin and Wesche 2002 for reviews). Additionally, in various cell types, NF-κB activation involves upstream PKC activation (Ghosh and Karin 2002; Su *et al.* 2002; Moscat *et al.* 2003).

### 4.1.1 EXPERIMENTAL STRATEGY

The results presented in the previous chapter have firmly established that IL-1 significantly induces the expression of C/EBPδ at both the mRNA and protein level in Hep3B cells. Although our preliminary results also indicate that the increase in C/EBPδ expression in response to IL-1 is a transcriptional response we were unable to detect any increase in promoter activation in response to this cytokine and potential reasons for this are discussed in section 3.4. Consequently, we decided to focus on studying the signalling mechanisms by which IL-1 modulates the expression of endogenous C/EBPδ mRNA and protein levels in Hep3B cells.

To initiate our investigations, we decided to make use of commercially available pharmacological inhibitors designed against various components of known IL-1 signalling pathways discussed in section 4.1. The inhibitors we selected for use have been used extensively in many studies, in a variety of cellular systems. Table 4.0 lists some studies that have made use of pharmacological inhibitors in their investigations

into the regulation of cytokine-mediated gene/protein expression. The table particularly focuses on the studies that have used pharmacological inhibitors to investigate IL-1-mediated gene/protein expression. It is also worthy of note, that some of these inhibitors have also been used *in vivo* and their actions confirmed in various animal models (Boehme *et al.* 1999; Han *et al.* 2001; van den Blink *et al.* 2001; Cheng *et al.* 2002; Semba *et al.* 2002; Gaddipati *et al.* 2003; Wang *et al.* 2004; Yamada *et al.* 2005). For example, apigenin has been used as an effective CK2 inhibitor in an animal model of glomerulonephritis. Inhibition of CK2 *in vivo* reduces the progression of this inflammatory disease in rats (Yamada *et al.* 2005). Aberrant inflammation also contributes towards the pathophysiology of arthritis and the JNK MAPK inhibitor, SP600125 has been used *in vivo* to treat this condition (Han *et al.* 2001).

Additionally, Table 4.1 describes the mode of action of the inhibitors we used in our investigation. The concentration of the inhibitors used in our experiments were based on those previously used in our laboratory and in published studies investigating hepatocyte gene expression and function (Ohigashi *et al.* 1999; Cavin *et al.* 2003; Giltiay *et al.* 2005; Wen-Sheng and Jun-Ming 2005; Cabrales-Romero Mdel *et al.* 2006; Lauricella *et al.* 2006; Wu *et al.* 2006; Dong *et al.* 2007; Harvey *et al.* 2007).

**TABLE 4.0. List of publications utilising pharmacological inhibitors to study signalling pathways regulating cytokine-mediated gene/protein expression.**

| INHIBITOR | SIGNALLING PATHWAY/COMPONENT | REFERENCES | CYTOKINE (S) | GENE OR PROTEIN ANALYSED |
|---|---|---|---|---|
| Apigenin | CK2 | Farah *et al.* (2003) | TNF-α | manganese superoxide dismutase |
| | | Cavin *et al.* (2003) | TGF-β | IκB-α |
| | | Mead *et al.* (2003) | IFN-γ | inducible cAMP early repressor |
| | | Singh and Ramji (2006) | TGF-β | apolipoprotein E |
| | | Harvey *et al.* (2007) | IFN-γ | monocyte chemoattractant protein-1 |
| Bisindolylmaleimide | PKC | Jordan *et al.* (1996) | IL-1 and TNF-α | monocyte chemoattractant protein-1 and RANTES |
| | | Mino *et al.* (1998) | IL-1 and TNF-α | IL-11 |
| | | Cafferata *et al.* (2000) | IL-1 | cystic fibrosis transmembrane conductance regulator |
| | | Molina-Holgado *et al.* (2000) | IL-1 | cyclooxygenase-2 and prostaglandin E(2) |
| | | Nagy *et al.* (2001) | IL-1 and TNF-α | IL-6 |
| | | Jang *et al.* (2004) | IL-1 | beta-defensin 2 |
| | | Duggan *et al.* (2007) | IL-1 | cyclooxygenase-2 |
| Curcumin | SAPK/JNK/AP-1 | Yokoo and Kitamura (1996) | IL-1 | matrix metalloproteinase-9 |
| | | Chaudhary and Avioli (1996) | IL-1 | IL-8 |
| | | Xu *et al.* (1997) | IL-1 and TNF-α | monocyte chemoattractant protein-1 |
| | | Terry *et al.* (1998) | IL-1 and TNF-α | heme oxygenase-1 |
| | | Li *et al.* (2002b) | IL-1 | Na(+)/taurocholate co-transporting polypeptide |
| | | Liacini *et al.* (2002) | IL-1 | matrix metalloproteinase-3 matrix metalloproteinase-13 |

| | | | | |
|---|---|---|---|---|
| | | Wuyts *et al.* (2003) | IL-1 | monocyte chemoattractant protein-1<br>monocyte chemoattractant protein-3 |
| | | Al-Sadi and Kreydiyyeh (2003) | IL-1 | Na(+)-K(+)ATPase |
| | | Lee *et al.* (2003b) | IL-1 and BMP-7 | monocyte chemoattractant protein-1 |
| | | Moon *et al.* (2005) | IL-1 | prostaglandin E synthase 1 |
| | | Zimmerman *et al.* (2006) | IL-1 | retinoid X receptor alpha |
| | | Moon *et al.* (2006) | IL-1 and TNF-α | galactose-alpha1,4-galactose-beta1,4-glucose ceramide synthase |
| | | Cho *et al.* (2007) | TNF-α | IL-1, IL-6, TNF-α and cyclin E |
| LY294002 | PI3K | Birkenkamp *et al.* (2000) | IL-1 | IL-6 |
| | | Weaver *et al.* (2001) | TNF-α | cyclo-oxygenase-2 |
| | | Varley *et al.* (2001) | IL-1 | PKC-ε |
| | | Kim *et al.* (2002b) | IL-1 | mucin 2 |
| | | Ruhul Amin *et al.* (2003) | IL-1 | matrix metalloproteinase-9 |
| | | Bian *et al.* (2004) | IL-1 | monocyte chemoattractant protein-1 |
| | | Jang *et al.* (2004) | IL-1 | beta-defensin 2 |
| | | Yamamoto *et al.* (2004) | IL-1 | claudin-2 |
| | | Gong *et al.* (2005) | IL-1 | growth hormone |
| | | Huang *et al.* (2006) | IL-1 | tissue type plasminogen activator |
| | | Nam (2006) | IL-1 | inducible nitric oxide synthase |
| | | Lin *et al.* (2007) | IL-1 | intercellular adhesion molecule-1 |
| PD98059 | MEK1-ERK1/2 MAPK | Kumar *et al.* (1998) | IL-1 and TNF-α | low density lipoprotein receptor |
| | | Lindroos *et al.* (1998) | IL-1 | alpha-platelet-derived growth factor receptor |
| | | Miwa *et al.* (1999) | IL-1 | IL-6 |

| | | | | |
|---|---|---|---|---|
| | | Jiang and Brecher (2000) | IL-1 | inducible nitric oxide synthase |
| | | Newton *et al.* (2000) | IL-1 | prostaglandin E2 |
| | | Fan *et al.* (2001) | IL-1 | cyclo-oxygenase-2 |
| | | Suzuki *et al.* (2001) | IL-1 and TNF-α | gastrin |
| | | Jiang *et al.* (2001) | IL-1 | inducible nitric oxide synthase |
| | | Hungness *et al.* (2002a; 2002b), | IL-1 | C/EBPβ and –δ |
| | | Liacini *et al.* (2002) | IL-1 | matrix metalloproteinase-3 |
| | | Yang *et al.* (2002) | IL-1 | cyclo-oxygenase-2 |
| | | Kim *et al.* (2002b) | IL-1 | mucin 2 |
| | | Luo *et al.* (2003) | IL-1 | IL-6 |
| | | Fleenor *et al.* (2003) | IL-1 | matrix metalloproteinase-3 |
| | | Jiang *et al.* (2004) | IL-1 | inducible nitric oxide cyclo-oxygenase-2 |
| | | Xie *et al.* (2004) | IL-1 | osteopontin |
| | | Qian *et al.* (2004) | IL-1 | hypoxic-inducible factor-1 |
| | | Lin *et al.* (2004) | IL-1 | cyclo-oxygenase-2 |
| | | Masuko-Hongo *et al.* (2004) | IL-1 | microsomal prostaglandin E synthase 1 |
| | | Lin *et al.* (2005) | IL-1 | intercellular adhesion molecule-1 |
| | | Takami *et al.* (2005) | IL-1 and IFN-γ | hepatocyte growth factor |
| | | Ma *et al.* (2005) | IL-1 | beta-amyloid precursor protein |
| | | Giltiay *et al.* (2005) | IL-1 | C/EBPβ |
| | | Nieminen *et al.* (2005) | IL-1 | cyclo-oxygenase-2 |
| | | Raymond *et al.* (2006) | IL-1 | matrix metalloproteinase-1 |
| | | Liu *et al.* (2006a) | IL-1 | renin |
| SB202190 | p38 MAPK | Kumar *et al.* (1998) | IL-1 and TNF-α | low density lipoprotein receptor |
| | | Tanaka *et al.* (2000) | IL-1 | vascular endothelial cell growth factor |
| | | Bian *et al.* (2001) | IL-1 and TNF-α | IL-8 and monocyte chemoattractant protein-1 |
| | | Degousee *et al.* (2001) | IL-1 | group IIa phospholipase A(2) |

| | | | | |
|---|---|---|---|---|
| | | Degousee *et al.* (2003) | IL-1 | cyclo-oxygenase-2 |
| | | Lin *et al.* (2004) | IL-1 | cyclo-oxygenase-2 |
| | | Wu *et al.* (2004) | IL-1 | matrix metalloproteinase-9 |
| | | Wilczynska *et al.* (2006) | IL-1 | tissue inhibitor of metalloproteinases-1 |
| | | Liang *et al.* (2007) | IL-1 | matrix metalloproteinase-9 |
| SB203580 | p38 MAPK | Ridley *et al.* (1997) | IL-1 | IL-6, prostaglandin H synthase-2, matrix metalloproteinase-1, matrix metalloproteinase-3 |
| | | Rovin *et al.* (1999) | IL-1 | monocyte chemoattractant protein-1 |
| | | Suzuki *et al.* (2000) | IL-1 and TNF-α | IL-6 and IL-8 |
| | | Waterhouse *et al.* (2001) | IL-1 | monocyte chemoattractant protein-1 |
| | | Jung *et al.* (2001) | IL-1 | vascular endothelial growth factor |
| | | Jung *et al.* (2002) | IL-1 | IL-8 |
| | | Parhar *et al.* (2003) | IL-1 | IL-8 |
| | | Lee *et al.* (2003a) | IL-1 | matrix metalloproteinase-13 |
| | | Patil *et al.* (2004) | IL-1 | IL-6 |
| | | Jang *et al.* (2004) | IL-1 | beta-defensin 2 |
| | | Masuko-Hongo *et al.* (2004) | IL-1 | prostaglandin E synthase 1 |
| | | Takami *et al.* (2005) | IL-1 and IFN-γ | hepatocyte growth factor |
| | | Brunius *et al.* (2005) | IL-1 | IL-8 |
| | | Demircan *et al.* (2005) | IL-1 and TNF-α | aggrecanase ADAMTS-9 |
| | | Rossa *et al.* (2005) | IL-1 | matrix metalloproteinase-13 |
| | | Huang *et al.* (2006) | IL-1 | tissue type plasminogen activator |
| | | Chen *et al.* (2006a) | IL-1 | cyclo-oxygenase-2 |
| | | Zhang *et al.* (2006) | IL-1 | tissue inhibitor of matrix metalloproteinase-1 |
| | | Rossa *et al.* (2006) | IL-1 and TNF-α | receptor-activated NF-κB ligand |
| | | Inatomi *et al.* (2007) | IL-1 and TNF-α | matrix metalloproteinase-3 |

| | | | | |
|---|---|---|---|---|
| SP600125 | SAPK/JNK MAPK | Oltmanns *et al.* (2003) | IL-1 | granulocyte-macrophage colony-stimulating factor, RANTES and IL-8 |
| | | Jang *et al.* (2004) | IL-1 | beta-defensin 2 |
| | | de Haij *et al.* (2005) | IL-1 | IL-6 |
| | | Ma *et al.* (2005) | IL-1 | beta-amyloid precursor |
| | | Nieminen *et al.* (2005) | IL-1 | cyclo-oxygenase-2 |
| | | Lee *et al.* (2006) | IL-1 | TGF-β1 |
| | | Wehkamp *et al.* (2006) | IL-1 | beta-defensin 2 |
| | | Zhang *et al.* (2006) | IL-1 | tissue inhibitor of matrix metalloproteinase-1 |
| | | Kwakkel *et al.* (2006) | IL-1 | deiodinase type 1 |
| | | Ishikawa and Morris (2006) | IL-1 | inducible nitric oxide synthase |
| | | Zhang *et al.* (2007a) | IL-1 | bradykinin B1 and B2 receptors |
| | | Liang *et al.* (2007) | IL-1 | matrix metalloproteinase-9 |

**TABLE 4.1. Action of pharmacological inhibitors used in this study.**

| INHIBITOR | CHEMICAL NAME | FORMULA | TARGET | MECHANISM | REFERENCE(S) |
|---|---|---|---|---|---|
| Apigenin | 4′,5,7-Trihydroxyflavone | $C_{15}H_{10}O_5$ | CK2 | Reversible ATP/GTP-competitive inhibitor of CK2 | Critchfield *et al.* (1997) |
| Bisindolylmaleimide I | 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide | $C_{25}H_{24}N_4O_2$ | PKC | Reversible ATP-competitive inhibitor of PKC | Gekeler *et al.* (1996) |
| Calphostin C | Carbonic acid, 2-(12-(2-(benzoyloxy)propyl)-3,10-dihydro-4,9-dihydroxy-2,6,7,11-tetramethoxy-3,10-dioxo-1-perylenyl)-1-methylethyl 4-hydroxyphenyl ester | $C_{44}H_{38}O_{14}$ | PKC | Competes with diacylglycerol and phorbol esters for binding at PKC regulatory domain | Kobayashi *et al.* (1989) |
| Curcumin | 1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione | $C_{21}H_{20}O_6$ | JNK MAPK and AP1 | Prevents JNK activation upstream of MEKK1. Inhibits expression of c-Jun and prevents AP1 binding | Chen and Tan (1998) Huang *et al.* (1991) |
| LY294002 | 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one | $C_{19}H_{17}NO_3$ | PI3K | Reversible ATP-competitive inhibitor of PI3K | Vlahos *et al.* (1994) |
| NF-κB Activation Inhibitor | 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline | $C_{22}H_{20}N_4O$ | NF-κB | Inhibits NF-κB transcriptional activation | Tobe *et al.* (2003) |
| PD98059 | 2′-Amino-3′-methoxyflavone | $C_{16}H_{13}NO_3$ | MEK1 | Selective non-competitive inhibitor of the ERK MAPK pathway | Dudley *et al.* (1995) |
| SB202190 | 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole | $C_{20}H_{14}FN_3O$ | p38 MAPK | Binds inactive p38 and inhibits its activation | Frantz *et al.* (1998) |
| SB203580 | 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole | $C_{21}H_{16}N_3OSF$ | p38 MAPK | ATP-competitive inhibitor of p38 MAPK | Frantz *et al.* (1998) |
| SP600125 | 1,9-Pyrazoloanthrone | $C_{14}H_8N_2O$ | JNK/SAPK | Reversible ATP-competitive inhibitor of JNK1, -2 and –3 | Bennett *et al.* (2001) |

#### 4.1.1.1 SPECIFIC AIMS

The main aim of the work presented in this chapter was to use pharmacological inhibitors to identify potential signalling pathways that may be involved in regulating the expression of C/EBPδ in response to IL-1. In order to initiate our investigations we decided to screen for candidate signalling pathways using semi-quantitative RT-PCR. This was because the increase in C/EBPδ mRNA levels in response to IL-1 were easily detected using this technique, as demonstrated in the previous chapter. In addition, this method has repeatedly been used successfully to initiate investigations of a similar nature in our laboratory (Mead *et al.* 2003; Singh and Ramji 2006; Harvey *et al.* 2007) and by others (Bian *et al.* 2001; Jang *et al.* 2004; Wu *et al.* 2004; Dong *et al.* 2007). Results in the previous chapter additionally show that the induction of C/EBPδ by IL-1 at the mRNA level is also mirrored at the protein level. Therefore, any candidate signalling pathway identified through the use of pharmacological inhibitors by RT-PCR would then be confirmed at the protein level using western blot analysis.

Figure 4.1 summaries our overall experimental approach that was designed to achieve the outlined experimental objectives.

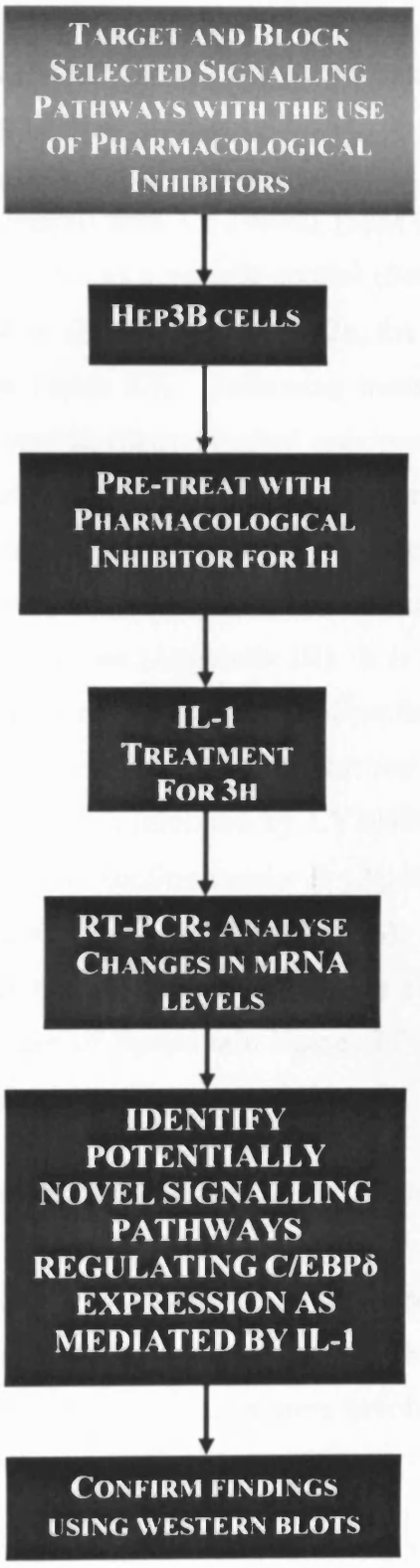



**FIGURE 4.1. Experimental strategy.** The aim of the experimental strategy was to investigate the involvement of various signalling pathways in the regulation of the IL-1-mediated induction of C/EBPδ gene expression. Having identified candidate pathways with semi-quantitative RT-PCR, these findings were then extended by western blot analysis.

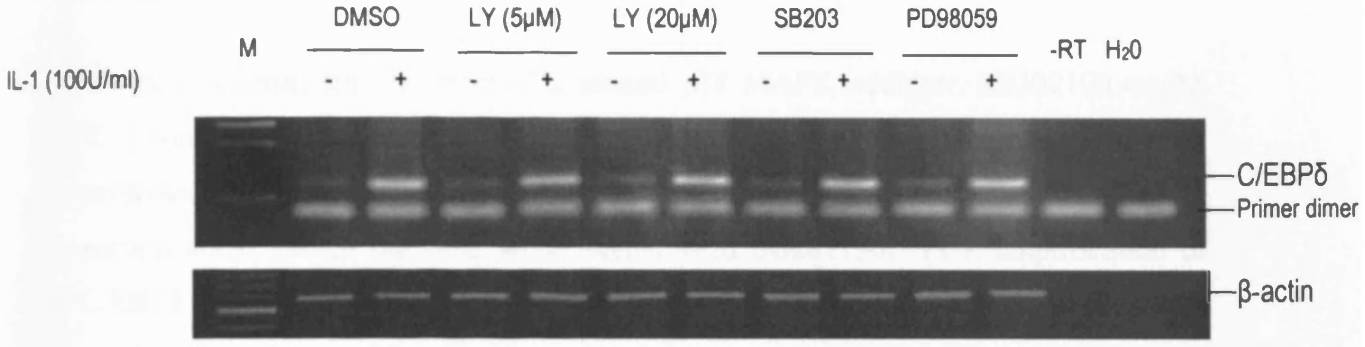
## 4.2 RESULTS

### 4.2.1 EFFECT OF LY294002, SB203580 AND PD98059 ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA LEVELS.

Hep3B cells were pre-treated with LY294002 (5μM and 20μM), SB203580 (5μM), PD98059 (50 μM) or DMSO as a vehicle control (Section 2.3.1.6). They were then cultured in the presence or absence of IL-1 for 3h, the time at which C/EBPδ exhibits maximal induction (see Figure 3.5). Following treatment, total RNA was extracted and RT-PCR analysis carried out as detailed previously. Figure 4.2 shows the PCR products for C/EBPδ and β-actin as resolved by agarose gel electrophoresis (Figure 4.2A) and the band intensities normalised to the β-actin control gene (Figure 4.2B). Where experiments were carried out three times or more a student's t-test was used to assess for statistical significance (Appendix III). It is worthy of note that the positive action of all three inhibitors has previously been confirmed in other studies conducted in our laboratory. In macrophages, the induction of monocyte chemoattractant protein-1 (MCP-1) by IFN-γ is inhibited by LY294002 (Harvey *et al.* 2007) and in monocytes the induction of apolipoprotein E (ApoE) by TGF-β is attenuated by SB203580 and SB202190 (Singh and Ramji 2006). Furthermore, the MEK1/ERK inhibitor, PD98059 has been shown to inhibit the phorbol 12-myristate 13-acetate (PMA)-mediated induction of lipoprotein lipase (LPL) during the differentiation of monocytes into macrophages (Mead *et al.* 2003).

As expected, treatment of Hep3B cells with IL-1 stimulated an increase in the levels of C/EBPδ mRNA. When combined with IL-1 treatment, neither LY294002, SB203580 nor PD98059 attenuated this response, at least at the specified concentrations of these inhibitors. These data suggested that neither the PI3K, p38 MAPK nor the ERK1/2 MAPK pathways were involved in regulating this response, respectively.

A.




B.

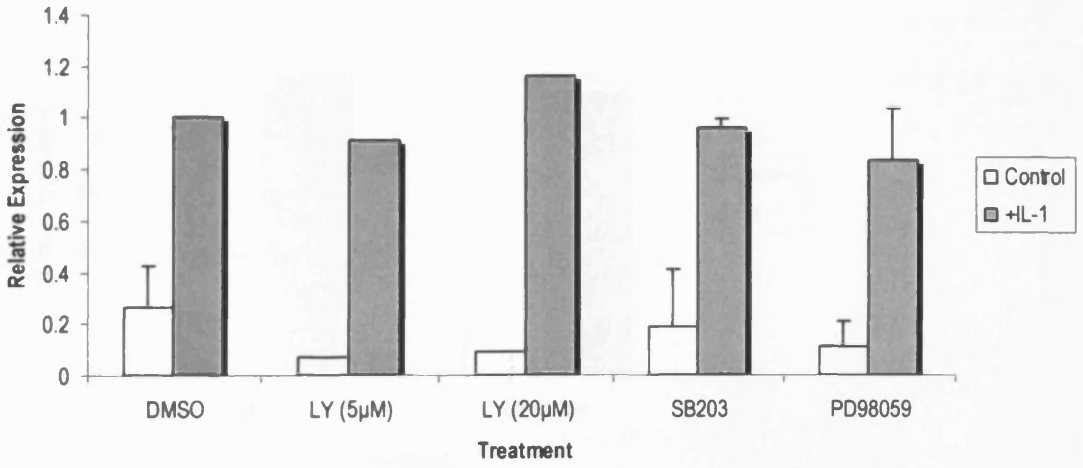



**FIGURE 4.2 Effect of LY294002, SB203580 and PD98059 on the induction of C/EBPδ mRNA expression as mediated by IL-1.** Hep3B cells were pre-treated with either LY294002; LY (5μM and 20μM), SB203580; SB203 (5μM), PD98059 (50μM) or with DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated (control) for 3h. Total RNA was then extracted and used for RT-PCR analysis (Section 2.6.3). The PCR amplification products for C/EBPδ and β-actin were resolved on 1.5% (w/v) agarose gels by electrophoresis (A). M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –IL-1 treated RNA). $H_20$ denotes a PCR in which cDNA was replaced with an equal volume of water. Panel B shows the graphical representation of the data as determined by densitometric analysis. The ratios of C/EBPδ:β-actin were normalised to the IL-1 treated DMSO control. The data shown for the LY294002 inhibitor is the mean from two independent experiments. The data shown for the SB203580 and PD98059 inhibitors is the mean ±SD from three independent experiments.

### 4.2.2 EFFECT OF SB202190 ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA LEVELS.

We next investigated the effect of a second p38 MAPK inhibitor, SB202190 on the IL-1-stimulated expression of C/EBPδ as an additional confirmation of the result presented in Figure 4.2. The experiment was carried out exactly as described in section 4.2.1, except the cells were treated with SB202190. PCR amplification of C/EBPδ and GAPDH was carried out as previously described. The data is presented graphically in Figure 4.3.

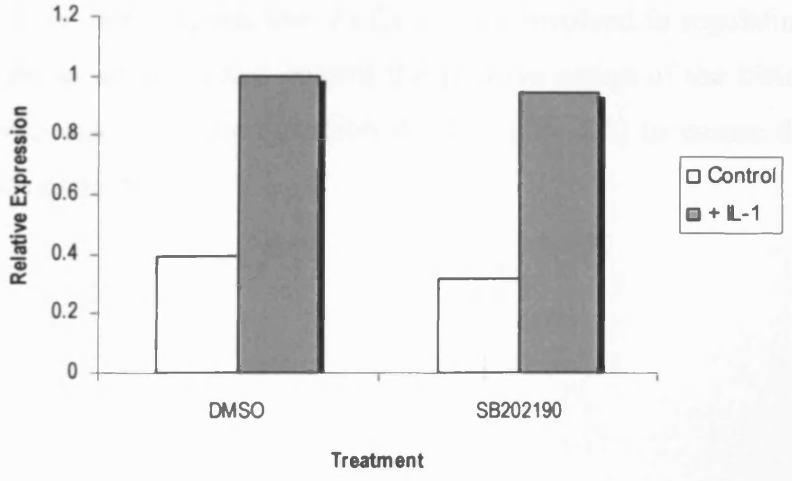



**FIGURE 4.3 Effect of SB202190 on the induction of C/EBPδ mRNA expression as mediated by IL-1.** Hep3B cells were pre-treated with SB202190 (10μM) or with DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated (control) for 3h. Total RNA was then extracted and used for RT-PCR analysis (Section 2.6.3). The PCR amplification products for C/EBPδ and GAPDH were resolved by electrophoresis. The histogram displays the ratios of C/EBPδ:GAPDH normalised to the IL-1 treated DMSO control as determined by densitometric analysis. These data are the mean from two independent experiments.

The results presented in Figure 4.3 shows that SB202190 does not impair the IL-1-stimulated increase in C/EBPδ mRNA levels in Hep3B cells (at least at the specified concentration). This further suggested that the p38 MAPK pathway was not involved in regulating this response, consistent with the data presented in Figure 4.2, where SB203580 also did not attenuate this response.

### 4.2.3 EFFECT OF BISINDOLYLMALEIMIDE AND CALPHOSTIN C ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA LEVELS.

To investigate the potential involvement of PKC in the regulation of the IL-1-mediated response, the PKC inhibitors bisindolylmaleimide and calphostin C were employed. The effect of these inhibitors on the IL-1-mediated induction of C/EBPδ mRNA levels was assessed by RT-PCR analysis as described in the above sections and results are presented in Figure 4.4.

The results show that pre-treatment of Hep3B cells with the specified PKC inhibitors does not attenuate the IL-1-mediated increase in C/EBPδ mRNA levels, compared to controls. This data suggests that PKCs are not involved in regulating this response. Furthermore, as an additional control the positive action of the bisindolylmaleimide inhibitor was also confirmed (section 4.2.4, Figure 4.5) to ensure the inhibitor was active in the experiments.

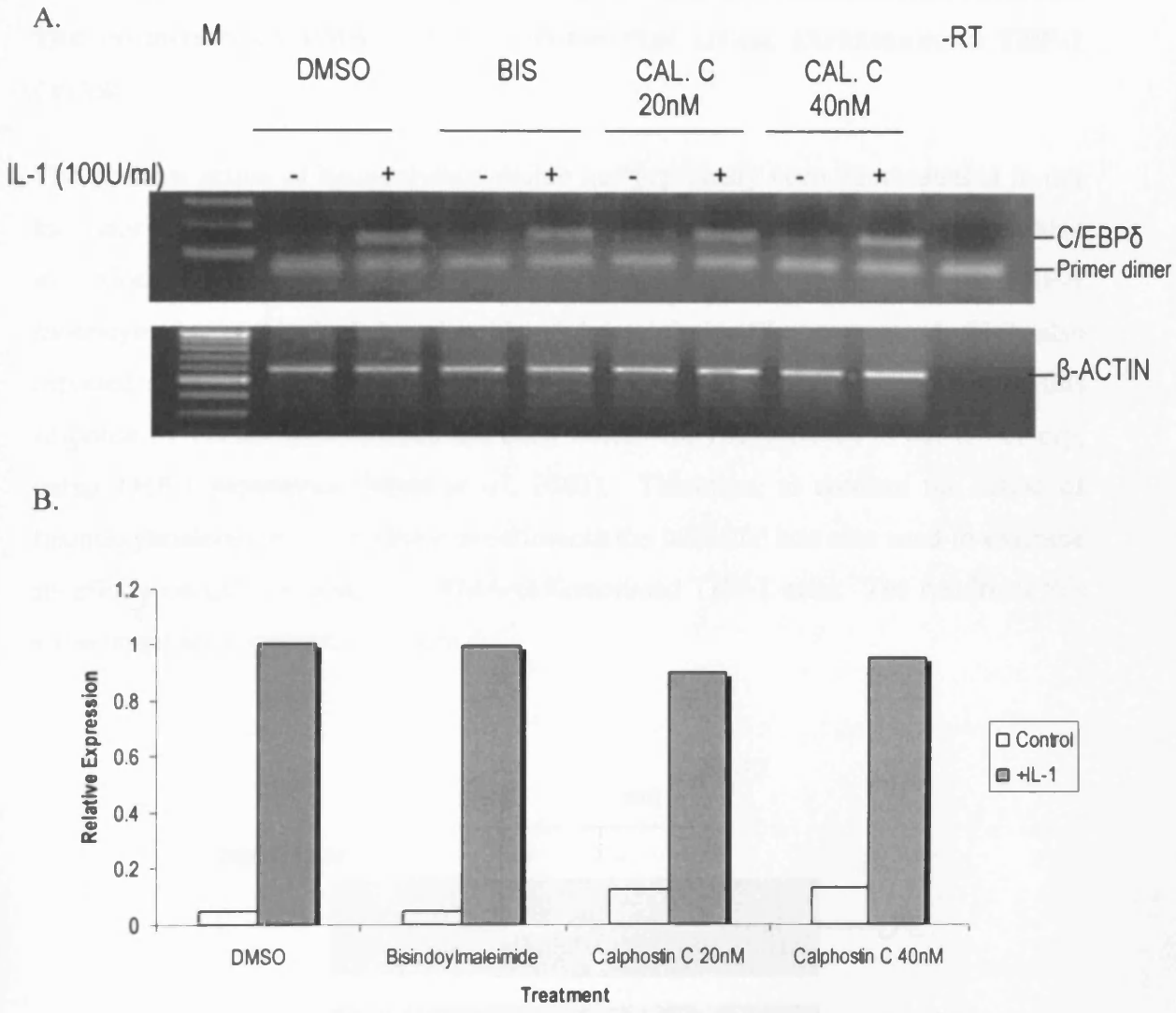



**FIGURE 4.4 Effect of bisindolylmaleimide and calphostin C on the induction of C/EBPδ mRNA expression as mediated by IL-1.** Hep3B cells were pre-treated with bisindolylmaleimide; BIS (2μM), calphostin C; CAL. C (20nM and 40nM) or with DMSO as a vehicle control for 1h. As before, following IL-1 treatment, the cells were harvested for RT-PCR analysis. The PCR amplification products for C/EBPδ and β-actin were resolved by electrophoresis (A). M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –IL-1 treated RNA). The ratios of C/EBPδ:β-actin were determined by densitometric analysis and normalised to the IL-1 treated DMSO control, as before (Panel B). The data shown for the bisindolylmaleimide inhibitor is the mean from two independent experiments. For calphostin C, the data shown is a result of densitometric analysis of the above experiment.

### 4.2.4 A POSITIVE CONTROL FOR THE ACTION OF BISINDOLYLMALEIMIDE THROUGH THE INHIBITION OF PMA-INDUCED LIPOPROTEIN LIPASE EXPRESSION IN THP-1 CELLS.

The positive action of bisindolylmaleimide has previously been demonstrated in our laboratory (Mead *et al.* 2003). A study by Auwerx *et al.* (1989) showed that induction of LPL gene expression during PMA-mediated differentiation of THP-1 monocytes, was blocked by the bisindolylmaleimide-like compound, H-7 also reported as a PKC inhibitor (Kawamoto and Hidaka 1984). The inhibition of this response by bisindolylmaleimide has been successfully reproduced in our laboratory, using THP-1 monocytes (Mead *et al.* 2003). Therefore, to confirm the action of bisindolylmaleimide in the above experiments the inhibitor was also used to examine its effects on LPL expression in PMA-differentiated THP-1 cells. The results of this experiment are presented in Figure 4.5.

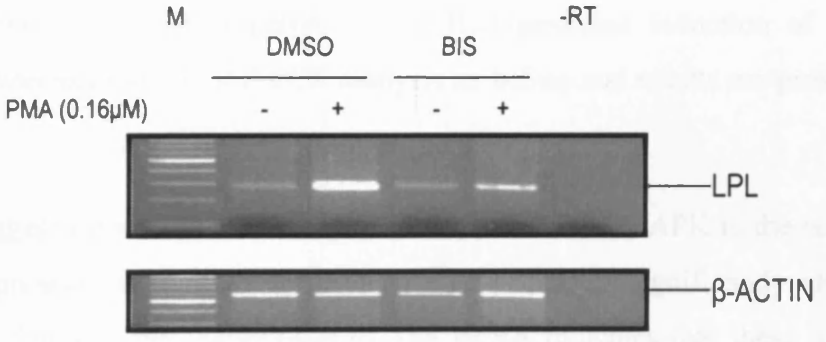



**FIGURE 4.5 Effect of bisindolylmaleimide on the induction of LPL mRNA expression as mediated by PMA in THP-1 cells.** THP-1 monocytes were pre-treated with bisindolylmaleimide; BIS (2μM) or with DMSO as a vehicle control for 1h. They were then either treated with PMA (0.16μM) or left untreated for 24h. Total RNA was then extracted and used for RT-PCR analysis. The primers and conditions used for the PCR amplification of LPL are specified in Tables 2.4 and 2.5 respectively. PCR amplification products for LPL and β-actin were resolved by electrophoresis as previously described. M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –PMA treated RNA).

As expected, bisindolylmaleimide attenuated the PMA-induced expression of LPL in THP-1 cells. Therefore, from this experiment we can conclude that the bisindolylmaleimide inhibitor used to examine the effects of PKC inhibition on IL-1-mediated induction of C/EBPδ was active because the same stock of inhibitor was used to determine this effect.

### 4.2.5 EFFECT OF CURCUMIN AND APIGENIN ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA LEVELS.

We next sought to determine any involvement of CK2 or JNK MAPK in the regulation of C/EBPδ mRNA expression by IL-1. For this we employed two inhibitors, apigenin and curcumin. Apigenin has been described as a CK2 inhibitor and work in our laboratory has confirmed this action in macrophages and hepatocytes (Mead *et al.* 2003; Harvey 2006; Singh and Ramji 2006; Harvey *et al.* 2007). Curcumin has been described as a JNK MAPK pathway inhibitor and indeed this action has been confirmed in our laboratory (Greenow 2004; Singh and Ramji 2006). The effect of curcumin and apigenin on the IL-1-mediated induction of C/EBPδ mRNA levels was assessed by RT-PCR analysis as before and results are presented in Figure 4.6.

The results suggest a potential involvement of CK2 and JNK MAPK in the regulation of C/EBPδ expression as induced by IL-1 as both inhibitors significantly attenuated this response. The constant expression of 28S rRNA indicates that these inhibitors were not having a global effect on cellular gene expression and therefore the observed effects were specific. Furthermore, we excluded the possibility that these effects were a result of the inhibitors compromising the viability of the cells with a trypan blue exclusion assay, using Hep3B cells treated with 20μM apigenin and 30μM curcumin (Section 2.3.1.7).

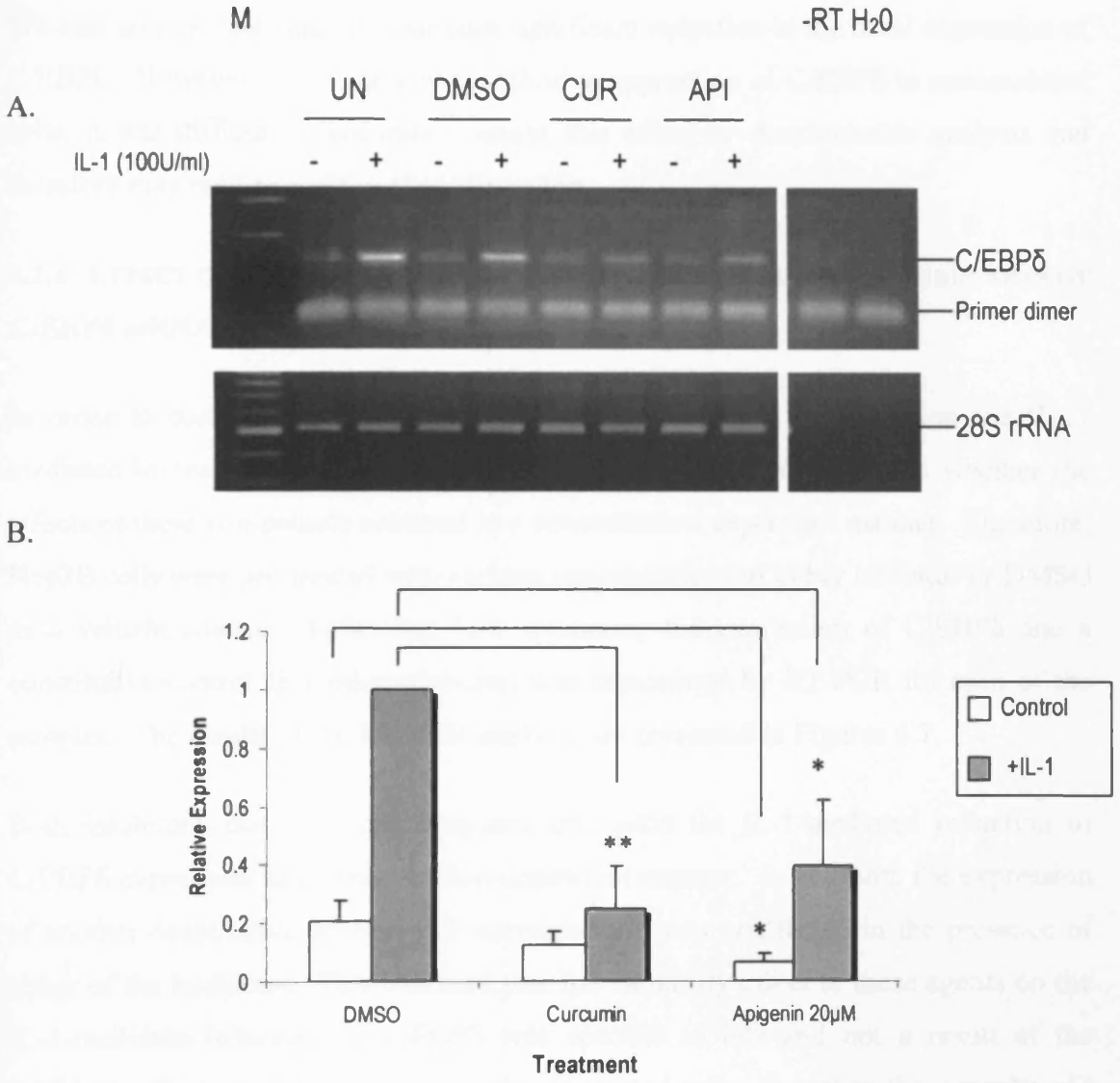



**FIGURE 4.6 Effect of curcumin and apigenin on the induction of C/EBPδ mRNA expression as mediated by IL-1.** Hep3B cells were pre-treated with curcumin; CUR (30μM), apigenin; API (20μM) or with DMSO as a vehicle control for 1h or left untreated (UN). Following stimulation with IL-1, the cells were harvested for RT-PCR analysis as before. The PCR amplification products for C/EBPδ and 28S rRNA were resolved by electrophoresis (A). M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using untreated RNA). $H_20$ denotes a PCR in which cDNA was replaced with an equal volume of water. Panel B displays the ratios of C/EBPδ:28S rRNA normalised to the IL-1 treated DMSO control as determined by densitometric analysis. The data shown is the mean ±SD from three independent experiments. *P<0.05, **P<0.01.

We also noticed that apigenin caused a significant reduction in the basal expression of C/EBPδ. However, given the low constitutive expression of C/EBPδ in unstimulated cells, it was difficult to accurately assess this effect by densitometric analysis and therefore may require additional confirmation.

### 4.2.6 EFFECT OF CURCUMIN AND APIGENIN ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA LEVELS IS CONCENTRATION DEPENDENT

In order to confirm the inhibitory effects of apigenin and curcumin on the IL-1-mediated increase in C/EBPδ mRNA levels it was necessary to assess whether the effects of these compounds occurred in a concentration dependent manner. Therefore, Hep3B cells were pre-treated with various concentrations of either inhibitor or DMSO as a vehicle control. Following IL-1 treatment, the expression of C/EBPδ and a constitutive control (β-2-microglobulin) was determined by RT-PCR for each of the samples. The results of the RT-PCR analysis are presented in Figures 4.7.

Both inhibitors, curcumin and apigenin attenuated the IL-1-mediated induction of C/EBPδ expression in a concentration-dependent manner. In addition, the expression of another constitutive control, β-2-microglobulin was unaffected in the presence of either of the inhibitors. This indicates that the inhibitory effect of these agents on the IL-1-mediated induction of C/EBPδ was specific to this and not a result of the inhibitors affecting global gene expression in treated cells. Together, these results add further support to our hypothesis, indicating a potential requirement for JNK MAPK and CK2 in the regulation of this response.

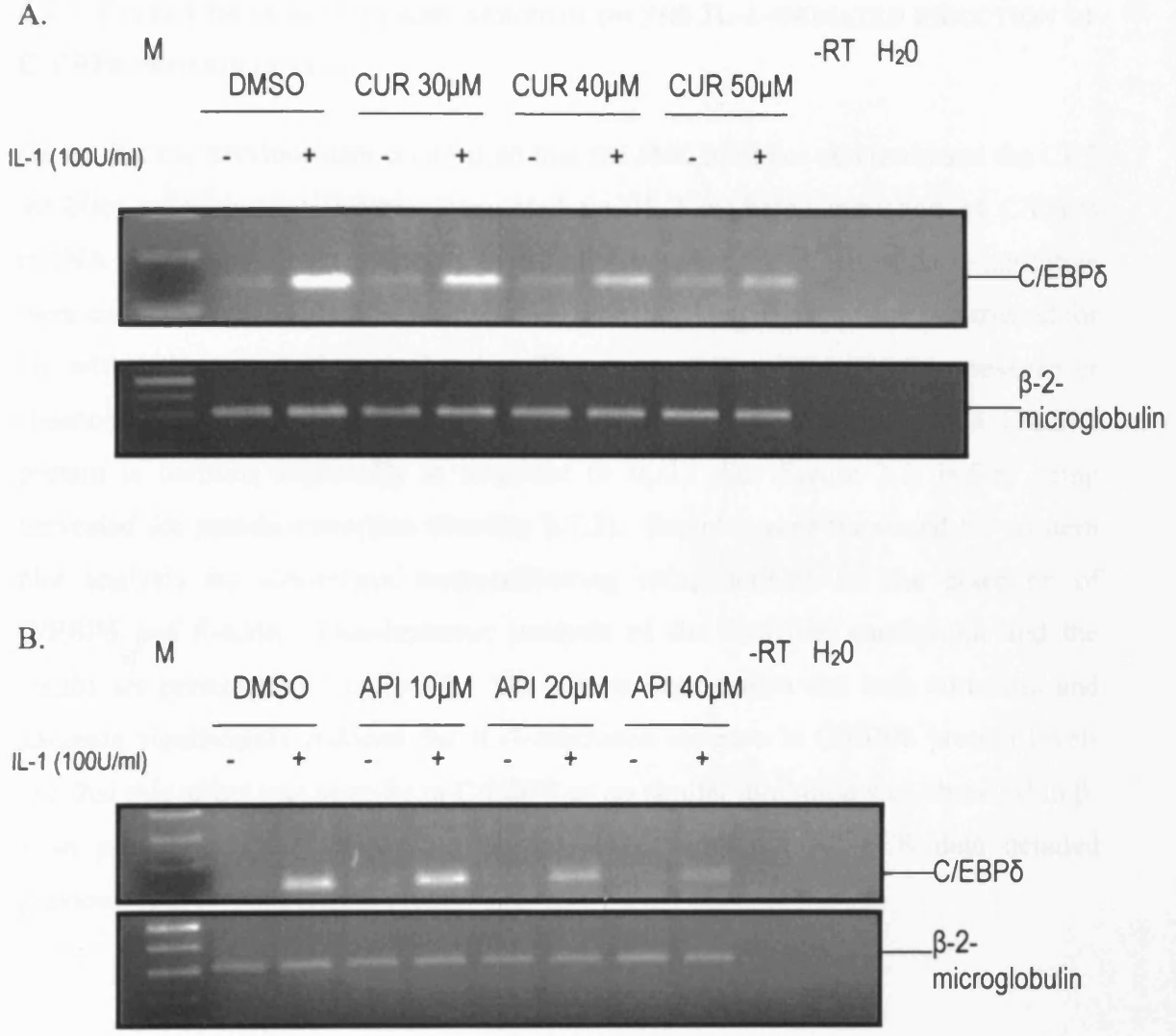



**FIGURE 4.7. Effect of various concentrations of curcumin (A) and apigenin (B) on the IL-1-mediated induction of C/EBPδ mRNA expression**. Hep3B cells were pre-treated with curcumin; CUR (30μM, 40μM and 50μM), apigenin; API (10μM, 20μM and 40μM) or with DMSO as a vehicle control for 1h. Following IL-1 treatment, the cells were harvested for RT-PCR analysis as before. The PCR amplification products for C/EBPδ and β-2-microglobulin were resolved by electrophoresis. M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –IL-1 treated RNA). $H_20$ denotes a PCR in which cDNA was replaced with an equal volume of water.

### 4.2.7 EFFECT OF CURCUMIN AND APIGENIN ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ PROTEIN LEVELS.

Given that our previous data established that the JNK inhibitor curcumin and the CK2 inhibitor apigenin significantly attenuated the IL-1-mediated induction of C/EBPδ mRNA levels, it was next necessary to establish whether the effects of these inhibitors were extended to the C/EBPδ protein level. For this, Hep3B cells were pre-treated for 1h, with either curcumin or apigenin. They were then cultured in the presence or absence of IL-1 for 3h (previously shown to be the time-point at which C/EBPδ protein is induced maximally in response to IL-1. See Figure 3.7) before being harvested for protein extraction (Section 2.7.2). Samples were then used for western blot analysis for subsequent immunoblotting using antisera for the detection of C/EBPδ and β-actin. Densitometric analysis of the data was carried out and the results are presented in Figure 4.8. The results demonstrate that both curcumin and apigenin significantly reduced the IL-1-mediated increase in C/EBPδ protein levels and that this affect was specific to C/EBPδ as no similar inhibition was observed in β-actin protein levels. These data are consistent with our RT-PCR data detailed previously.

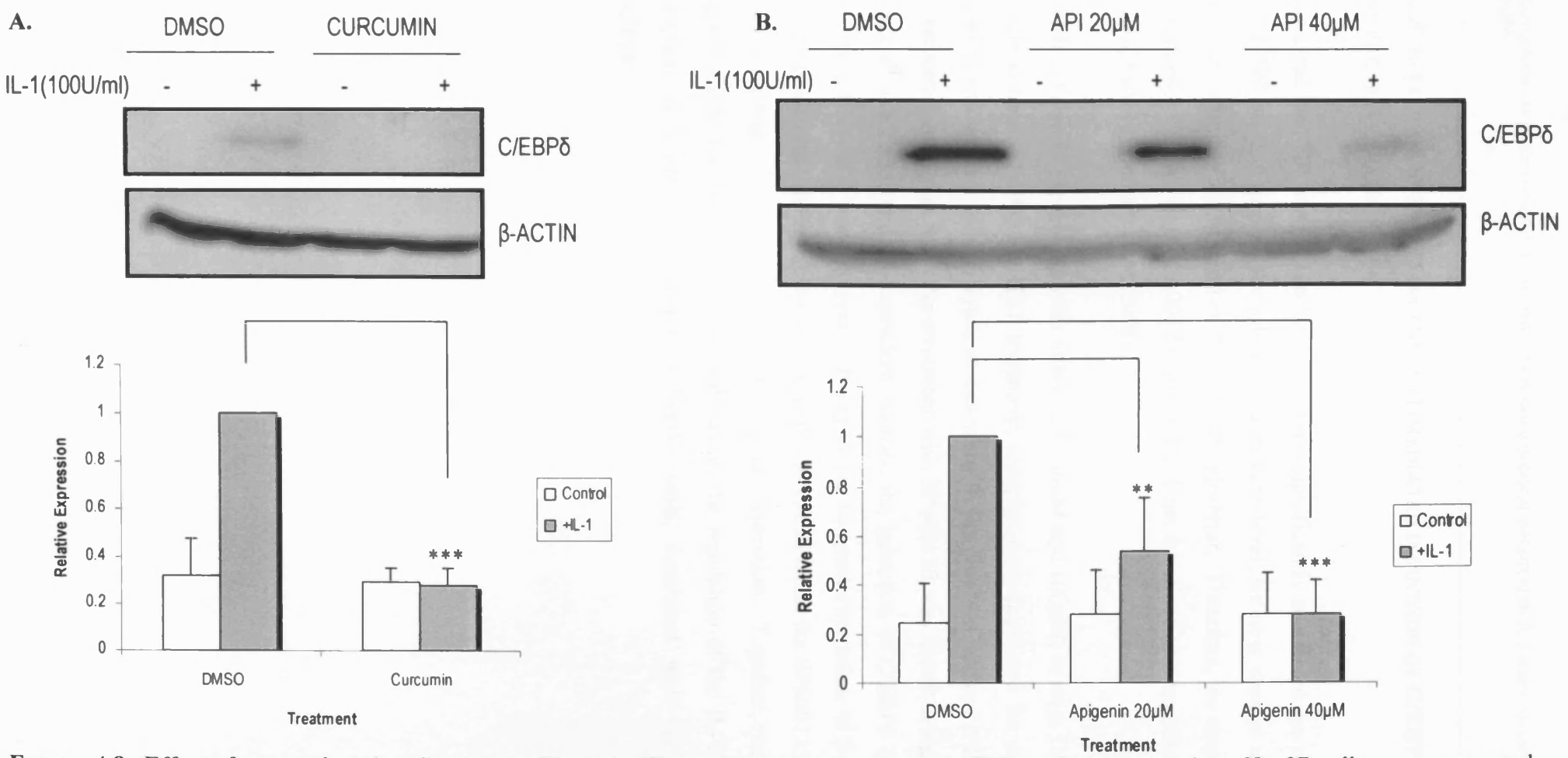



**FIGURE 4.8. Effect of curcumin (A) and apigenin (B) on the IL-1-mediated induction of C/EBPδ protein expression.** Hep3B cells were pre-treated with either curcumin (30μM), apigenin (20μM and 40μM) or DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated (control) for 3h at which point they were harvested for protein extraction (Section 2.7.2). Protein extracts were used for western blot analysis with 60μg of each sample subjected to 10% (w/v) SDS PAGE. Blots were probed with an anti-C/EBPδ antibody and visualised using ECL detection reagent. The same blot was then re-probed with an anti-β-actin antibody to ensure equal loading. Graphs display the ratios of C/EBPδ:β-actin, normalised to the IL-1 treated DMSO control as determined by densitometric analysis. Data shown for the inhibitor curcumin is the mean ± SD from three independent experiments and for the inhibitor apigenin, data shown is the mean ± SD from four independent experiments.
**P<0.01, ***P<0.001

### 4.2.8 EFFECT OF SP600125 ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA AND PROTEIN LEVELS

Given that the JNK MAPK inhibitor curcumin significantly attenuated the expression of C/EBPδ by IL-1 at both the mRNA and protein level, we next wanted to confirm these data with the use of another JNK MAPK inhibitor. Therefore, we employed the pharmacological inhibitor SP600125, which has been described as a specific inhibitor of JNK MAPK (Bennett *et al.* 2001).

Hep3B cells were pre-treated with SP600125 (50μM and 100μM) or with DMSO as a vehicle control. Following IL-1 treatment, samples were harvested for subsequent RT-PCR and western blot analysis, as described in the previous sections. The results are presented in Figure 4.9. Pre-treatment with SP600125 was found to significantly decrease, in a concentration-dependent manner, the induction of C/EBPδ by IL-1 at both the mRNA and protein level. Owing to the constant expression of β-actin, we also concluded that these results were specific to C/EBPδ and the SP600125 inhibitor was not having a global effect on cellular gene expression. Together, these results support a role for the JNK MAPK pathway in the regulation of the IL-1-mediated induction of C/EBPδ expression in Hep3B cells, consistent with our previous findings.

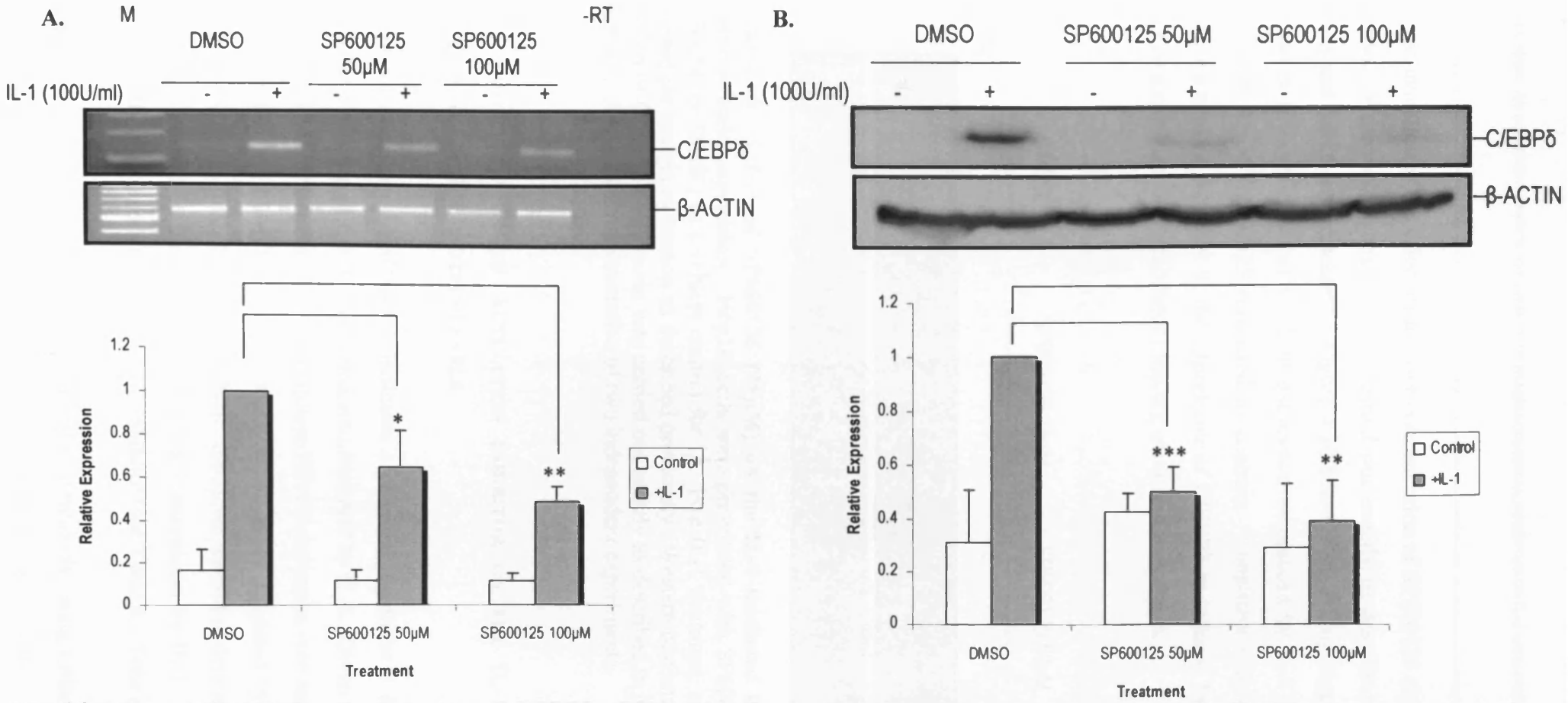



**FIGURE 4.9 Effect of SP600125 on the induction of C/EBPδ mRNA (A) and protein (B) expression as mediated by IL-1.** Hep3B cells were pre-treated with SP600125 (50μM and 100μM) or with DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated (control) for 3h at which point samples were harvested for RT-PCR and western blot analysis as described previously. The PCR amplification products for C/EBPδ and β-actin were resolved by electrophoresis (**A**). M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –IL-1 treated RNA). Ratios of C/EBPδ:β-actin were normalised to the IL-1 treated DMSO control as determined by densitometric analysis. The RT-PCR data shown is the mean ±SD from three independent experiments. Protein extracts were used for western blot analysis exactly as described in the legend of Figure 4.8 (**B**). Ratios of C/EBPδ:β-actin were normalised to the IL-1 treated DMSO control as determined by densitometric analysis. The western blot data shown is the mean ± SD from three independent experiments. *P<0.05, ** P<0.01, ***P<0.001.

We next investigated the effect of a lower concentration of SP600125 (25μM) on this response. Western blot analysis was carried out exactly as described in previous sections and results are presented in Figure 4.10 below. We did not note any marked decrease in the expression of C/EBPδ expression as induced by IL-1 in cells pre-treated with 25μM SP600125 compared to controls. Consistent with our previous results, we noted a decrease in the expression of C/EBPδ as induced by IL-1 at the higher concentration of the inhibitor (50μM), compared to controls.

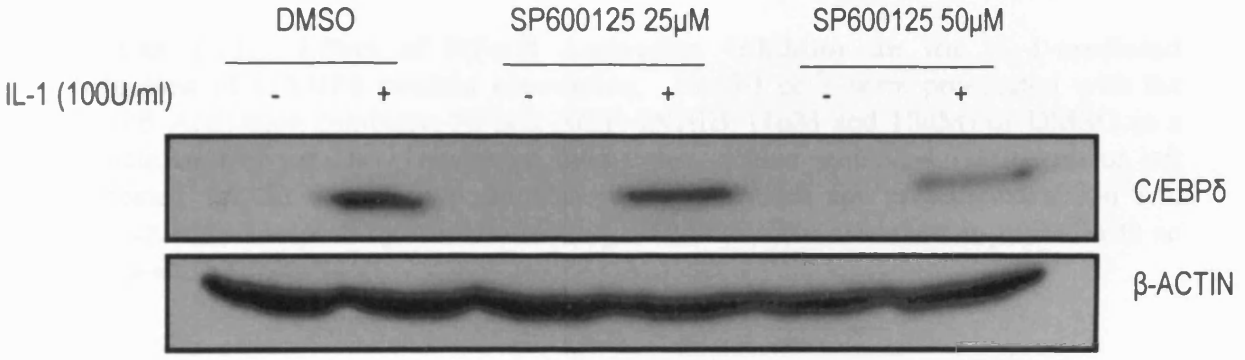



**FIGURE 4.10. Effect of SP600125 (25μM) on the IL-1-mediated induction of C/EBPδ protein expression**. Hep3B cells were pre-treated with SP600125 (25μM and 50μM) or DMSO as a vehicle control for 1h. Post IL-1 treatment, samples were harvested for protein extraction as described previously. Western blot analysis for the detection of C/EBPδ and β-actin was carried out exactly as described in the legend of Figure 4.8. Results are representative of two independent experiments.

### 4.2.9 EFFECT OF NF-κB ACTIVATION INHIBITOR ON THE IL-1-MEDIATED INDUCTION OF C/EBPδ PROTEIN LEVELS

Our previous data presented above, indicates a potential requirement for CK2 and JNK in the regulation of C/EBPδ expression as mediated by IL-1. Given that both the CK2 and JNK MAPK are known to modulate NF-κB activation (see section 4.3 for details) and because NF-κB is a major transcription factor regulated by IL-1 action (Martin and Wesche 2002; Dunne and O'Neill 2003), we wanted to determine whether NF-κB was involved in the regulation of C/EBPδ expression by IL-1. For this we used a recently described NF-κB activation inhibitor (see Table 4.1 Tobe *et al.* 2003).

Western blot analysis was carried out as specified previously, using antibodies against C/EBPδ and β-actin. Figure 4.11 shows the result of this western blot.

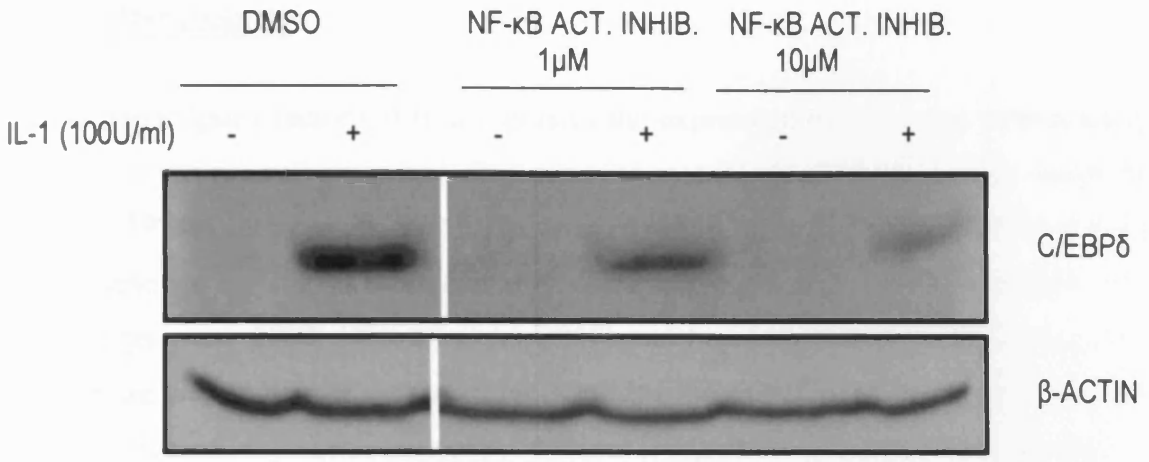



**FIGURE 4.11. Effect of NF-κB Activation Inhibitor on the IL-1-mediated induction of C/EBPδ protein expression**. Hep3B cells were pre-treated with the NF-κB Activation Inhibitor; NF-κB ACT. INHIB. (1μM and 10μM) or DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated for 3h at which point they were harvested for protein extraction and subsequently analysed by western blotting. The same blot was then re-probed with an anti-β-actin antibody.

The results presented above suggest that NF-κB is potentially involved in regulating the IL-1-mediated expression of C/EBPδ. The NF-κB activation inhibitor attenuates the IL-1-induced expression of C/EBPδ protein compared to the control at both specified concentrations and the inhibition appears to occur in a concentration-dependent manner. In addition, the inhibitor does not appear to affect the expression of the β-actin control, suggesting that the results are specific to C/EBPδ expression.

## 4.3 DISCUSSION

The transcription factor C/EBPδ regulates the expression of numerous inflammatory genes in various cell types including macrophages (Godambe *et al.* 1994a; Godambe *et al.* 1994b; Plevy *et al.* 1997; Hu *et al.* 1998; Liu *et al.* 2006b), epithelial cells (Hungness *et al.* 2002a; Hungness *et al.* 2002b; Svotelis *et al.* 2005), mesangial cells (Granger *et al.* 2000; Miyoshi *et al.* 2007) and hepatocytes (Poli 1998). However, there are limited studies devoted to determining the signalling pathways by which the expression/activity of this transcription factor is regulated by inflammatory stimuli.

Thus the primary aim of this project was to identify signalling pathways by which the pro-inflammatory cytokine, IL-1 may regulate C/EBPδ expression in hepatocytes. We hoped that these investigations would provide further insight into the molecular mechanisms by which C/EBPδ expression is regulated. The data presented in the previous chapter firmly establishes that IL-1 induces the expression of C/EBPδ at both the mRNA and protein levels in Hep3B cells. These results therefore provided a good basis for further study. Therefore, to initiate our investigations, we analysed the action of pharmacological inhibitors targeting key signalling pathways on the IL-1-mediated induction of C/EBPδ expression. Table 4.2 summarises the findings from these investigations.

**Table 4.2. Summary of experimental findings from investigations into the effect of pharmacological inhibitors on the IL-1-mediated induction of C/EBPδ expression.**

| INHIBITOR | CONCENTRATION | EFFECT ON IL-1-MEDIATED INDUCTION OF C/EBPδ MRNA AND PROTEIN | FIGURE(S) |
|---|---|---|---|
| Apigenin | 10-40μM | Inhibits mRNA and protein levels at 20 and 40μM | Figure 4.6<br>Figure 4.7(B)<br>Figure 4.8 |
| Bisindolylmaleimide | 2μM | No effect on mRNA levels | Figure 4.4 |
| Calphostin C | 20 and 40nM | No effect on mRNA levels | Figure 4.4 |
| Curcumin | 30-50μM | Inhibits mRNA and protein levels between 30-50μM | Figure 4.6<br>Figure 4.7(A)<br>Figure 4.8 |
| LY294002 | 5 and 20μM | No effect on mRNA levels | Figure 4.2 |
| NF-κB activation inhibitor | 1 and 10μM | Inhibits at protein level at both concentrations | Figure 4.11 |
| PD98059 | 50μM | No effect on mRNA levels | Figure 4.2 |
| SB202190 | 10μM | No effect on mRNA levels | Figure 4.3 |
| SB203580 | 5μM | No effect on mRNA levels | Figure 4.2 |
| SP600125 | 25-100μM | Inhibits mRNA and protein at 50 and 100μM | Figure 4.9<br>Figure 4.10 |

The inhibitors against the JNK MAPK, CK2 and NF-κB signalling pathways successfully attenuated the IL-1-mediated induction of C/EBPδ expression at the mRNA and/or protein level in Hep3B cells, all novel findings. Therefore, further investigations were undertaken to analyse in more detail the role of these pathways in the regulation of this response, this being the focus of the next three chapters.

The JNK MAPK pathway is known to be activated by a diverse group of extracellular signals, for example, UV light, inflammatory cytokines (including IL-1) and a variety of other cellular stresses (see section 5.1). The transcription factor c-Jun is a downstream target of the JNK MAPK pathway. A role for c-Jun in the regulation of C/EBPδ expression has recently been described (Huang *et al.* 2007). Peptidoglycan induces the expression of C/EBPδ in murine macrophages and this increase is

mediated at least in part, by the transcription factor c-Jun. In addition, another study has also suggested a role for the JNK MAPK pathway in the regulation of C/EBPδ expression as mediated by IL-1 in the HepG2 cell line (Dong *et al.* 2007). The experimental findings of the Dong *et al.* (2007) study are discussed in detail in chapter 8 of this thesis. With the use of the inhibitor SP600125, the authors demonstrate that the IL-1-mediated increase in C/EBPδ expression is reduced in cells pre-treated with this inhibitor. We also report similar findings in relation to the expression of C/EBPδ by IL-1 in the Hep3B cell line. In addition, we provide further evidence for a role of the JNK MAPK pathway in the regulation of this response with the use of another JNK MAPK inhibitor, curcumin.

The role of CK2 in the regulation of C/EBPδ expression by IL-1 was investigated with the inhibitor apigenin. Apigenin inhibited, in a concentration-dependent manner, the IL-1-mediated induction of C/EBPδ expression at both the mRNA and protein level [Figures 4.6, 4.7(B) and 4.8]. These results are potentially novel because to our knowledge, no study has analysed a role for CK2 in the regulation of C/EBPδ expression during the inflammatory response. We did note however that apigenin also significantly inhibited the constitutive expression of C/EBPδ mRNA (see Figure 4.6). Unfortunately, the low basal level of C/EBPδ mRNA and protein expression in unstimulated cells made it difficult to accurately assess this effect by semi-quantitative RT-PCR and western blotting. Nevertheless, this observation potentially suggests that CK2 may also function to regulate the constitutive expression of C/EBPδ. A role for CK2 in constitutive gene expression is plausible given the known properties of this enzyme. CK2 is ubiquitously expressed and is constitutively active (although its activity is also reported to be inducible. See section 5.1). It is therefore possible, that CK2 may have dual roles in the regulation of both the constitutive and IL-1-induced expression of C/EBPδ. Indeed CK2 has been reported to modulate transcription of mRNA produced by RNA polymerse II by regulating the activity of the basal transcription machinery (Cabrejos *et al.* 2004; Lewis *et al.* 2005b) and CK2 has also been implicated in the regulation of inducible gene/protein expression through the regulation of other transcription factors such as Egr-1, STAT1, AP-1 and ICER (Zdunek *et al.* 2001; Mead *et al.* 2003; Singh and Ramji 2006; Harvey *et al.* 2007). Further studies would be required to determine the role (if any) of CK2 in the constitutive expression of C/EBPδ. A potential role for CK2 in the regulation of

C/EBPδ activity has previously been suggested (Osada *et al.* 1996). Given this, the fact that CK2 action has previously been implicated during the inflammatory response (Li *et al.* 2005; Yamada *et al.* 2005) and that IL-1 has been reported to stimulate CK2 (Bird *et al.* 1997), we decided that determining the role of CK2 in IL-1-mediated induction of C/EBPδ warranted further investigation (Chapters 5 and 6).

Our results also suggest a potential role for NF-κB in the regulation of C/EBPδ expression by IL-1 (Figure 4.11). Numerous studies have demonstrated a key role for NF-κB activation during IL-1 signalling (see Martin and Wesche 2002; Dunne and O'Neill 2003 and references therein). However, a role for NF-κB in the regulation of C/EBPδ expression as mediated by IL-1 is yet to be reported. Recently, NF-κB has been implicated in the regulation of C/EBPδ expression as mediated by LPS and peptidoglycan in murine macrophages (Liu *et al.* 2006b; Huang *et al.* 2007). The role of NF-κB in the regulation of C/EBPδ expression by IL-1 was investigated further and the results of these investigations are presented in chapter 7 of this thesis.

Although the JNK MAPK, CK2 and NF-κB pathways have been discussed separately with regards to their potential role in regulating IL-1-mediated expression of C/EBPδ, one cannot rule out the possible relevance of cross-talk between the pathways in relation to the regulation of this response. Cross-talk between CK2 and the JNK MAPK pathways has previously been described (Fleming *et al.* 2000; Singh and Ramji 2006). Fleming *et al.* (2000) showed that CK2 catalysed the phosphorylation of JNK2α2 at serine$^{407}$ and threonine$^{404}$, at least *in vitro*. Although the role of serine$^{407}$ and threonine$^{404}$ phosphorylation was not determined in this study, it was suggested that phosphorylation of these two residues could play a role in regulating stability of JNK2α2, or mediating its interactions with other proteins. In a separate study conducted in our laboratory, a role for CK2 in JNK MAPK signalling was also suggested (Singh and Ramji 2006). TGF-β induces the expression of ApoE in human monocytes and this induction is regulated by three principle pathways involving JNK, p38 MAPK and CK2. In relation to this response, the action of CK2 and the JNK MAPK pathway converged on c-Jun/AP-1 (Singh and Ramji 2006). However, no experiments were carried out to determine the role for CK2 in the regulation of TGF-β-mediated activation of JNK MAPK. These studies are particularly interesting in relation to this study, because they suggest a possible link between these two

pathways and therefore provided us with a basis for future experiments (Chapters 5 and 6).

In addition, cross-talk between the JNK MAPK and the NF-κB pathways has also been described (Schulze-Osthoff *et al.* 1997; Nakano *et al.* 2006; Wullaert *et al.* 2006; Liu *et al.* 2006c; Tirumurugaan *et al.* 2007). Furthermore, a number of studies have also presented evidence for CK2 in the regulation of NF-κB activity (McElhinny *et al.* 1996; Landesman-Bollag *et al.* 2001; Romieu-Mourez *et al.* 2001; Romieu-Mourez *et al.* 2002; Cavin *et al.* 2003; Yu *et al.* 2006). Given that our data suggests a potential requirement for JNK MAPK, CK2 and NF-κB in the regulation of IL-1-mediated induction of C/EBPδ expression, the possibility that cross-talk or convergence between the pathways may occur was not ruled out and therefore was investigated further (Chapters 5-8).

The other results presented in this chapter suggest that neither the ERK1/2 MAPK nor the p38 MAPK are involved in regulating the IL-1-mediated induction of C/EBPδ expression, at least as assessed by RT-PCR (see Figures 4.2 and 4.3). This response was not attenuated by PD98059, SB203580 or SB202190. Since the positive action of these inhibitors has previously been confirmed in our laboratory (see section 4.2.1) these data were unlikely to be a result of the inhibitors themselves being inactive. Based on these results, we did not pursue detailed investigations into the role of these MAPKs in the regulation of this response. Roles for the p38 and ERK1/2 MAPK pathway in the IL-1-mediated regulation of C/EBPδ have been suggested in two separate studies. IL-1-mediated activation of C/EBPδ is reported to be regulated by p38 MAPK, but not the ERK1/2 MAPK in IEC-6 intestinal epithelial cells (Svotelis *et al.* 2005). The other study indicates a role for the ERK1/2 MAPK in modulating the activity/expression of C/EBPδ by IL-1 in the Caco-2 intestinal epithelial cell line (Hungness *et al.* 2002b). Because both groups having utilised intestinal epithelial cells for their studies, a cell line type-specific mechanism of C/EBPδ regulation by IL-1 is suggested.

Our results also suggest that the PI3K pathway is also not required in the regulation of this response, given that the specific inhibitor LY294002 did not attenuate the expression of C/EBPδ mRNA as determined by RT-PCR (see Figure 4.2). Again, as the positive action of this inhibitor has previously been confirmed in our laboratory

(section 4.2.1) these data are unlikely to be a result of the inhibitor itself being inactive. However, a role for the PI3K pathway has been suggested in the regulation of C/EBPδ as induced by EGF. The induction of C/EBPδ expression by EGF was found to be mediated via a PI3K/p38 MAPK/CREB dependent pathway in human epidermoid carcinoma A431 cells (Wang *et al.* 2005).

Finally, our data also suggests that the PKCs are not required for the regulation of C/EBPδ by IL-1 (Figure 4.4). To add further support to this result, we also confirmed the action of the generic PKC inhibitor, bisindolylmaleimide in our experiments (Figure 4.5). The PMA-mediated induction of LPL expression was successfully blocked by bisindolylmaleimide in THP-1 cells, as previously described (Mead *et al.* 2003). This ruled-out the possibility the inhibitor we used in our experiments was inactive.

In conclusion, the results in this chapter have successfully identified three signal transduction pathways (JNK MAPK, CK2 and NF-κB) that may be involved in regulating the IL-1-mediated induction of C/EBPδ expression. These data are novel and provide a basis for the further investigation of these pathways in the regulation of this response, which is the focus of the remainder of this thesis. The following chapters will examine the potential role of each of these pathways in more detail.

# CHAPTER FIVE: AN INVESTIGATION INTO THE EFFECTS OF IL-1 ON JNK AND CK2 ACTIVATION AND THEIR ROLES IN IL-1-MEDIATED INDUCTION OF C/EBPδ EXPRESSION

## 5.1 INTRODUCTION

The results presented in the previous chapter suggest a potential role for the JNK MAPK and CK2 signalling pathways in the regulation of IL-1-mediated induction of C/EBPδ expression. As the JNK MAPK module and CK2 have not been described in detail previously in this thesis, a description of these signalling components is given below.

***JNK MAPK***- As mentioned earlier in sections 1.4.4 and 4.1, JNK is one of three major MAPK modules found in mammalian systems and all MAPK pathways are organised into a central three-tiered 'core signalling system'. A summary of the events resulting in MAPK activation is presented in Figure 5.1.

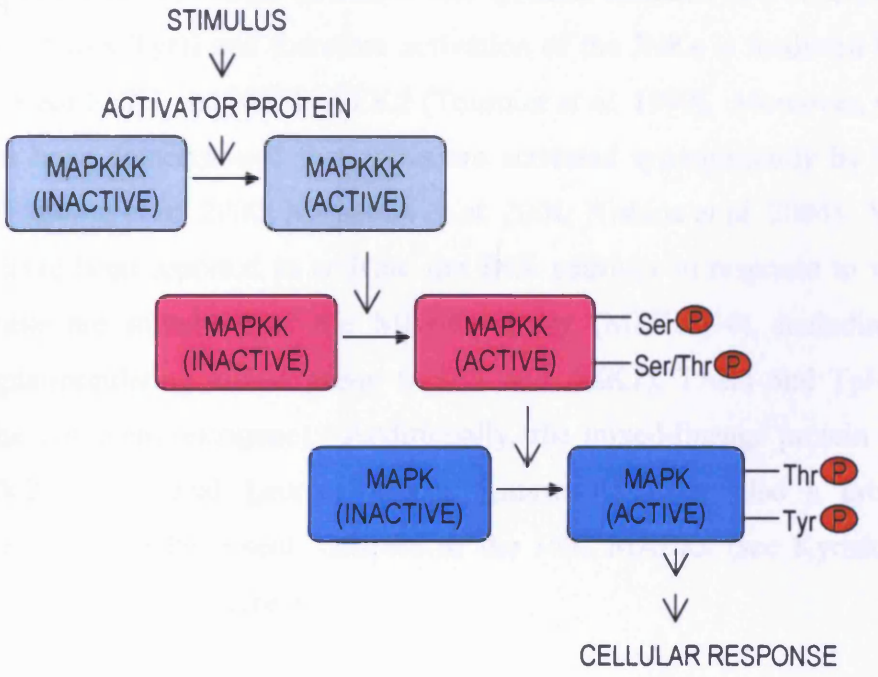



**FIGURE 5.1. A schematic representation of the core MAP kinase signalling module (adapted from Kyriakis and Avruch 2001).** A variety of extracellular signals feed into the core module, activating the pathway. Activated MAPKKKs catalyse the serine/threonine phosphorylation of downstream effectors, the MAPKK. The phosphorylation of MAPKK concomitantly activates their kinase activity and results in the phosphorylation of the MAPK substrates. The threonine and tyrosine phosphorylation of the MAPK activates these enzymes, which in turn phosphorylate their target proteins. The downstream targets of this cascade then coordinate the appropriate cellular response.

The stress-activated protein kinases (SAPKs) or JNK MAPKs are strongly induced in cells that have been exposed to a variety of 'stress-inducing' treatments. These include heat shock, ionising radiation, oxidant stress, DNA damaging chemicals (topisomerase inhibitors and alkylating agents) and protein synthesis inhibitors such as cycloheximide and anisomycin (Hibi *et al.* 1993; Derijard *et al.* 1994; Kyriakis *et al.* 1994; Kyriakis and Avruch 1996; Pombo *et al.* 1996). JNKs are also strongly activated in response to inflammatory cytokines such as IL-1 and TNF-α (see Davis 1999; Kyriakis and Avruch 2001 and references therein; Li *et al.* 2001a). At least twelve separate JNK isoforms have been identified as the products of three genes (JNK1-3) (Kallunki *et al.* 1994; Kyriakis *et al.* 1994; Gupta *et al.* 1996; Dreskin *et al.* 2001; Kyriakis and Avruch 2001). Differential mRNA splicing leads to the generation of two predominant forms of JNK, p54 and p46 (Kyriakis and Avruch 2001). Dual phosphorylation [at threonine and tyrosine residues in a conserved tri-peptide motif (Thr-X-Tyr)] and therefore activation of the JNKs is mediated by two MAPKKs, MKK4/SEK1 and MKK7/SEK2 (Tournier *et al.* 1999). Moreover, several recent studies have demonstrated that JNKs are activated synergistically by MKK4 and MKK7 (Fleming *et al.* 2000; Matsuoka *et al.* 2004; Nishina *et al.* 2004). Several MAPKKKs have been reported to activate the JNK pathway in response to various stimuli. These are members of the MEKK family (MEKK1-4), including, the apoptosis signal-regulating kinase group (ASK1 and ASK2), TAK1 and Tpl-2 (the product of the *col* proto-oncogene). Additionally, the mixed-lineage protein kinase family (MLK2-3 and Dual Leucine zipper Kinase-DLK) are also a group of MAPKKKs reported to be potent inducers of the JNK MAPKs (see Kyriakis and Avruch 2001 and references therein).

JNK protein kinases were originally described as activators of the transcription factor c-Jun. JNKs phosphorylate the $NH_2$-terminal activation domain of this transcription factor at serine$^{63}$ and serine$^{73}$ resulting in increased c-Jun transcriptional activity (Pulverer *et al.* 1991; Smeal *et al.* 1991; Adler *et al.* 1992; Franklin *et al.* 1992; Hibi *et al.* 1993). c-Jun is a major component of the AP-1 transcription factor complex and the JNKs regulate its recruitment/activation in response to extracellular stimuli. AP-1 is a heterodimer comprised of bZIP transcription factors, typically c-Jun, JunD, members of the *fos* (usually c-Fos) and activating transcription factor (ATF-2)

families (Kyriakis and Avruch 2001). A summary of the major components in JNK MAPK signalling is presented in Figure 5.2.

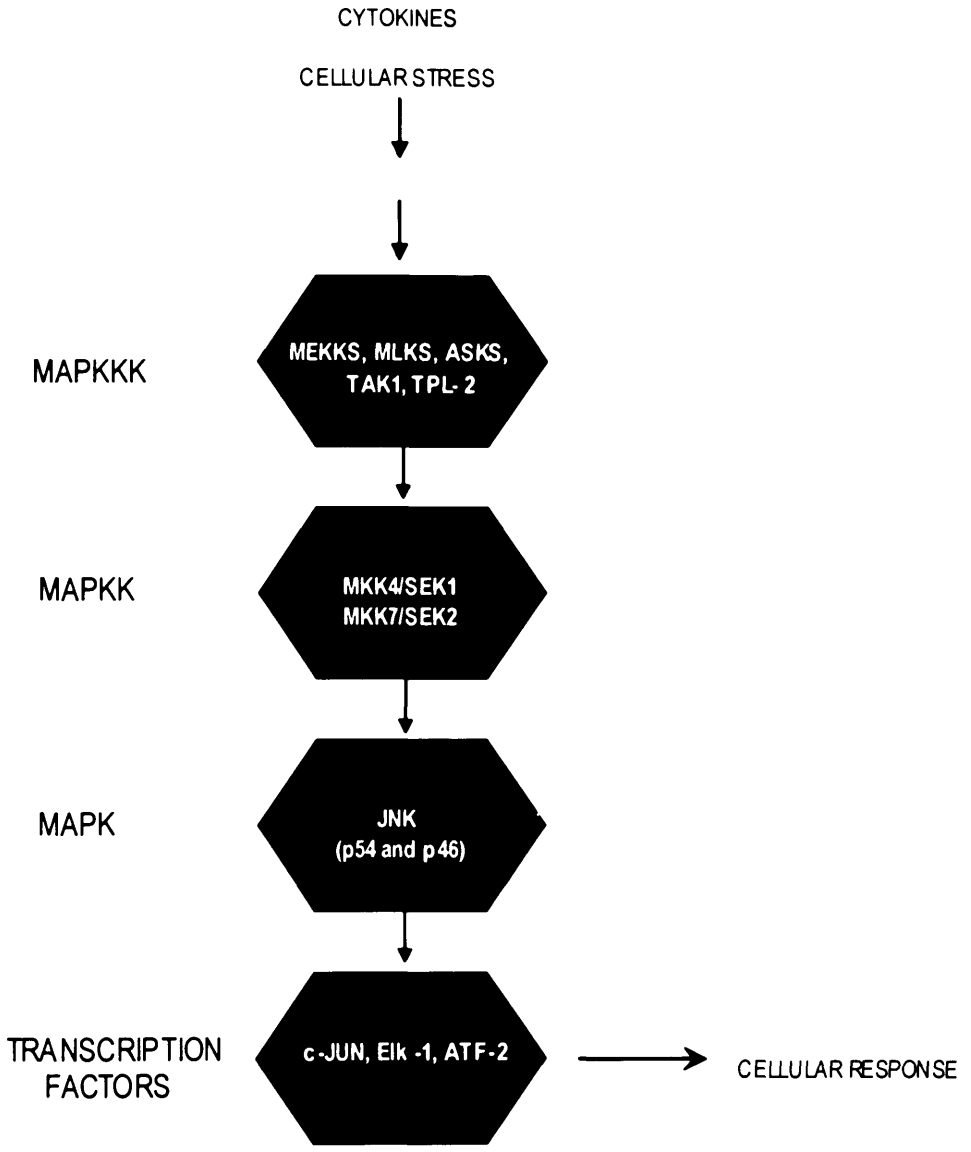



**Figure 5.2. A schematic representation of the JNK signalling cascade.** Abbreviations: SEK, SAPK/ERK kinase; Elk-1, Ets-domain transcription factor.

***CK2***-As mentioned briefly in section 4.1, CK2 is a highly conserved, pleiotropic protein serine/threonine kinase. This enzyme is ubiquitously expressed, constitutively active and regulates a variety of cellular processes. These include, cell cycle regulation, circadian rhythms, apoptosis, transformation, tumorigenesis, inflammation and recently a role for CK2 in the regulation of cell morphology has emerged (Pinna

1997; Pinna 2002; Litchfield 2003; Li *et al.* 2005; Yamada *et al.* 2005; Canton and Litchfield 2006). Typically, the enzyme exists as a tetrameric holoenzyme consisting of two catalytic subunits (in any combination of α, α' or α'') and two regulatory β subunits. However, catalytic isoforms are active in isolation and as part of the holoenzyme. Although the catalytic subunits are structurally similar, α and α' are reported to exhibit some differential functions (Litchfield 2003). A body of work indicates that the β subunit regulates the assembly of tetrameric CK2 complexes, enhances the catalytic activity and stability of the kinase and is involved in regulating substrate selectivity of CK2 (see Litchfield 2003 and references therein). CK2β is also proposed to have functions independent of the CK2 tetramer because firstly, this subunit does not exclusively co-localise with the catalytic subunits of the kinase inside cells. Also, CK2β interacts with and regulates the activity of a number of other serine/threonine kinases in the absence of CK2 catalytic subunits (Bibby and Litchfield 2005). In terms of cellular localisation, CK2 has been found in the nucleus, the cytoplasm and in association with the plasma membrane, golgi and endoplasmic reticulum (Faust *et al.* 2001; Faust *et al.* 2002; Filhol *et al.* 2003).

The mechanisms by which CK2 is regulated are poorly understood. Despite being constitutively active, the kinase is subject to inducible regulation by a variety of stimuli, including IFN-γ (Mead *et al.* 2003), EGF (Pepperkok *et al.* 1991), TNF-α (Sayed *et al.* 2000), TGF-β (Zdunek *et al.* 2001; Singh and Ramji 2006), UV-B light (Brenneisen *et al.* 2002) and LPS (Lodie *et al.* 1997). Other mechanisms that have also been proposed to contribute towards the regulation of CK2 include; regulated expression and assembly of the enzyme, control by covalent modification and modulation through interactions with other proteins and/or non-protein molecules (see Litchfield 2003 and references therein).

There is an ever growing list of CK2 substrates with over 300 documented in 2003 (Meggio and Pinna 2003). They include signalling molecules, transcription factors, proteins affecting DNA/RNA functions and protein synthesis, viral proteins, cytoskeleton-structural proteins and metabolic enzymes (Meggio and Pinna 2003). Given the diversity of its substrates and its role in a number of global cellular processes, CK2 is likely to regulate a variety of signal transduction pathways.

### 5.1.1 EXPERIMENTAL STRATEGY

Work in this chapter was carried out with the aim of determining in more detail, the role of JNK MAPK and CK2 in the regulation of C/EBPδ gene expression by IL-1. In relation to JNK MAPK signalling, this was firstly to be achieved by analysing the changes in the phosphorylation of JNK in response to IL-1 treatment. In conjunction, the activity of this kinase in response to cytokine treatment was also analysed. The effect of pharmacological inhibitors on IL-1-mediated JNK activation was also assessed to determine potential mechanism(s) by which IL-1 stimulates JNK activation. Dominant negative (DN) mutants against selected components of the JNK pathway were then used to determine their effect on IL-1-mediated induction of C/EBPδ mRNA levels. Using a similar approach, we also examined the effect of IL-1 on the activation of CK2 and used a DN CK2 mutant to elucidate a role for this kinase in regulating C/EBPδ expression by IL-1.

Figure 5.3 summaries our overall experimental approach that was designed to achieve the outlined experimental objectives.

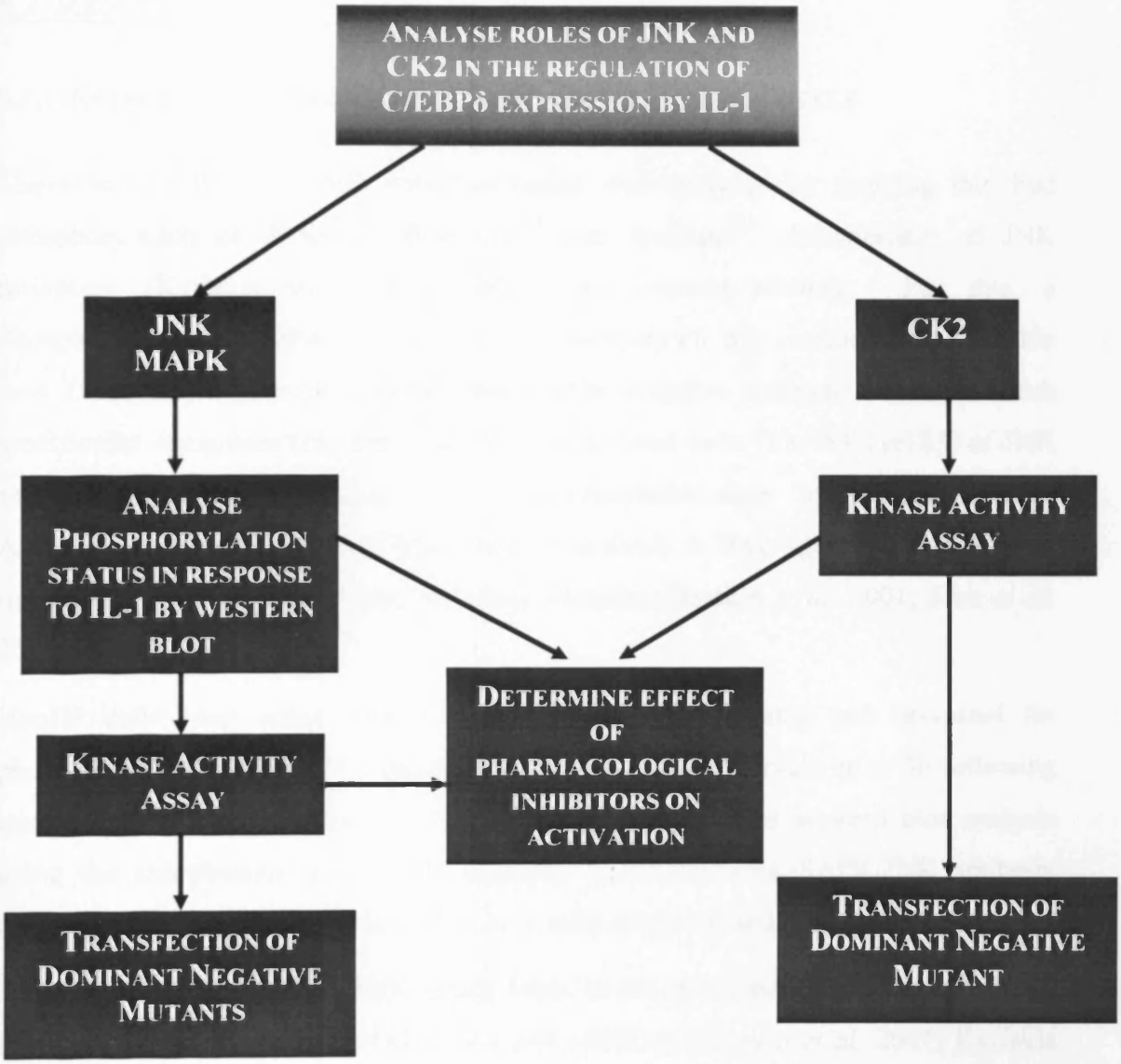



**FIGURE 5.3. Experimental strategy.** Potential roles of JNK and CK2 during IL-1 signalling in relation to C/EBPδ expression were investigated by determining the effect of cytokine treatment on the activation of these kinases. Possible mechanisms of regulation were determined with the on going use of pharmacological inhibitors and with the use of DN mutants.

## 5.2 RESULTS

### 5.2.1 EFFECT OF IL-1 ON JNK PHOSPHORYLATION IN HEP3B CELLS

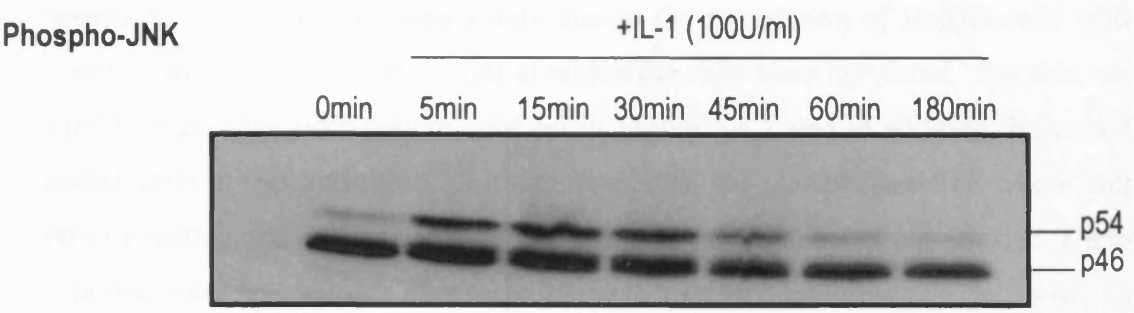
The effect of IL-1 on JNK phosphorylation was analysed by studying the dual phosphorylation of JNKs at threonine[183] and tyrosine[185] characteristic of JNK activation (Kyriakis and Avruch 2001), by western blotting. For this, a PhosphoPlus® SAPK/JNK (Thr183/Tyr185) antibody kit was commercially available (see Table 2.0). This kit contains two highly selective antibodies, one of which specifically recognises only the dually phosphorylated form (Thr183/Tyr185) of JNK and the other detects total JNK (phosphorylation-state independent) levels. Antibodies such as these have been used extensively to investigate the role of JNK signalling in different cell types including Hep3Bs (Dreskin *et al.* 2001; Kim *et al.* 2004; Yeh and Yen 2005).

Hep3B cells were either treated with IL-1 or left untreated and harvested for phosphatase-free whole cell protein extraction at various intervals up to 3h following stimulation by IL-1 (Section 2.7.2). Extracts were used for western blot analysis using the anti-phospho-SAPK/JNK antibody or the anti-total-SAPK/JNK antibody (Section 2.7.7-2.7.10 and Table 2.9). The results are presented in Figure 5.4.1.

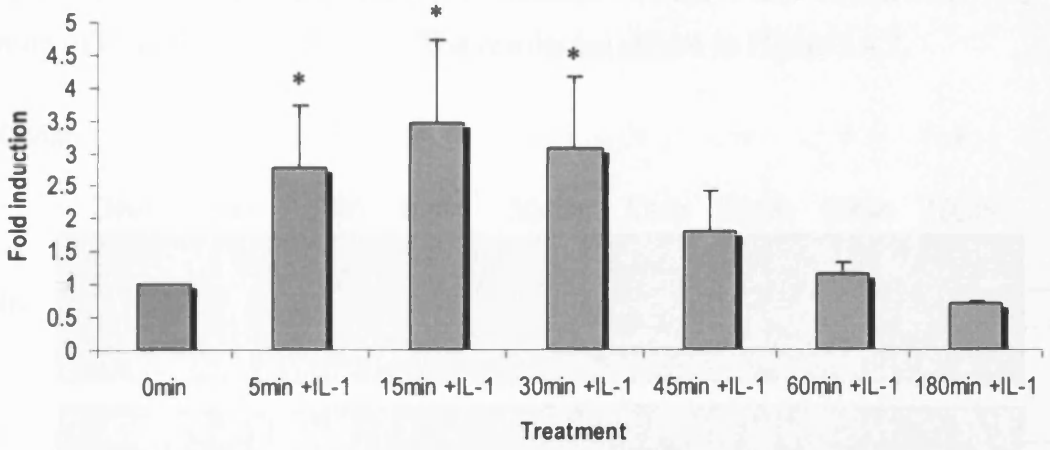
Differential splicing of the JNK genes leads to the generation of two predominant forms of this enzyme, p54 (54kDa) and p46 (46kDa) (Dreskin *et al.* 2001; Kyriakis and Avruch 2001). Western blot analysis of protein extracts from human cells, using antibodies specific for JNK detection, reveal that the p46 JNK isoforms often appear as more than one band (often a doublet) and the p54 JNK isoforms as a single band when these proteins are size fractionated by SDS-PAGE (Dreskin *et al.* 2001). Our results show that IL-1 induces rapid phosphorylation of mainly p54 JNK, after only 5 minutes of treatment. IL-1-mediated phosphorylation of p54 JNK peaks with 3.5-fold induction at 15min. The phosphorylated p54 isoform can be clearly detected up to 45 minutes following IL-1 treatment, with levels returning to basal at 180 minutes (Figure 5.4.1, Panel A). Total-JNK levels do not vary between each of the samples examined (Figure 5.4.1, Panel C), indicating that the changes observed in Panel A are due to phosphorylation alone and not increased expression of the JNK proteins.

**FIGURE 5.4.1. Time dependent effect of IL-1 on phospho- and total-JNK MAPK levels in Hep3B cells over a period of 3h.** Hep3B cells were either treated with IL-1 or left untreated (0min) and harvested for phosphatase-free protein extraction (Section 2.7.2) at each of the indicated time-points. Western blot analysis was carried out by SDS-PAGE using 10% (w/v) gels as previously described with 80μg of each sample and blots were probed using an anti-phospho-JNK antibody (A) or an anti-total-JNK antibody (C). The phosphorylated p54 protein level for each time-point was determined by densitometric analysis, normalised to the 0min control and plotted as a histogram (B). The data shown in Panel B is the mean ± SD from three independent experimental series (*$P<0.05$). Similarly, the total p54 protein level for each time-point was also determined by densitometric analysis, normalised to the 0min control and plotted as a histogram (Panel D). The data in Panel D shown is the mean from two independent experimental series.

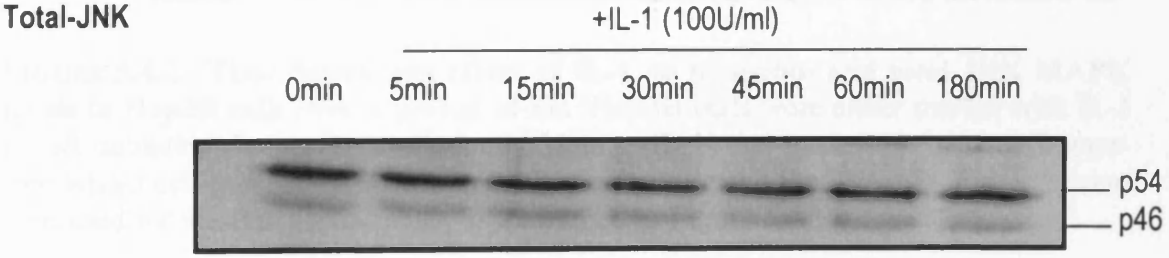
**A.**


Phospho-JNK
+IL-1 (100U/ml)
0min
5min
15min
30min
45min
60min
180min
p54
p46

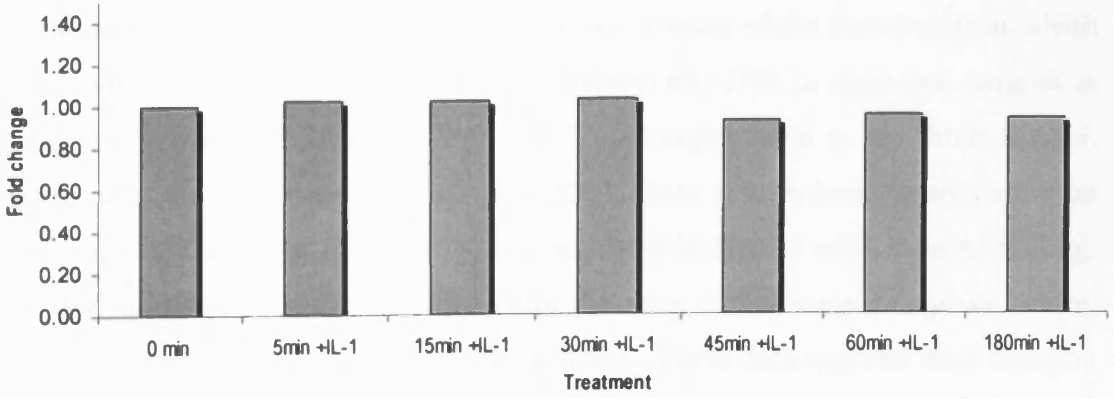
**B.**


Fold induction
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
*
*
*
0min
5min +IL-1
15min +IL-1
30min +IL-1
45min +IL-1
60min +IL-1
180min +IL-1
Treatment

**C.**


Total-JNK
+IL-1 (100U/ml)
0min
5min
15min
30min
45min
60min
180min
p54
p46

**D.**


Fold change
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
0 min
5min +IL-1
15min +IL-1
30min +IL-1
45min +IL-1
60min +IL-1
180min +IL-1
Treatment

We next sought to show that the observed phosphorylation of p54 JNK as demonstrated in Figure 5.4.1 was solely due to the stimulation of Hep3B cells with IL-1 and not a result of the time-point at which the cells were harvested. For this, we decided to repeat the time-course detailed in Figure 5.4.1 and in addition, harvested untreated cells at the 5min and 30min time-points, for phosphatase-free whole cell protein extraction and subsequent western blot analysis as detailed previously. These time-points were specifically chosen to harvest untreated cells because as shown in Figure 5.4.1, p54 JNK phosphorylation is induced at both 5 and 30min following stimulation of Hep3B cells with IL-1. The results are shown in Figure 5.4.2.

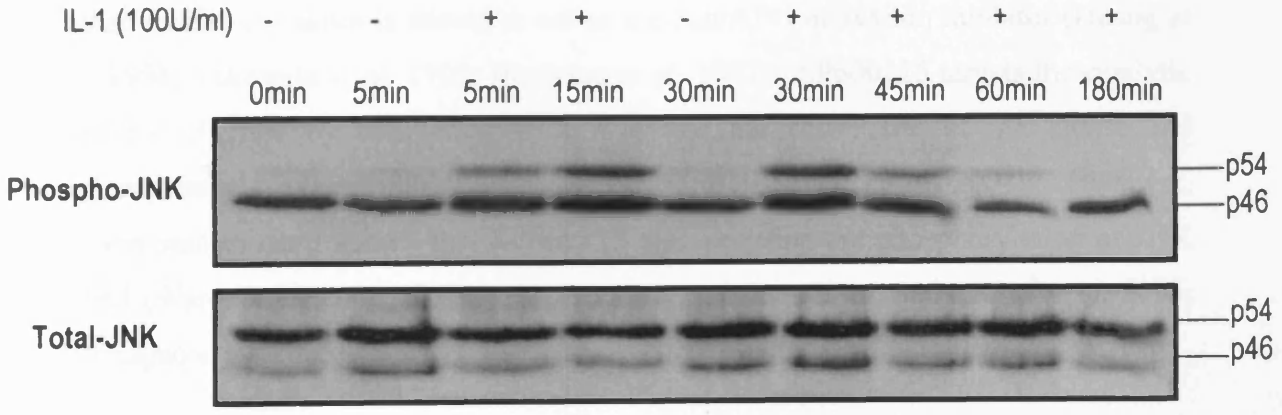



**FIGURE 5.4.2. Time dependent effect of IL-1 on phospho- and total-JNK MAPK levels in Hep3B cells over a period of 3h.** Hep3B cells were either treated with IL-1 or left untreated (0min, 5min –IL-1 and 30min –IL-1) and harvested for phosphatase-free whole cell protein extraction at each of the indicated time-points. Extracts were then used for western blot analysis as described in Figure 5.4.1.

The results clearly show that the noted increase in phosphorylation of JNK is due to the stimulation of Hep3B cells with IL-1 and not a result of the time-points at which the cells were harvested. The levels of phosphorylated JNK in untreated samples at 5min (5min –IL-1) and 30min (30min –IL-1) are comparable to the 0min control. Together with the data presented in Figure 5.4.1, these results demonstrate that upon stimulation with IL-1, the JNK pathway is activated in Hep3B cells, a novel finding. Total-JNK levels were also examined from the same set of samples and as before, there was minimal variation between the samples. These data together with our data presented in the previous chapter supports our hypothesis for a role of the JNK MAPK pathway in the regulation of C/EBPδ expression by IL-1.

### 5.2.2 EFFECT OF CURCUMIN, APIGENIN AND SP600125 ON IL-1-MEDIATED JNK PHOSPHORYLATION

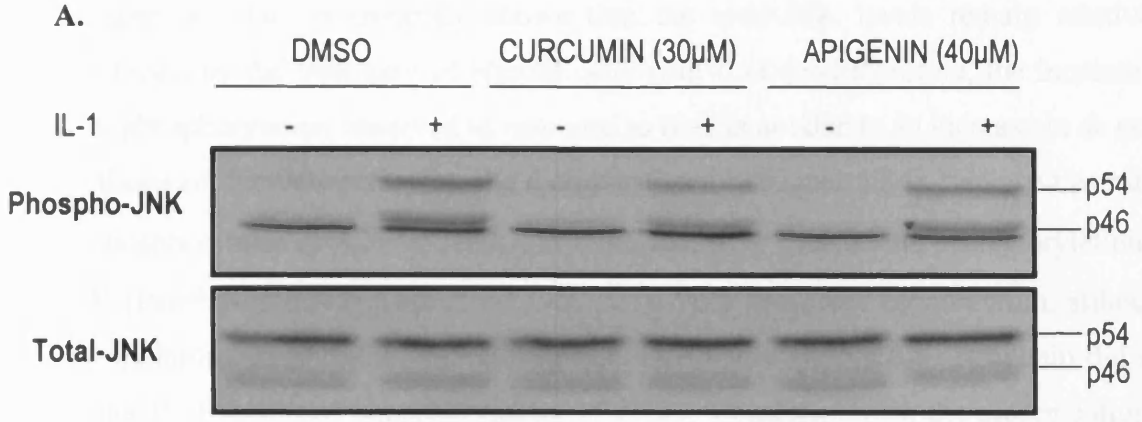
The results from chapter 4 show that the JNK inhibitors, SP600125 and curcumin significantly attenuate the IL-1-mediated induction of C/EBPδ mRNA and protein expression. Therefore, we wanted to confirm the activity of both curcumin and SP600125 as JNK inhibitors in Hep3B cells. A study by Chen and Tan (1998) suggested that curcumin acts as an upstream inhibitor of the JNK pathway. This compound is believed to act at the MAPKKK level, probably through the inhibition of MEKK1 (Chen and Tan 1998) and therefore may block JNK phosphorylation. In other studies curcumin is shown to act as a c-Jun/AP-1 activation inhibitor (Huang *et al.* 1991; Takeshita *et al.* 1995; Bierhaus *et al.* 1997). SP600125 targets the catalytic activity of JNK by competing with ATP for the active site of the kinase and subsequently prevents phosphorylation of c-Jun (Bennett *et al.* 2001). However, recent studies have shown that SP600125 also prevents the phosphorylation of JNK itself (Wang *et al.* 2002; Tokuda *et al.* 2003; Nakahara *et al.* 2004; Cho *et al.* 2005; Tamagno *et al.* 2005).

The CK2 inhibitor apigenin also inhibits the expression of C/EBPδ by IL-1 (See chapter 4). Given that CK2 has previously been suggested to function as an upstream component of the JNK pathway (Fleming *et al.* 2000; Min *et al.* 2003) we wanted to investigate whether apigenin inhibited JNK phosphorylation.

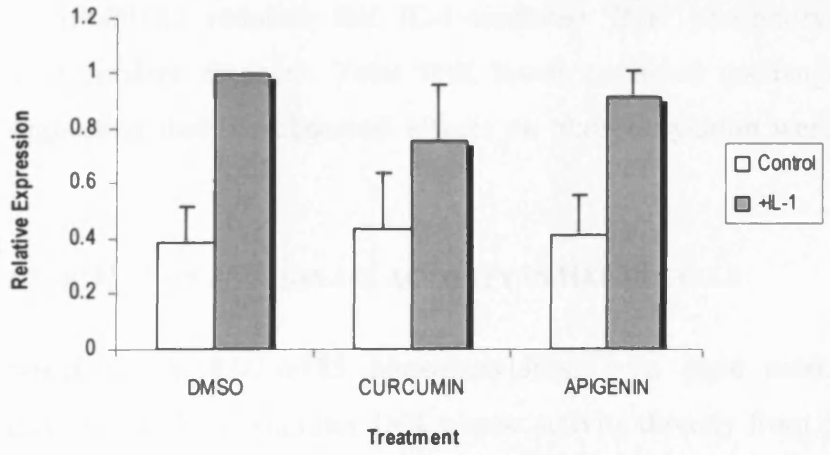
All the above mentioned inhibitors were used to examine their effect on IL-1-mediated induction of JNK phosphorylation. Hep3B cells were pre-treated with curcumin (30μM), apigenin (40μM) or SP600125 (50μM and 100μM) in addition to DMSO (vehicle-control), for 1h prior to the addition of IL-1. The cells were then harvested at 15min, following cytokine treatment for phosphatase-free whole cell protein extraction as before. The cells were harvested at 15min because it is at this time-point that maximum JNK phosphorylation was observed in response to IL-1 (Figures 5.4.1 and 5.4.2). Western blot analysis was carried out on protein extracts as specified above, immunoblotting with the specific antibodies raised against phospho-JNK and total-JNK. Results are presented in Figure 5.5.

**Figure 5.5. The effect of inhibitors on IL-1-mediated JNK phosphorylation in Hep3B cells.** Hep3B cells were treated with the indicated inhibitor at the specified concentrations in addition to DMSO (vehicle-control), for 1h prior to the addition of IL-1 (100U/ml). Phosphatase-free protein extracts were prepared from cells harvested at 15min following the addition of IL-1 and used for western blot analysis as described in Figure 5.4.1. The images are shown in Panels A and C. The phosphorylated and total p54 protein level for each sample was determined by densitometric analysis. Panels B and D show the ratios of phosphorylated p54 JNK:total p54 JNK for each sample, normalised to the IL-1 treated DMSO control that has been assigned as 1. The data shown in Panel B is the mean ±SD from three independent experimental series. The data shown in Panel D is representative of two independent experiments.

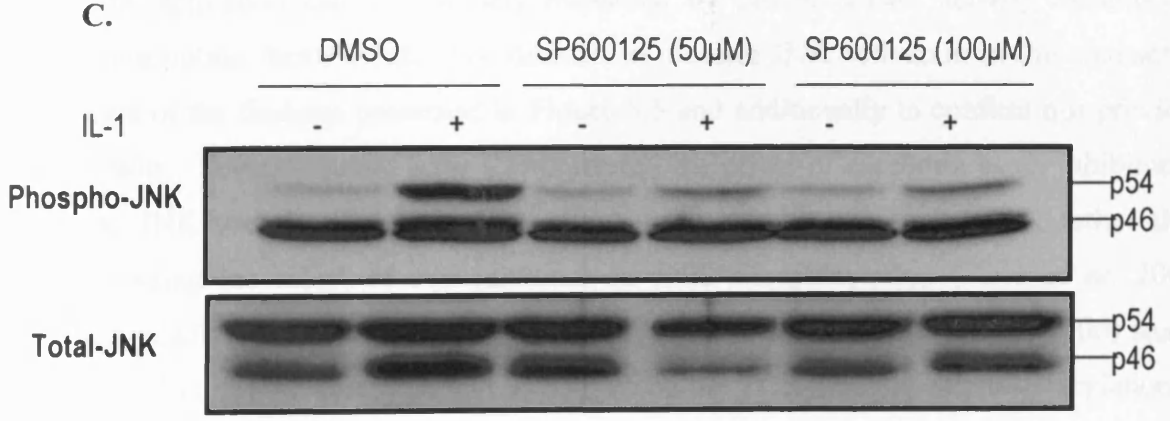
**A.**


DMSO
CURCUMIN (30μM)
APIGENIN (40μM)
IL-1
- + - + - +
Phospho-JNK
Total-JNK
p54
p46
p54
p46

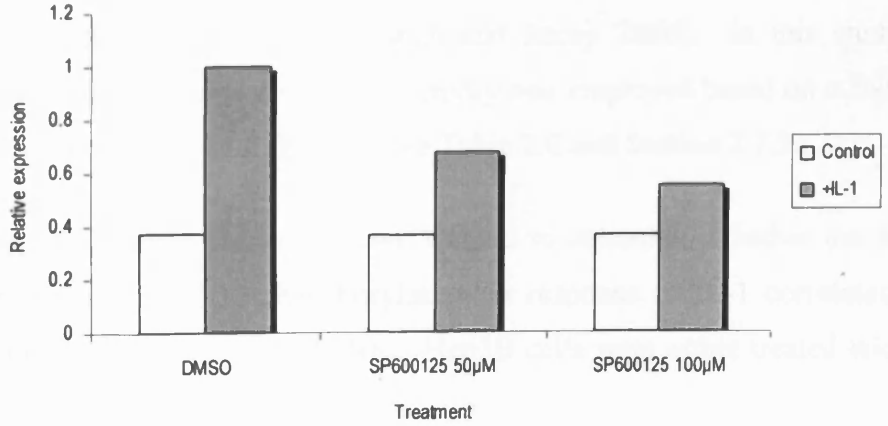
**B.**


1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression
Control
+IL-1
DMSO
CURCUMIN
APIGENIN
Treatment

**C.**


DMSO
SP600125 (50μM)
SP600125 (100μM)
IL-1
- + - + - +
Phospho-JNK
Total-JNK
p54
p46
p54
p46

**D.**


1.2
1
0.8
0.6
0.4
0.2
0
Relative expression
Control
+IL-1
DMSO
SP600125 50μM
SP600125 100μM
Treatment

Because our data consistently shows that the total-JNK levels remain relatively unaffected by the treatment of Hep3B cells with IL-1 (and therefore, the increase in JNK phosphorylation observed in response to IL-1 is not due to an increase in *de novo* synthesis of the JNK proteins), the data presented in Figure 5.5 is displayed as ratios of phosphorylated JNK:total JNK. As expected, IL-1 induced the phosphorylation of JNK (Panel A and C). This effect was marginally prevented by curcumin, although this inhibition in phosphorylation was not statistically significant. Apigenin did not inhibit IL-1-mediated phosphorylation of JNK. Consistent with the aforementioned publications, SP600125 reduced the IL-1-mediated JNK phosphorylation in a concentration-dependent manner. Total JNK levels remained unchanged by these inhibitors, suggesting that the observed effects on phosphorylation were specific to this.

### 5.2.3 EFFECT OF IL-1 ON JNK KINASE ACTIVITY IN HEP3B CELLS

Although detecting Thr183/Tyr185 phosphorylation is a good marker of JNK activation, it is also useful to monitor JNK kinase activity directly from cell extracts. JNK activation can be routinely measured by protein kinase activity towards the transcription factor c-Jun. We decided to monitor JNK activation in this manner in light of the findings presented in Figure 5.5 and additionally to confirm our previous results. Several studies have demonstrated the effect of curcumin as an inhibitor of the JNK pathway by monitoring the activity of this kinase *in vitro*, rather than assessing the effect of this inhibitor on JNK phosphorylation (Parra *et al.* 2000; Comalada *et al.* 2003; Singh and Ramji 2006). In the Singh and Ramji (2006) study, the effect of both curcumin and SP600125 on the TGF-β-mediated phosphorylation of JNK was analysed by western blot analysis. Neither inhibitor affected this phosphorylation but both significantly attenuated the TGF-β-stimulated increase in JNK kinase activity (Singh 2003; Singh and Ramji 2006). In this study, a non-radioactive method of measuring JNK activity was employed based on a JNK activity assay kit from New England Biolabs (see Table 2.0 and Section 2.7.5).

Therefore, as an initial experiment we wanted to determine whether the previously observed increase in JNK phosphorylation in response to IL-1 correlated with an increase in the kinase activity of JNK. Hep3B cells were either treated with IL-1 or

left untreated and harvested at 15min and 30min post treatment for phosphatase-free whole cell protein extraction (Section 2.7.2). Kinase assays were performed as described in section 2.7.5, whereby c-Jun fusion proteins linked to agarose beads were used to 'pull-down' the active JNKs present in the protein extracts. Addition of ATP, results in the phosphorylation of the c-Jun substrate by active JNKs present in the sample. Phosphorylated c-Jun was subsequently detected by western blot analysis. The primary antibody used for phospho-c-Jun detection specifically recognises phospho-serine[63], the residue that is characteristically phosphorylated by activated JNKs (Hibi *et al.* 1993). The detection of β-actin by western blotting was used as a control for equal loading of samples, as described previously. The results are shown in Figure 5.6.

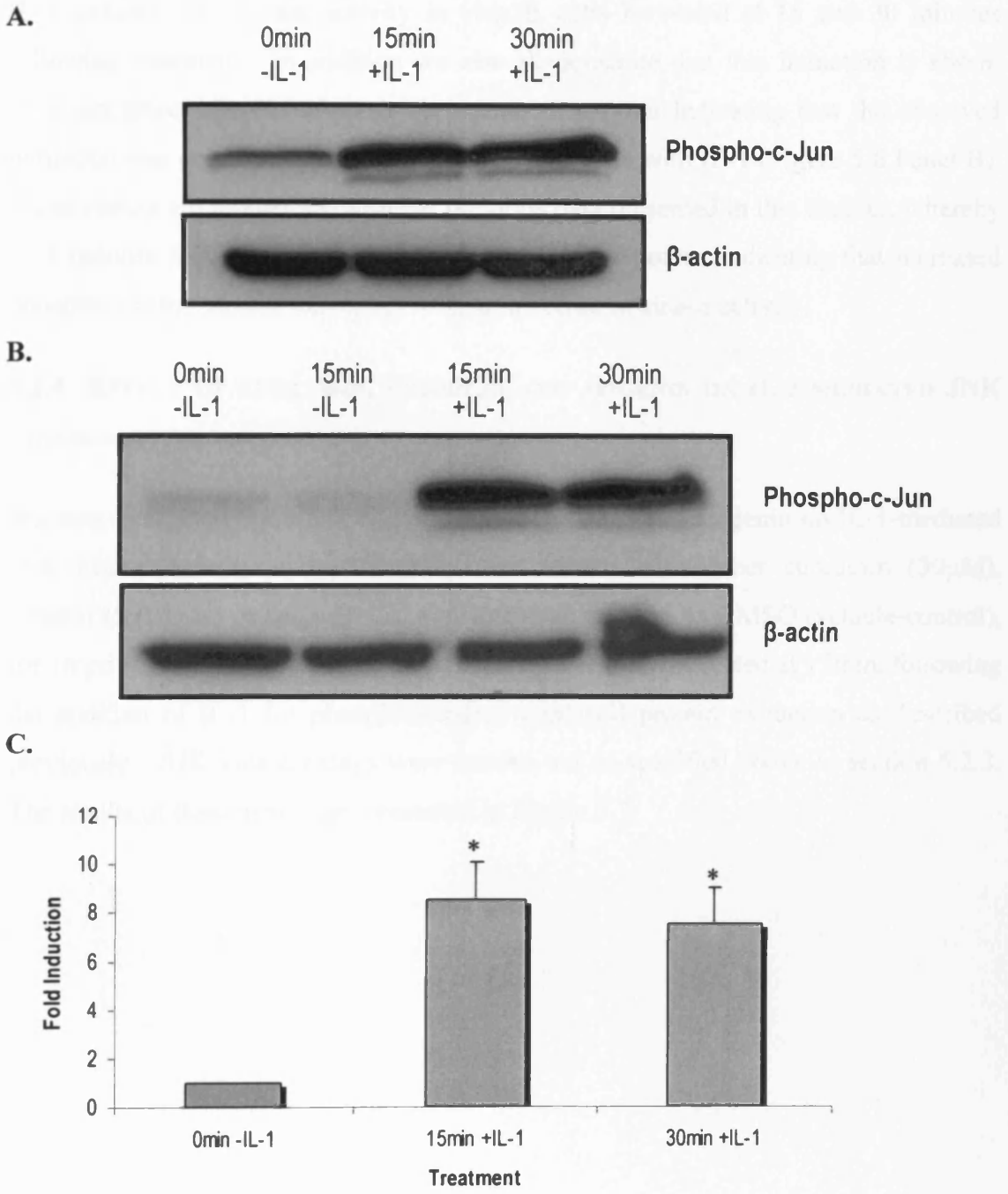



**Figure 5.6. The effect of IL-1 on JNK activity in Hep3B cells.** Hep3B cells were either treated with IL-1 (100U/ml) or left untreated (0min -IL-1, 15min –IL-1) and harvested for phosphatase-free protein extraction at each of the indicated time-points. JNK kinase assays were carried out using 250μg of the protein extracts as described in Section 2.7.5 and blotted membranes were probed with the phospho-c-Jun (serine$^{63}$) antibody as shown in Panel A and B. For β-actin western blots, 10μg of each protein extract was subjected to western analysis as described previously (A and B). The phospho-c-Jun and β-actin protein level for each sample was determined by densitometric analysis. Panel C displays the ratios of Phospho-c-Jun:β-actin normalised to the 0min control. The data shown in Panel C is the mean ±SD from three independent experimental series. *P<0.05.

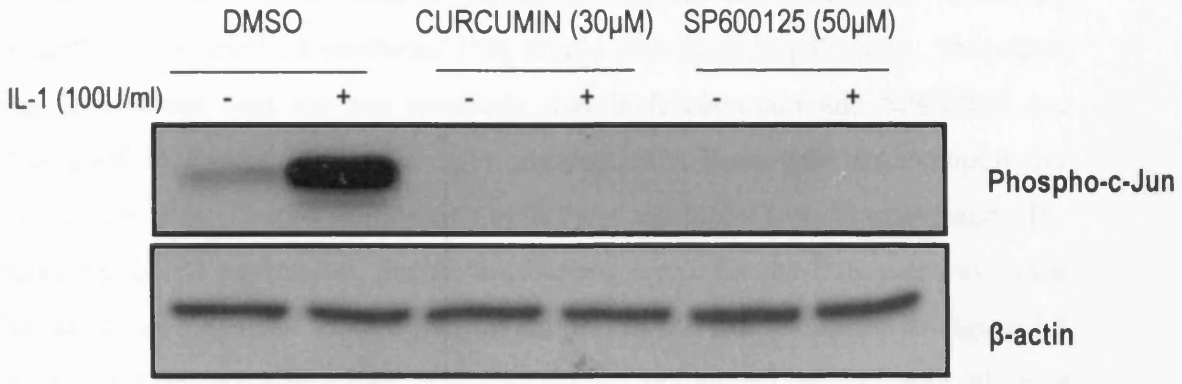
IL-1 induces JNK kinase activity in Hep3B cells harvested at 15 and 30 minutes following treatment. In addition we also demonstrate that this induction is absent from untreated samples taken at the 15min time-point indicating that the observed induction was only due to stimulation of Hep3B cells with IL-1 (Figure 5.6 Panel B). These results are in keeping with the previous data presented in this chapter, whereby IL-1 induces JNK phosphorylation at the same time-points, indicating that increased phosphorylation of JNK correlates with an increase in kinase activity.

### 5.2.4 EFFECT OF CURCUMIN, SP600125 AND APIGENIN ON IL-1-MEDIATED JNK KINASE ACTIVITY

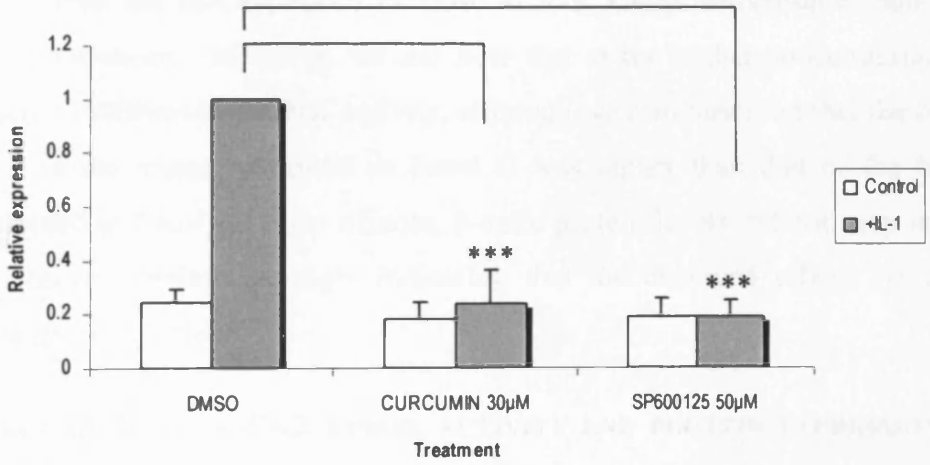
We next examined the effect of curcumin, SP600125 and apigenin on IL-1-mediated JNK kinase activity. Hep3B cells were treated with either curcumin (30μM), SP600125 (50μM) or apigenin (20 and 40μM) in addition to DMSO (vehicle-control), for 1h prior to the addition of IL-1. The cells were then harvested at 15min, following the addition of IL-1 for phosphatase-free total cell protein extraction as described previously. JNK kinase assays were carried out as specified above in section 5.2.3. The results of these assays are presented in Figure 5.7.

**Figure 5.7. The effect of inhibitors on IL-1-mediated JNK kinase activity in Hep3B cells.** Hep3B cells were treated with each of the specified inhibitors in addition to DMSO (vehicle-control) as indicated, for 1h prior to the addition of IL-1. Phosphatase-free protein extracts were prepared from cells harvested at 15min following the addition of IL-1. The JNK kinase assays and β-actin westerns were carried out as specified in Figure 5.6 and are shown in Panels A and C. Panels B and D show the ratios of Phospho-c-Jun:β-actin for each sample, normalised to the IL-1 treated DMSO control that has been assigned as 1. The data shown in Panel B is the mean ±SD from three independent experimental series, ***P<0.001. The data shown in Panel D is the mean of two independent experiments.

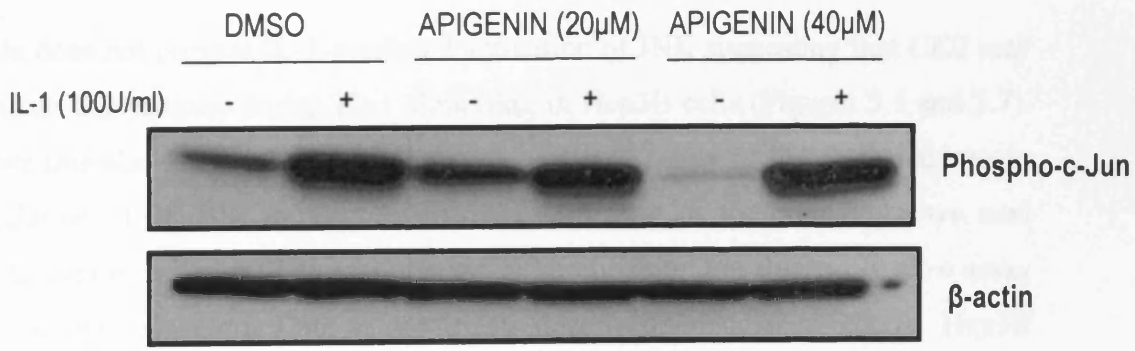
**A.**


DMSO
CURCUMIN (30μM)
SP600125 (50μM)
IL-1 (100U/ml)
- + - + - +
Phospho-c-Jun
β-actin

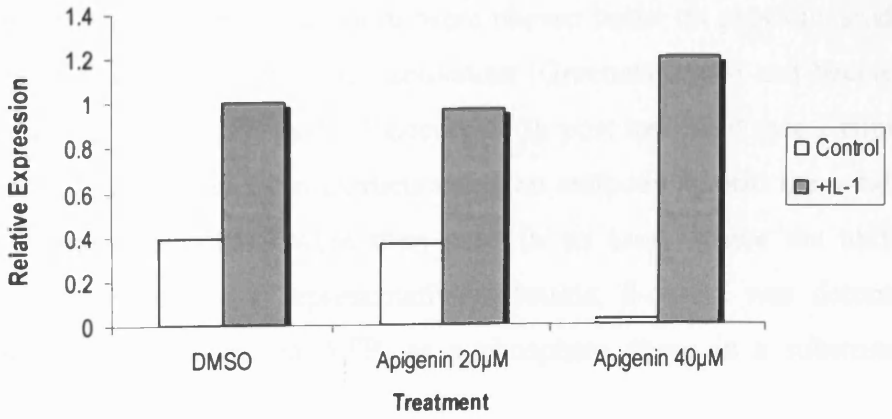
**B.**


Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Control
+IL-1
DMSO
CURCUMIN 30μM
SP600125 50μM
Treatment

**C.**


DMSO
APIGENIN (20μM)
APIGENIN (40μM)
IL-1 (100U/ml)
- + - + - +
Phospho-c-Jun
β-actin

**D.**


Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
+IL-1
DMSO
Apigenin 20μM
Apigenin 40μM
Treatment

The data presented above, clearly shows that both curcumin and SP600125 significantly inhibit the IL-1-mediated JNK kinase activity in Hep3B cells. Therefore, judging from these data we can conclude that both curcumin and SP600125 are effective JNK inhibitors in Hep3B cells. Importantly, these data also support our previous results from chapter 4 in which both these inhibitors significantly impair IL-1-mediated C/EBPδ expression, further implicating a role for the JNK pathway in the regulation of this response. In addition, in support of the data presented in Figure 5.5 where apigenin did not affect JNK phosphorylation as induced by IL-1, this inhibitor also did not impair the IL-1-mediated increase in JNK kinase activation at both the specified concentrations. However, we did note that at the higher concentration of 40μM, apigenin inhibited basal JNK activity, although we also observed that the basal JNK activity in the image presented in Panel C was higher than that of the basal activity presented in Panel A. Also of note, β-actin protein levels did not vary in the presence of these inhibitors strongly indicating that the observed effects on JNK activity were specific to this.

### 5.2.5 EFFECT OF IL-1 ON CK2 KINASE ACTIVITY AND PROTEIN EXPRESSION IN HEP3B CELLS

Apigenin does not prevent IL-1-mediated activation of JNK suggesting that CK2 may not regulate this enzyme during IL-1 signalling in Hep3B cells (Figures 5.5 and 5.7). Therefore this also suggests that CK2 may act independently of the JNK pathway in the regulation of C/EBPδ expression by IL-1. In light of these findings, we next wanted to explore whether IL-1 stimulated CK2 activation. For this, an *in vitro* assay for CK2 activity was carried out as previously described (Mead *et al.* 2003). Hep3B cells were either treated with IL-1 or left untreated for various time-points over 3h and harvested for phosphatase-free whole cell protein extraction. For initial experiments, the 30min, 60min and 180min time-points were chosen based on previous studies in our laboratory analysing inducible CK2 activation (Greenow 2004) and because the maximum induction of C/EBPδ by IL-1 occurs at 3h post treatment (see Chapter 3). CK2 was immunoprecipitated from extracts using an antibody against the α catalytic subunit and immunoprecipitates were then used in an assay where the ability of CK2/CK2α to phosphorylate a representative substrate, β-casein was determined. This assay utilised $\gamma$-$^{32}$-P-labelled ATP, as a phosphate donor in a substrate mix

containing β-casein. Following the incubation of samples with the substrate mix, the content of each was size-fractionated by SDS-PAGE. Phosphorylated-β-casein was then detected by autoradiography (See section 2.7.6 for details). As an additional measure, the expression of the CK2α subunit was also determined in extracts by immunoblotting, to assess for any action of IL-1 on *de novo* synthesis of the protein. The results are shown in Figure 5.8.

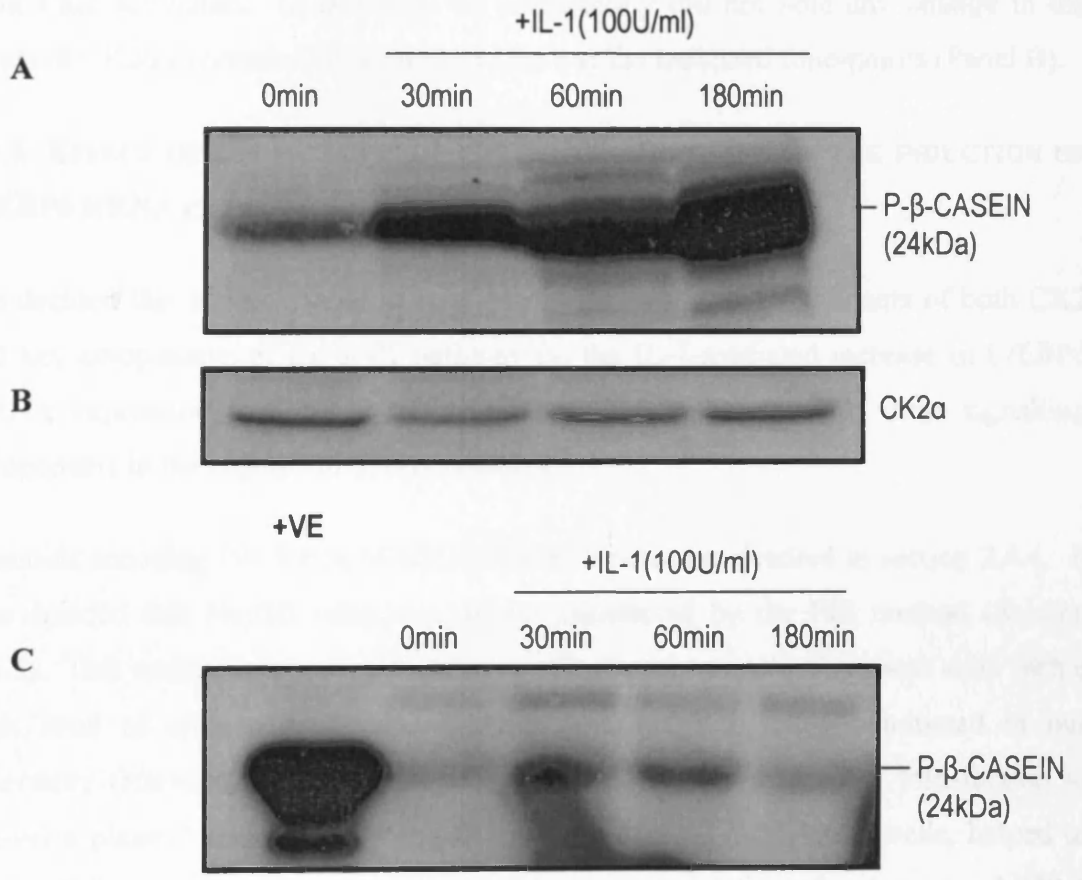



**Figure 5.8. Effect of IL-1 on CK2 activity and protein expression in Hep3B cells.** Hep3B cells were either treated with IL-1 or left untreated at each of the indicated time-points and harvested for protein extraction. The CK2 kinase assays (A and C) were carried out using 150μg of phosphatase-free whole cell extracts as detailed in section 2.7.6. Also shown is a positive control for the kinase assay in Panel C using purified CK2 enzyme (1U; Promega) which was incubated separately with the β-casein substrate mix during the kinase assay. Protein extracts were also used to detect CK2α by immunoblotting (B). Western blot analysis was carried out by SDS-PAGE using 10% (w/v) gels as described previously with 35μg of each sample and blots were probed using an anti-CK2α antibody. Experiments were carried out at least three times.

Results from initial experiments were encouraging (Figure 5.8A), indicating that IL-1 may indeed induce CK2 activation, however in all subsequent experiments IL-1 produced a variable and inconsistent effect on CK2 activation (compare Figure 5.8A and C, as an example). Nevertheless, as can be seen from Panel C in Figure 5.8, the positive control sample, containing purified CK2, produced a dramatic phosphorylation of β-casein, indicating that the assay itself was reliable. Therefore, based on these data alone we could not make any firm conclusions on the effect of IL-1 on CK2 activation. In addition, we consistently did not note any change in the levels of CK2α expression in response to IL-1 at the indicated time-points (Panel B).

### 5.2.6 EFFECT OF DN MUTANTS OF CK2, JNK AND C-JUN ON THE INDUCTION OF C/EBPδ MRNA EXPRESSION BY IL-1

We decided that it was crucial to next assess the action of DN mutants of both CK2 and key components of the JNK pathway on the IL-1-mediated increase in C/EBPδ mRNA expression in order to directly assess the involvement of these signalling components in the regulation of this response.

Plasmids encoding DN forms of CK2, JNK and c-Jun are detailed in section 2.4.4. It was decided that Hep3B cells were to be transfected by the PEI method (Section 2.4.1). This method has proven to be very effective for transfecting these cells with a high level of efficiency as demonstrated in a previous study conducted in our laboratory (Harvey 2006). In this study, the use of this method of transfection to deliver a plasmid specifying for the DN mutant of CK2 into Hep3B cells, helped to successfully identify a potential role for CK2 in the regulation of endogenous MCP-1 expression by IFN-γ in these cells (Harvey 2006; Harvey *et al.* 2007). It is also worthy of note that all these plasmids have been used in previous studies to identify potential roles of CK2 and JNK in a variety of responses (see section 2.4.4, Singh and Ramji 2006; Greenow 2004; Harvey *et al.* 2007).

Hep3B cells were transfected with each of the plasmids specifying for DN forms of CK2, JNK, c-Jun and with the empty plasmid expression vector pcDNA3 (Section 2.4.1). The cells were then either stimulated with IL-1 for 3h or left untreated, at which point samples were prepared for RT-PCR analysis as previously described. RT-PCR was carried out using primers specific for C/EBPδ and β-actin. PCR

products were resolved by agarose gel electrophoresis and the results are presented in Figure 5.9.

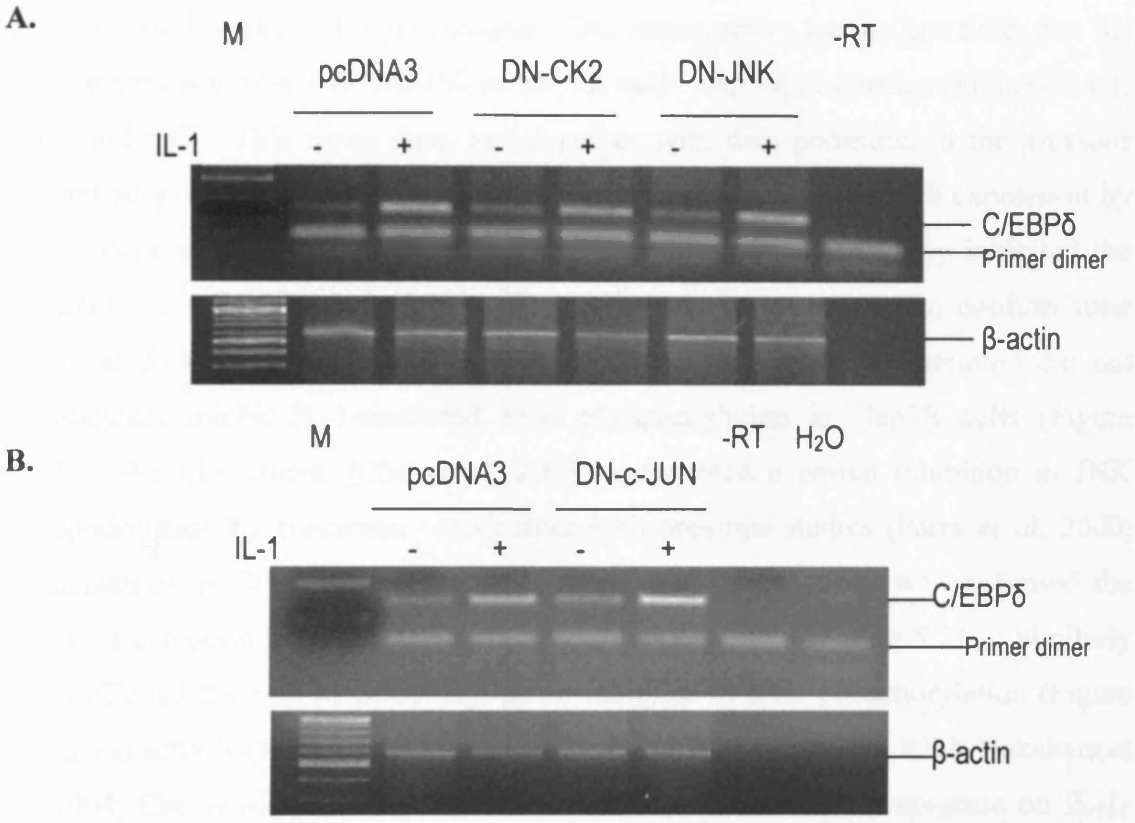



**FIGURE 5.9. Effect of DN inhibitors of CK2, JNK and c-Jun on the induction of C/EBPδ mRNA expression by IL-1.** Hep3B cells were transfected with plasmids specifying for DN mutant forms of CK2, JNK and c-Jun. As a control, cells were also transfected with pcDNA3. All plasmids were transfected using 10μg of plasmid DNA by the PEI method (Section 2.4.1). Hep3B cells were then either treated with IL-1 (100U/ml) or left untreated for 3h and harvested for RT-PCR analysis. Primers against C/EBPδ and β-actin were used to PCR-amplify the respective products as described previously. PCR products were then size-fractionated by electrophoresis as before. The size of each PCR-amplified product was determined by comparing it to standard DNA markers, denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using pcDNA3 transfected –IL-1 RNA). $H_2O$ denotes a PCR in which cDNA was replaced with an equal volume of water. Results in Panel A are representative of two independent experiments.

As expected, IL-1 produced an increase in the levels of C/EBPδ mRNA. However, none of the plasmids specifying for DN forms of CK2, JNK or c-Jun inhibited the induction of C/EBPδ expression by IL-1 compared to the pcDNA3 transfected controls. The results of these experiments, together with the other findings presented in this chapter are discussed in detail below.

## 5.3 DISCUSSION

The potentially novel roles of JNK and CK2 in the regulation of C/EBPδ expression by IL-1 were investigated in this chapter. Our results show, for the first time, that IL-1 transiently activates JNK MAPK in Hep3B cells with rapid kinetics (Figures 5.4.1, 5.4.2 and 5.6). This novel data, and together with data presented in the previous chapter supported a role for this pathway in the regulation of C/EBPδ expression by IL-1. Because JNK inhibitors, SP600125 and curcumin significantly inhibited the induction of C/EBPδ expression by IL-1 (Chapter 4), we wanted to confirm their action as JNK inhibitors in our cellular system. We found that curcumin did not significantly inhibit IL-1-mediated JNK phosphorylation in Hep3B cells (Figure 5.5A). We like others, (Cho *et al.* 2005) only noted a partial inhibition in JNK phosphorylation by curcumin. Consistent with previous studies (Parra *et al.* 2000; Comalada *et al.* 2003; Greenow 2004; Singh and Ramji 2006) we confirmed the action of curcumin as an effective inhibitor of JNK activity (Figure 5.7A). Similarly we confirmed the role of SP600125 as an inhibitor of JNK phosphorylation (Figure 5.5C) and activity (Figure 5.7A) (Bennett *et al.* 2001; Tokuda *et al.* 2003; Nakahara *et al.* 2004; Cho *et al.* 2005; Tamagno *et al.* 2005). The effect of apigenin on IL-1-mediated JNK phosphorylation was also examined in an effort to elucidate a possible role for CK2 in the regulation of the JNK MAPK during IL-1 signalling, given that JNK is a known substrate of CK2 (Fleming *et al.* 2000; Meggio and Pinna 2003). Apigenin did not attenuate IL-1-mediated JNK phosphorylation or activity (Figures 5.5A and 5.7C respectively). Notably however, apigenin (40μM) reduced basal JNK activity (Figure 5.7C). The precise reasons for this observation are presently unclear but since CK2 is a constitutively active enzyme (Pinna 2002) it may contribute slightly to the constitutive activity of JNK either directly or indirectly through kinases that phosphorylate JNK or other factors that modulate JNK activity. This however is distinct from IL-1-mediated regulation of JNK as this is not affected by apigenin at this concentration.

Because apigenin did not attenuate IL-1-mediated JNK activation we hypothesised that CK2 may act independently of the JNK pathway in relation to its regulation of C/EBPδ expression by IL-1. Therefore we next investigated the action of IL-1 on CK2 activation. IL-1 produced a variable and inconsistent effect on CK2 activation in

Hep3B cells (Figure 5.8). Therefore based on these data it is tempting to speculate that IL-1 does not regulate CK2 activation in these cells. However, no definitive conclusions can be made based on these data alone. Further experiments would have to be conducted to firmly establish whether IL-1 does modulate CK2 activity in these cells. Our *in vitro* kinase assay was limited to the isolation of CK2 from cell extracts by immmunoprecipitation using an anti-CK2α antibody only. Considering that this is not the only catalytic subunit identified for this enzyme (Litchfield 2003), it would be interesting to determine whether immunoprecipitation with anti-CK2α' and anti-CK2α'' antibodies produced similar results in the kinase assay in relation to IL-1 action on CK2. This would be particularly intriguing since differential functions for the catalytic subunits have been described and because individual catalytic subunits have been identified both in isolation and as part of the holoenzyme in cells (see Litchfield 2003 and references therein). Moreover, the CK2α'' has been found to be differentially expressed in the liver and not other tissues, indicating a cell-type specific role for this isoform in hepatocytes (Shi *et al.* 2001; Hilgard *et al.* 2004). In order to decipher whether IL-1 differentially regulates the activity of individual CK2 catalytic subunits, our *in vitro* kinase assay could be modified slightly. There are CK2 substrates, a prototype being calmodulin, that are preferentially phosphorylated by individual CK2 catalytic subunits but not by tetrameric CK2 (Marin *et al.* 1999; Arrigoni *et al.* 2004). Therefore substituting calmodulin for β-casein in the kinase assay may determine whether IL-1 preferentially influences the action of individual catalytic subunits over the tetrameric enzyme.

Another potential explanation for our data is that IL-1 may not regulate activation of CK2 directly but instead influence its association with other signalling proteins. This theory is supported by the findings of Bird *et al.* (1997). These authors investigated the activation of NF-κB by IL-1 in the HepG2 cell line (a closely related cell line to Hep3B), and report that this activation is accompanied by CK2-mediated phosphorylation of the p65 NF-κB subunit. Similar to our findings, these authors report little apparent stimulation of CK2 activity by IL-1 in treated cells, although an increased association of CK2 with NF-κB was noted in response to cytokine treatment. Given that a potential role for NF-κB has been suggested in the regulation of C/EBPδ expression by IL-1 by our previous data (Chapter 4) and because our pharmacological inhibitor data also suggests an involvement for CK2, the results

presented by Bird *et al.* (1997) may bare particular significance in relation to this project and provide a basis for future work (See chapter 8).

In order to further determine a role of the JNK pathway and CK2 in IL-1-mediated C/EBPδ expression, DN inhibitors of components of the JNK pathway and CK2 were used. The induction of C/EBPδ expression by IL-1 was not impaired by transfecting Hep3B cells with plasmids specifying for DN-CK2, DN-JNK or DN-c-Jun (Figure 5.9). These results could suggest that some functional redundancy may exist between the pathways that regulate C/EBPδ expression by IL-1 in Hep3B cells. Similar results have been reported by Mestre *et al.* (2001). The transcriptional up-regulation of cyclooxygenase-2 (COX-2) gene expression by LPS was examined in RAW 264.7 and THP-1 macrophages. Whilst, ERK2, p38, JNK MAPKs and PKC-ζ were all shown to regulate this response, transfection of DN mutant forms of any one of these signalling components failed to abrogate the LPS-mediated increase in human COX-2 promoter activity. In addition, three functional promoter elements consisting of an NF-κB, C/EBP and CRE sites were all shown to regulate the LPS-mediated induction of COX-2 promoter activity. However, mutation of any one of these *cis*-acting elements at the promoter was not enough to abrogate the response. The response was only impaired when all three sites were mutated together or two of the three sites were mutated simultaneously. Furthermore, expression plasmids encoding wild-type proteins for each of the MAPKs and PKC-ζ had a synergistic effect with LPS to induce luciferase activity driven by COX-2 promoter-reporter constructs containing a single wild-type sequence of NF-κB, C/EBP or CRE. However, expression plasmids encoding DN mutant plasmids for MAPKs and PKC-ζ did not diminish LPS-mediated COX-2 promoter activation from the same promoter-reporter constructs. Therefore, this suggests that COX-2 gene transcription through each promoter element (i.e. NF-κB-, C/EBP-, or CRE-driven transcription) is supported by at least two different signalling pathways (Mestre *et al.* 2001). Given this apparent redundancy, transcriptional repression of COX-2 could not be achieved by targeting a single pathway or transcription factor. Functional redundancy has also been described during Notch signalling (Krebs *et al.* 2000), between the Src family of tyrosine kinases (Lowell *et al.* 1994), between transcription factors such as the STATs (Ramana *et al.* 2005), AP-1 family members (Mechta-Grigoriou *et al.* 2001) and Nur77 steroid nuclear receptors (Cheng *et al.* 1997).

Another plausible explanation for our results in Figure 5.9 is the possibility that the DN plasmids were delivered into the cells with low efficiency. However, we consider this unlikely because our chosen method of transfection has previously been demonstrated to transfect Hep3B cells with a high level of efficiency. For example, transfecting the plasmid specifying for the DN-CK2 mutant protein into Hep3B cells using the same method, is sufficient to attenuate the IFN-γ-induced MCP-1 expression in these cells (Harvey 2006). Alternatively, it may be such that neither CK2, JNK or c-Jun are necessary to regulate IL-1-mediated regulation of C/EBPδ in isolation. Therefore, to address these issues and to investigate the potential existence of redundancy between the pathways, we decided to extend our investigations with the use of siRNA technology, this being the focus of the next chapter.

To summarise, the results in this chapter have firmly established that IL-1 activates JNK MAPK and that both curcumin and SP600125 are effective inhibitors of this kinase in Hep3B cells. Furthermore, our results also suggest CK2 may not be an upstream regulator of this pathway during IL-1 signalling. In addition, we suggest that the role of CK2 in IL-1 signalling may not be regulated at the level of activity, although further experiments are required in this area of investigation. Finally, our data also suggests the possibility that some functional redundancy may exist between the pathways that regulate C/EBPδ expression by IL-1 and this possibility will be explored in the following chapter.

# CHAPTER SIX: THE USE OF SIRNA TO INVESTIGATE SIGNALLING MEDIATORS REGULATING IL-1- AND IL-6-MEDIATED INDUCTION OF C/EBPδ EXPRESSION

## 6.1 INTRODUCTION

The introduction of double-stranded RNA (dsRNA) into a cell activates the RNA interference (RNAi) pathway which triggers the degradation of mRNAs that show sequence complementarity to the exogenous dsRNA. This phenomenon whereby gene expression is silenced by homologous sequences was first described in transgenic plants and was termed post-transcriptional gene silencing. Attempts to deepen the violet colour of Petunias, by introducing multiple copies of a transgene encoding chalcone synthase into these plants (an enzyme responsible for the production of the violet pigment), unexpectedly resulted in the production of white flowers (Napoli *et al.* 1990). The introduction of extra copies of the chalcone synthase gene caused an unexplained decrease in the expression of its mRNA, rather than the anticipated increase. Then in 1998, Fire and colleagues made a breakthrough finding. Using *Caenorhabditis elegans* as a model, the group discovered that injecting dsRNA into the nematode worm was more effective at inducing gene silencing, than injecting either sense or antisense RNA alone, against a target gene. Similar gene silencing phenomena have since been described in multiple organisms (see Dykxhoorn *et al.* 2003; Stevenson 2003 and references therein).

In eukaryotic cells, RNAi represents an evolutionarily conserved cellular defence mechanism regulating the expression of 'foreign genes'. When viruses infect eukaryotic cells or when transposons and transgenes are randomly integrated into the host genome, dsRNA is frequently produced from the foreign genes. The RNAi pathway acts as a surveillance system that responds to the presence of dsRNA by activating the cellular processes that inhibit gene expression (post-transcriptionally) to silence the expression of invading genes (Tuschl and Borkhardt 2002).

The discovery that dsRNA produced RNAi, prompted a series of investigations into elucidating the mechanism by which this occurs (see Tuschl and Borkhardt 2002; Dykxhoorn *et al.* 2003; Stevenson 2003 and references therein). The process occurs in the cell cytoplasm (Zeng and Cullen 2002) and is ATP dependent (Nykanen *et al.* 2001). When dsRNA is introduced into a cell, it is processed into ~22 nucleotide segments by an RNAse-III-like dsRNA-specific ribonuclease termed Dicer (Zamore *et al.* 2000; Bernstein *et al.* 2001). These short fragments of cleaved RNA are then

incorporated into a multiprotein RNA-induced silencing complex, RISC (Hammond *et al.* 2000). As a prerequisite for incorporation by RISC, dsRNA must be 5' phosphorylated and short dsRNAs that lack a 5' phosphate are rapidly phosphorylated by an endogenous kinase (Schwarz *et al.* 2002). The dsRNA is then unwound, leaving the antisense strand to 'guide' RISC to the homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved at a single site in the centre of the duplex region between the guide RNA and the target RNA and the resulting RNA fragments are subsequently degraded (Elbashir *et al.* 2001a).

Initial attempts to recreate RNAi in mammalian cells were unsuccessful. This is because in mammalian cells, the introduction of dsRNA greater than 30 nucleotides in length, induces an interferon response (Elbashir *et al.* 2001b). Interferon mediates the degradation of mRNA by inducing 2'-5' oligoadenylate synthase which subsequently activates RNAse L. Furthermore, interferon also activates protein kinase R (PKR), which ultimately leads to global inhibition of mRNA translation (Stark *et al.* 1998). However, further studies showed that the introduction of chemically synthesised dsRNAs between 21-22 nucleotides in length; containing 3' overhangs (and therefore mimicking the structure of the Dicer cleavage products) were effective at causing RNAi in mammalian cells without activating the interferon response. These short strands of dsRNA were collectively termed small interfering RNAs or siRNAs (Elbashir *et al.* 2001b). The known cellular events leading to RNAi with the use of siRNAs are summarised in Figure 6.1.

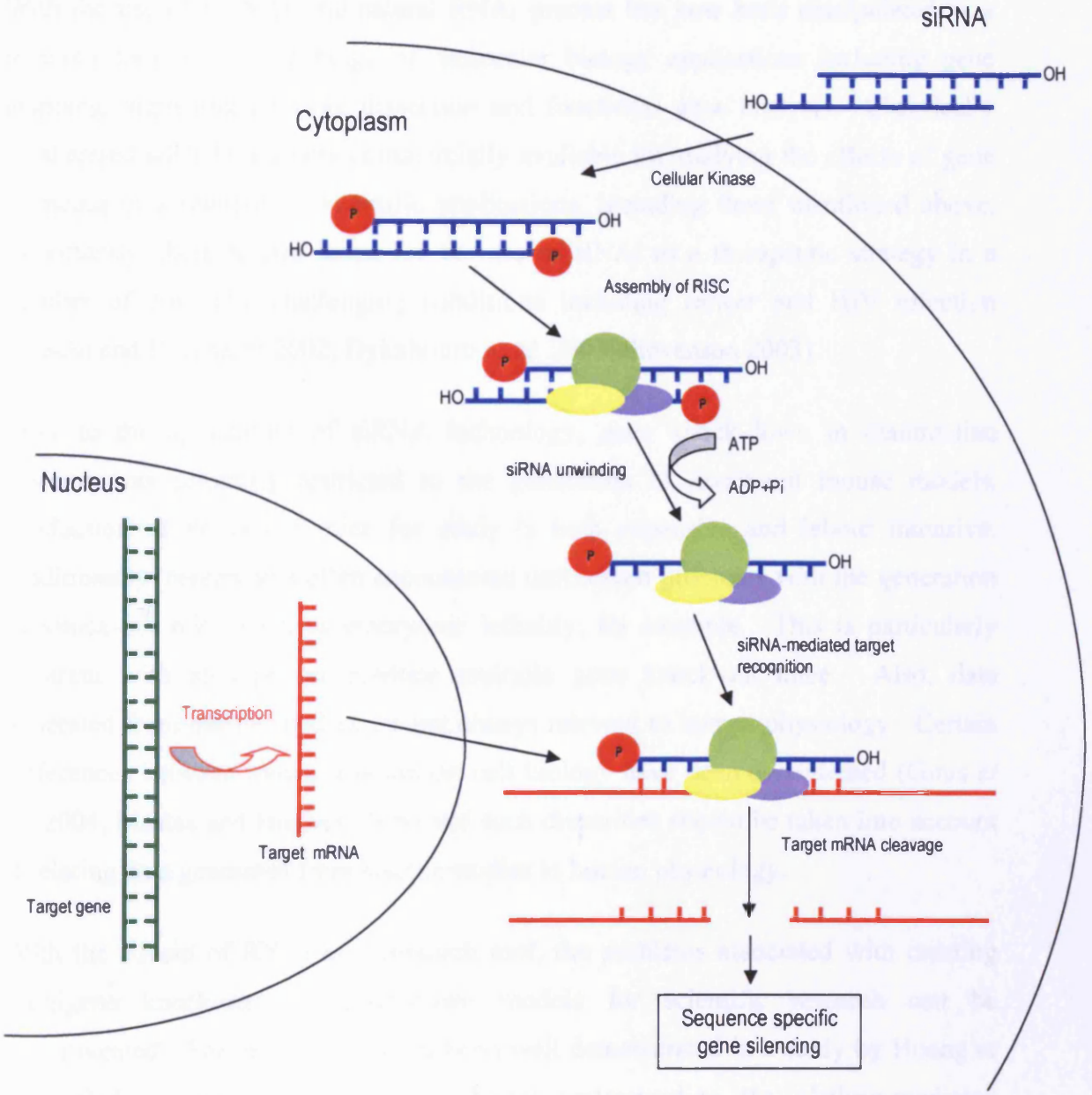



**FIGURE 6.1. Gene silencing by RNA interference using siRNA (adapted from Lewis 2005a).** Artificial siRNAs introduced into the cell are phosphorylated on their 5' ends by a cellular kinase. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC) which contains both helicase and ribonuclease activity. Although the uptake of siRNAs by RISC is an ATP independent process, the unwinding of the siRNA duplex requires ATP. RISC is directed by the antisense strand of the unwound siRNA to the target mRNA through sequence complementarity and the target mRNA is subsequently cleaved and degraded.

With the use of siRNAs, the natural RNAi process has now been manipulated as a research tool in a wide range of molecular biology applications including gene mapping, signalling pathway dissection and functional gene analysis. Chemically synthesised siRNAs are now commercially available for studying the effects of gene silencing in a number of scientific applications, including those mentioned above. Importantly, there is also scope for the use of RNAi as a therapeutic strategy in a number of clinically challenging conditions including cancer and HIV infection (Tuschl and Borkhardt 2002; Dykxhoorn *et al.* 2003; Stevenson 2003).

Prior to the application of siRNA technology, gene knock-down in mammalian systems was primarily restricted to the generation of knock-out mouse models. Production of knock-out mice for study is both expensive and labour intensive. Additionally, researchers often encountered unforeseen problems with the generation of knock-out mice such as embryonic lethality, for example. This is particularly apparent with attempts to produce multiple gene knock-out mice. Also, data generated from murine studies are not always relevant to human physiology. Certain differences between mouse and human cell biology have been documented (Ginis *et al.* 2004; Mestas and Hughes 2004) and such disparities should be taken into account if relating data generated from murine studies to human physiology.

With the advent of RNAi as a research tool, the problems associated with creating multigene knock-out or knock-down models for scientific research can be circumvented. For instance, this has been well demonstrated in a study by Huang *et al.* (2004). This study aimed at better understanding the clathrin-mediated endocytosis of the EGF receptor. To assess the contribution of the Rab5 family of endosomal proteins in this process, the group used siRNAs directed against the various family members. Individually, none of the three Rab5 isoforms (Rab5a, b and c) were found to contribute to this form of receptor internalisation. However, when the expression of all three genes was simultaneously depleted by RNAi, the contribution of this family was clarified. These results represent just one example that illustrates the strength of RNAi-induced gene silencing as a tool in pathway dissection. Furthermore, as also demonstrated by the Huang *et al.* (2004) study, the use of this technology also permits the researcher to overcome barriers associated with genetic redundancy.

### 6.1.1 EXPERIMENTAL STRATEGY

Already the use of siRNA technology has advanced our understanding of signal transduction pathways that regulate cellular growth, proliferation and function (Tuschl and Borkhardt 2002; Brummelkamp and Bernards 2003; Moffat and Sabatini 2006).

Certainly, siRNAs that target the message of JNK MAPK or components of this pathway, have been used successfully in studies to implicate this pathway in the regulation of various responses (Gururajan *et al.* 2005; Hocevar *et al.* 2005; Kuntzen *et al.* 2005; Gao *et al.* 2006). Similarly, siRNAs directed against the catalytic subunits of CK2 (mainly CK2α) have also been used successfully to elucidate a role for this kinase in various cellular activities (Di Maira *et al.* 2005; Yanagawa *et al.* 2005; Di Maira *et al.* 2007).

Therefore, in light of recent advances in the field we decided to make use of this technology and examine the effects of silencing the message of components of the JNK MAPK pathway and CK2 on IL-1-mediated induction of C/EBPδ expression. In theory, silencing the expression of such genes should interrupt these signalling pathways and therefore impair any responses that are primarily regulated by the JNK MAPK and CK2 in transfected cells. Indeed, this has been shown to be the case in the studies listed above. In addition, with the use of RNAi we were also able to assess if potential redundancy between JNK and CK2 exists in mediating the induction of C/EBPδ expression by IL-1. This was particularly important in light of the findings presented in the previous chapter.

Additionally, in conjunction with this study, a similar study was also initiated in our laboratory in relation to IL-6 signalling. Dr S.A. Rogers (personal communication) examined the effect of a panel of pharmacological inhibitors on the IL-6-mediated induction of C/EBPδ expression in an effort to initiate more detailed investigations into the signalling pathway(s) by which IL-6 up-regulates C/EBPδ expression in Hep3B cells. As determined from Dr Rogers' data, the principle pathways identified in regulating this response also involved JNK and CK2. These data were particularly relevant to this study and suggested the possibility that common signalling pathways may regulate pro-inflammatory cytokine-mediated induction of C/EBPδ expression.

As mentioned in chapter 3 and section 1.3.1.3, IL-6 is reported to increase the transcriptional expression of the C/EBPδ gene and the principle transcription factor reported to regulate this response is STAT3. However, the majority of studies that indicate this are primarily restricted to promoter analysis and *in vitro* DNA binding assays (Yamada *et al.* 1997; Cantwell *et al.* 1998; Sanford and DeWille 2005). We also showed that the human C/EBPδ gene promoter is responsive to IL-6 (Chapter 3). However, to our knowledge no study has of yet determined whether the STAT3 transcription factor regulates the expression of endogenous C/EBPδ levels as mediated by IL-6. With the use of RNAi, we also sought to determine a role for this transcription factor in the regulation of endogenous C/EBPδ levels (mRNA and protein) as induced by IL-6.

Because a study by Ramana *et al.* (2005) suggested that some redundancy exists between STAT1 and STAT3 transcription factors and because redundancy has been suggested to exist between other components of the gp130/JAK/STAT signalling pathway (Carbia-Nagashima and Arzt 2004), we designed our experiments to take this into consideration. Using siRNA directed against STAT1 and STAT3, we co-transfected Hep3B cells with both siRNAs and subsequently assessed the relative contribution of these transcription factors in producing the IL-6-mediated increase in C/EBPδ mRNA and protein levels. In conjunction, because Dr Rogers' data suggested a requirement for JNK MAPK and CK2 in the regulation of this response, we also assessed the role of these signalling mediators in regulating STAT1 and STAT3 activation by IL-6 with the use of pharmacological agents, in an attempt to determine a possible mechanism of regulation. Work in these areas could lead to the generation of potentially novel data in relation to IL-6-mediated regulation of C/EBPδ expression. Therefore, studies presented in this chapter are split into two lines of investigation. In the first part of this chapter we present our RNAi data in relation to determining a role for JNK MAPK and CK2 in the regulation of the IL-1-mediated induction of C/EBPδ expression. And in the second part of this chapter we present data relevant to the IL-6-mediated regulation of C/EBPδ expression. Figure 6.2 summarises our overall experimental approach that was designed to achieve the outlined experimental objectives.

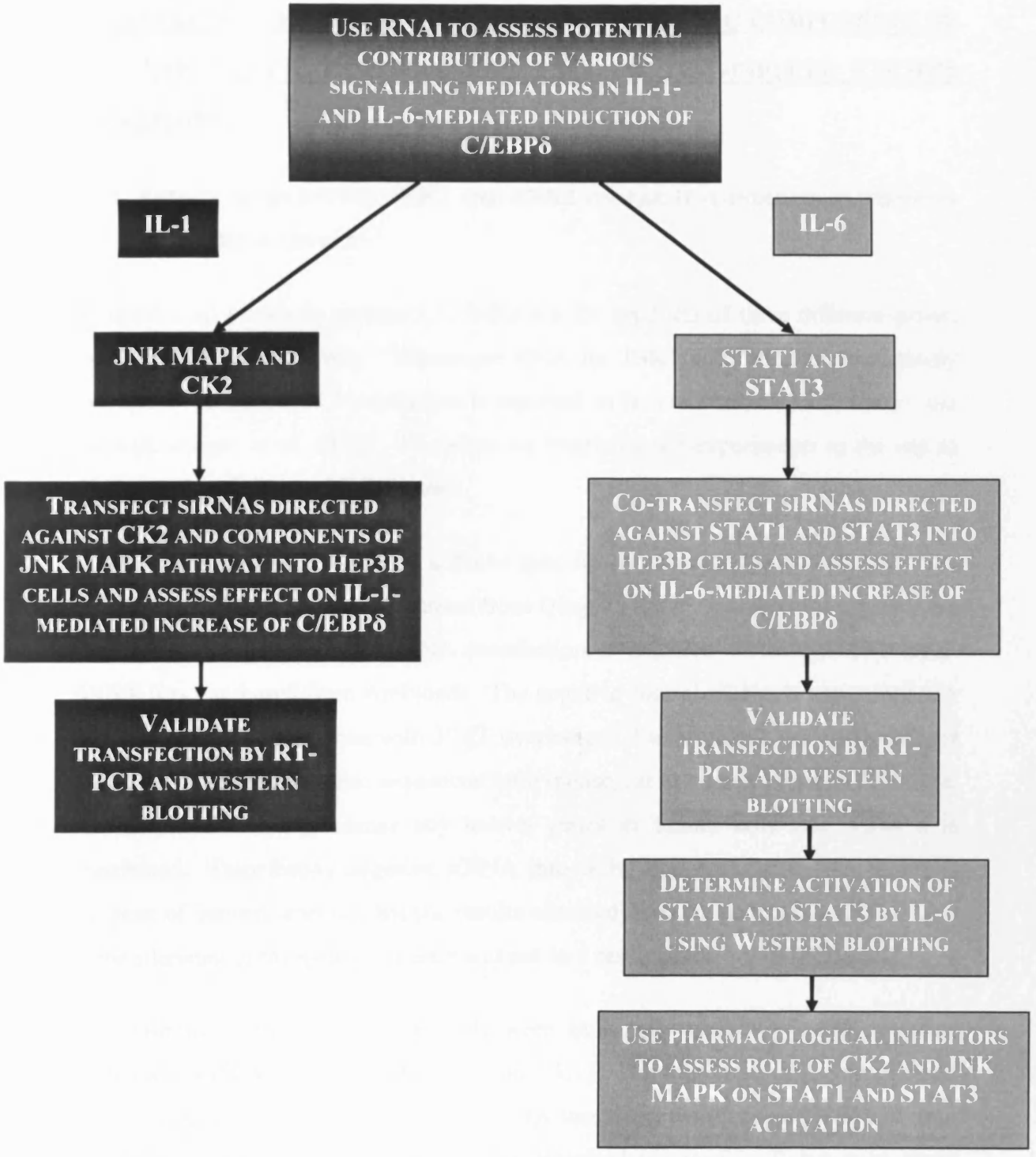



**FIGURE 6.2 Experimental strategy.** Potential roles of various signalling mediators in the regulation of IL-1- and IL-6-mediated increase of C/EBPδ expression were assessed by RNAi. siRNA directed against CK2 and components of the JNK MAPK was used to determine the requirement of these signalling pathways in the regulation of C/EBPδ expression by IL-1 and to assess any functional redundancy between the pathways in the regulation of the response. A potential requirement of the STAT transcription factors in the regulation of C/EBPδ expression by IL-6 was also assessed using RNAi. In association with this, activation of STAT1 and STAT3 by IL-6 was examined by western blot analysis. Because previous data from our laboratory suggested a role of JNK MAPK and CK2 in the regulation of C/EBPδ expression by IL-6, the effects of pharmacological agents against these signalling mediators on STAT activation were also monitored by western blot analysis.

## 6.2 INVESTIGATIONS INTO THE EFFECT OF SILENCING COMPONENTS OF THE JNK MAPK PATHWAY AND CK2 ON IL-1-INDUCED C/EBPδ EXPRESSION

### 6.2.1 EFFECT OF SILENCING JNK1 AND JNK2 ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ MRNA LEVELS

As mentioned briefly in section 5.1, JNKs are the products of three different genes, JNK1, 2 and 3 respectively. Messenger RNA for JNK1 and JNK2 is ubiquitously expressed, whereas JNK3 expression is reported to be predominant in the brain and testis (Kumagae *et al.* 1999). Therefore we restricted our experiments to the use of siRNA against JNK1 and JNK2 genes.

Commercially available validated siRNAs specifically targeting the mRNA of human JNK1 and JNK2 genes were purchased from Qiagen (Table 2.1). Additionally, for the purpose of controlling for the siRNA transfection, a 'negative' or 'scrambled-control' siRNA was purchased from Ambion®. The negative control siRNA is comprised of a 19bp non-targeting sequence with 3' dT overhangs. The sequence has no significant similarity to any known gene sequences from mouse, rat or human origins. Therefore, this siRNA should not silence any known genes in human cells into which it is transfected. Transfecting negative siRNA into cells, alongside the siRNA targeting the gene of interest, ensures that the results obtained from experiments are solely due to the silencing of the gene of interest and not as a result of the transfection itself.

For preliminary analysis, Hep3B cells were transiently transfected with negative siRNA and siRNA targeting JNK2 (Section 2.5.1). The cells were then either treated with IL-1 or left untreated for 3h. Total RNA was extracted (Section 2.6.2) and used for semi-quantitative RT-PCR analysis as described previously. Polymerase chain reactions for the amplification of C/EBPδ, JNK2 and β-actin were carried out with primers and conditions detailed in Tables 2.4 and 2.5. Figure 6.3 shows the PCR products for C/EBPδ, JNK2 and β-actin respectively, as size fractionated by agarose gel electrophoresis.

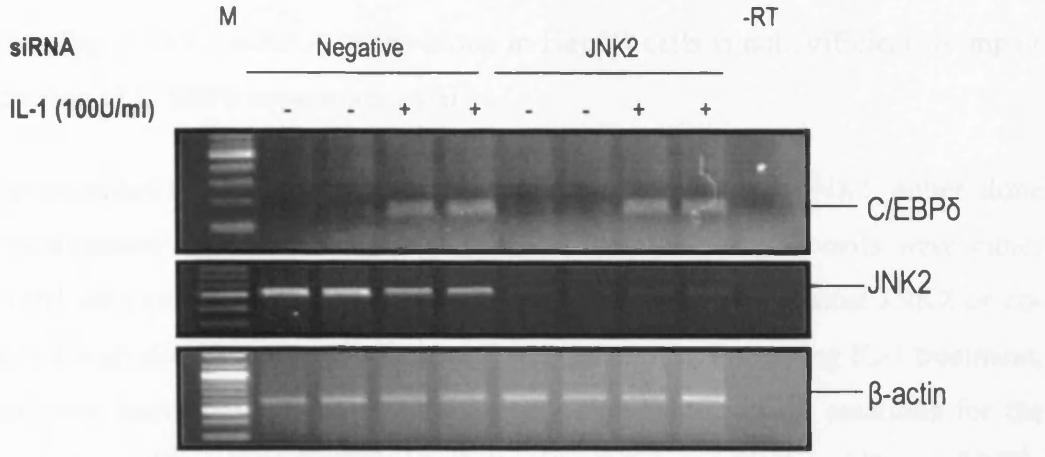


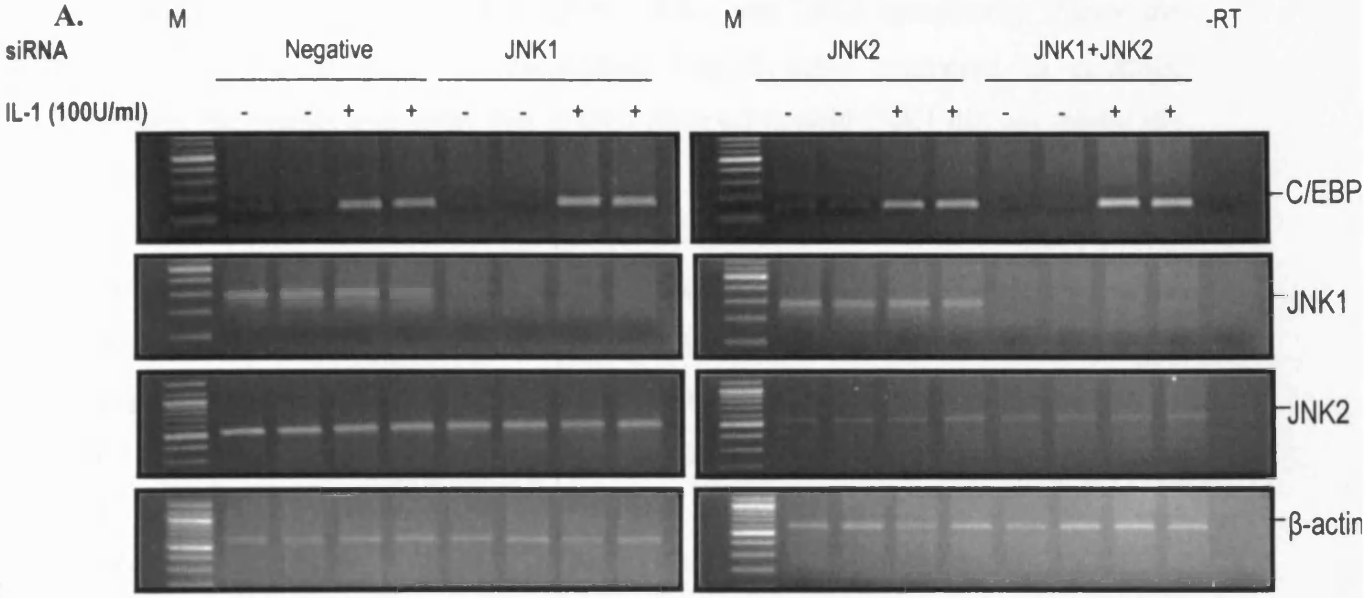

**FIGURE 6.3. Effect of silencing JNK2 on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with negative siRNA (6.25nM) or siRNA designed against JNK2 (6.25nM) using DharmaFECT reagent as described in Section 2.5.1, with each transfection carried out in duplicate. Post transfection, the media was changed on the cells to DMEM complete-medium minus antibiotics and the cells were either treated with IL-1 (100U/ml) or left untreated for 3h. Total RNA was extracted from cells and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, JNK2 (amplified using primer set 2 in Table 2.4) and β-actin were resolved on 1.5% (w/v) agarose gels by electrophoresis. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using JNK2 siRNA transfected, -IL-1 RNA).

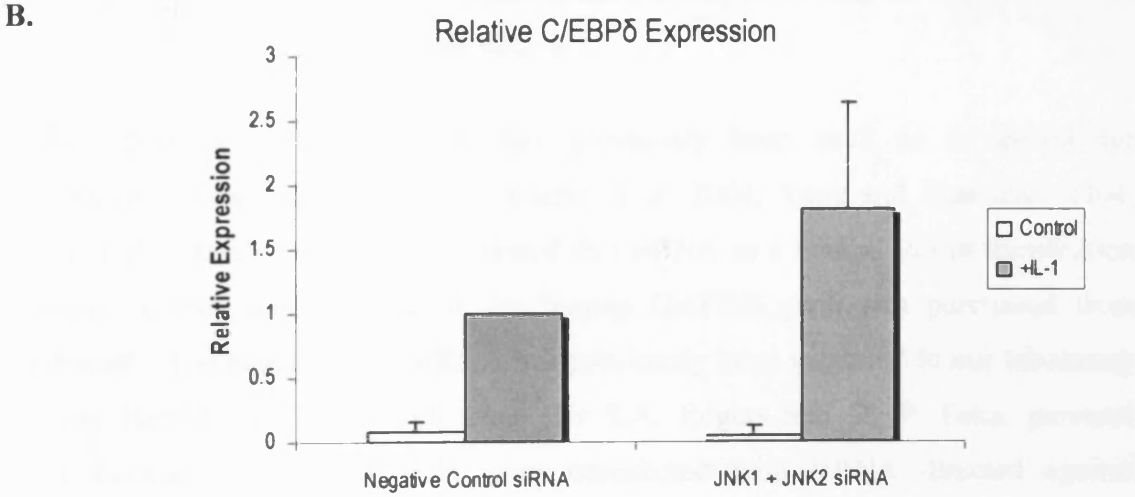
As shown in Figure 6.3, siRNA directed against JNK2 specifically silenced this gene, as only a very small amount of JNK2 PCR product was detected in samples transfected with the corresponding siRNA, in contrast to cells transfected with negative siRNA. These results suggest that levels of JNK2 mRNA in cells transfected with the corresponding siRNA are dramatically depleted compared to control cells. In addition, the constant expression of β-actin in all the samples suggests that neither siRNA was having a global effect on gene expression in cells, further indicating that the results are indeed specific. Additionally, as previously observed, IL-1 induces the expression of C/EBPδ in Hep3B cells, and this is also the case for those cells transfected with negative siRNA, suggesting that the presence of this siRNA did not affect this previously observed induction. Furthermore, the induction of C/EBPδ expression by IL-1 in cells transfected with JNK2 siRNA is comparable with the induction observed in cells transfected with negative siRNA. These results indicate

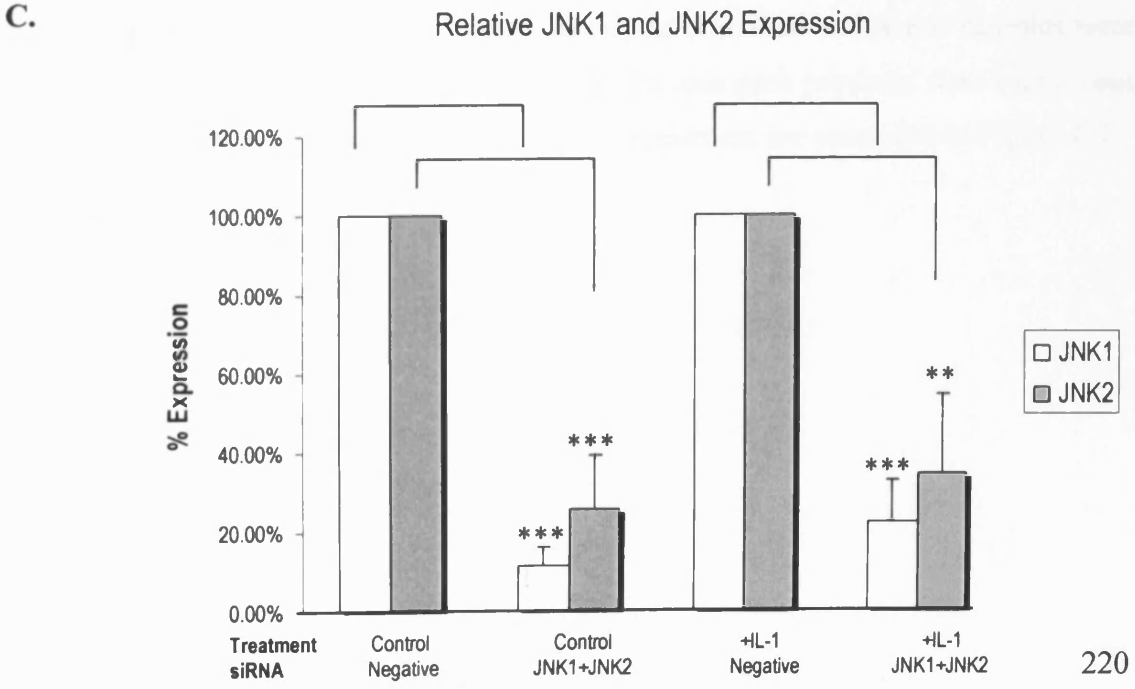
that depletion of JNK2 mRNA levels alone in Hep3B cells is not sufficient to impair the induction of C/EBPδ expression by IL-1.

We next extended the above experiment to include siRNA against JNK1, either alone or in combination with siRNA against JNK2. Therefore, Hep3B cells were either transfected with negative siRNA, siRNA against JNK1, siRNA against JNK2 or co-transfected with siRNA against JNK1 and JNK2, as above. Following IL-1 treatment, samples were harvested for RT-PCR analysis. Polymerase chain reactions for the various gene products were carried out as described above, with the addition of PCR-amplification of JNK1 (see Tables 2.4 and 2.5 for primers and conditions). The results are presented in Figure 6.4.

**FIGURE 6.4. Effect of silencing JNK1 and JNK2 on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with negative siRNA (12.5nM) or siRNA designed against JNK1 (12.5nM), JNK2 (12.5nM) or co-transfected with siRNA against both JNK1 and JNK2 (6.25nM of each siRNA) as described before, with each transfection carried out in duplicate. Post transfection, the cells were either treated with IL-1 (100U/ml) or left untreated for 3h as described previously. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, JNK1, JNK2 (amplified using primer set 2 in Table 2.4) and β-actin were resolved by electrophoresis as before. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using negative siRNA transfected, -IL-1 RNA), Panel A. Although images for each PCR product in Panel A are separated by a gap, it should be noted that PCR products for each gene were resolved on the same agarose gel. Panel B displays the ratios of C/EBPδ:β-actin, averaged for duplicate samples, normalised to the IL-1-treated negative siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratios of JNK1:β-actin and JNK2:β-actin, were determined for the specified samples and the percentage (%) reduction in expression for both JNK1 and JNK2 was calculated by normalising their expression in negative siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio $\pm$SD from three independent experimental series. **P<0.01, ***P<0.001.


A.
M
M
-RT
siRNA
Negative
JNK1
JNK2
JNK1+JNK2
IL-1 (100U/ml)
- - + + - - + +
- - + + - - + +
C/EBP
JNK1
JNK2
β-actin


B.
Relative C/EBPδ Expression
Relative Expression
3
2.5
2
1.5
1
0.5
0
Control
+IL-1
Negative Control siRNA
JNK1 + JNK2 siRNA


C.
Relative JNK1 and JNK2 Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
***
***
***
**
JNK1
JNK2
Treatment
siRNA
Control
Negative
Control
JNK1+JNK2
+IL-1
Negative
+IL-1
JNK1+JNK2

The results show siRNAs directed against JNK1 and JNK2 specifically silence the expression of these genes in transfected Hep3B cells, compared to controls. Moreover, the results also show that siRNA directed against JNK1 did not inhibit the expression of JNK2 and vice versa, producing further evidence for the specificity of the siRNA used in our experiments. Co-transfecting JNK1 and JNK2 together also produced similar effects, resulting in knock-down of JNK1 and JNK2 expressions comparable to that observed when either siRNA was transfected alone (Panel A). On average, the knock-down in expression of JNK1 was between 80-90% and that of JNK2 was between 70-80% in three experiments (Panel C). In addition, depletion of JNK1 and JNK2 expression did not result in a decrease in C/EBPδ expression as induced by IL-1 (Panels A and B). In fact, co-transfecting siRNAs against JNK1 and JNK2 produced a slight increase in C/EBPδ expression (Panel B). To eliminate the possibility this effect was due to negative control siRNA in use, the experiment was repeated with an alternative control siRNA.

siRNA directed against GAPDH has previously been used as a control for experiments involving RNAi (Larigauderie *et al.* 2004; Yang and Sharrocks 2004; Chen *et al.* 2005a). Therefore, we tested this siRNA as a control in our transfection system. siRNA directed against the human GAPDH gene was purchased from Ambion®. The action of this siRNA has previously been validated in our laboratory in both Hep3B and THP-1 cell lines (Dr S.A. Rogers and Dr P. Foka, personal communications). Hep3B cells were transfected with siRNA directed against GAPDH or co-transfected with siRNA against JNK1 and JNK2. The cells were then either treated with IL-1 or left untreated exactly as described before and samples were harvested for RT-PCR analysis. PCRs for the various gene products were carried out as described previously and the results of this experiment are presented in Figure 6.5.

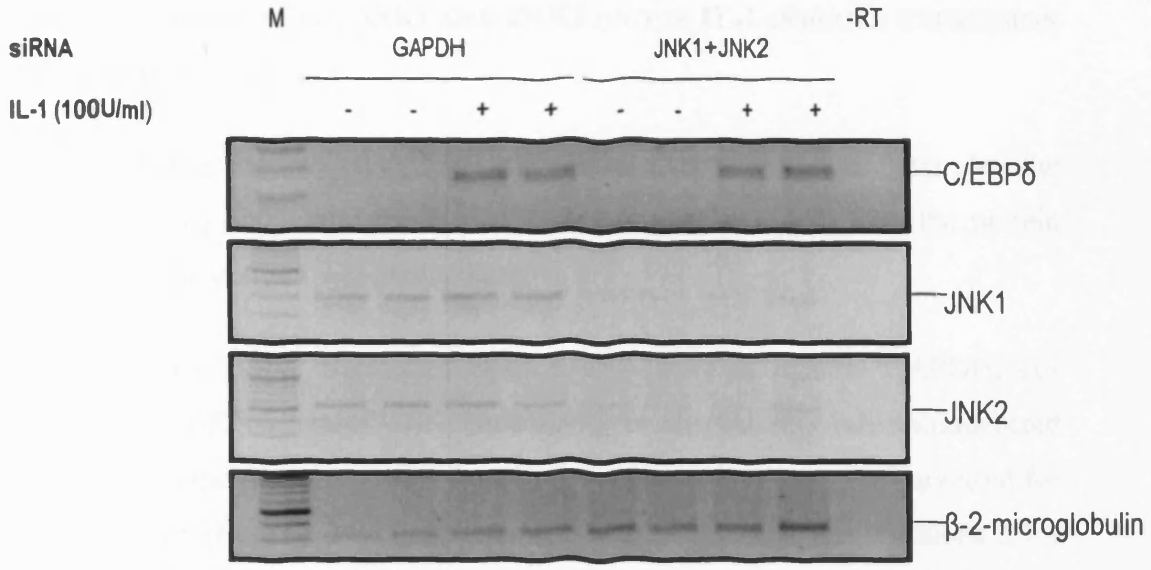



**FIGURE 6.5. Effect of simultaneously silencing JNK1 and JNK2 on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (12.5nM) or co-transfected with siRNA against JNK1 and JNK2 (6.25nM of each), as before. The cells were then either treated with IL-1 (100U/ml) or left untreated for 3h. Total RNA was extracted from cells and used for semi-quantitative RT-PCR analysis. The PCR amplification products for the indicated genes were then size fractioned by electrophoresis. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected, -IL-1 RNA). Inverted images are shown for clarity.

The induction of C/EBPδ in response to IL-1 was apparent in cells transfected with GAPDH siRNA, at a level comparable to that observed in cells transfected with negative siRNA from previous experiments. This induction was also comparable to that observed from cells co-transfected with JNK1 and JNK2 in this experiment. In addition, as previously observed, the messages for both JNK1 and JNK2 genes were depleted in cells that were co-transfected with the corresponding siRNAs.

Together, data presented in this section indicates that knock-down of JNK MAPK alone is not sufficient to impair the expression of C/EBPδ by IL-1, at least at the mRNA level.

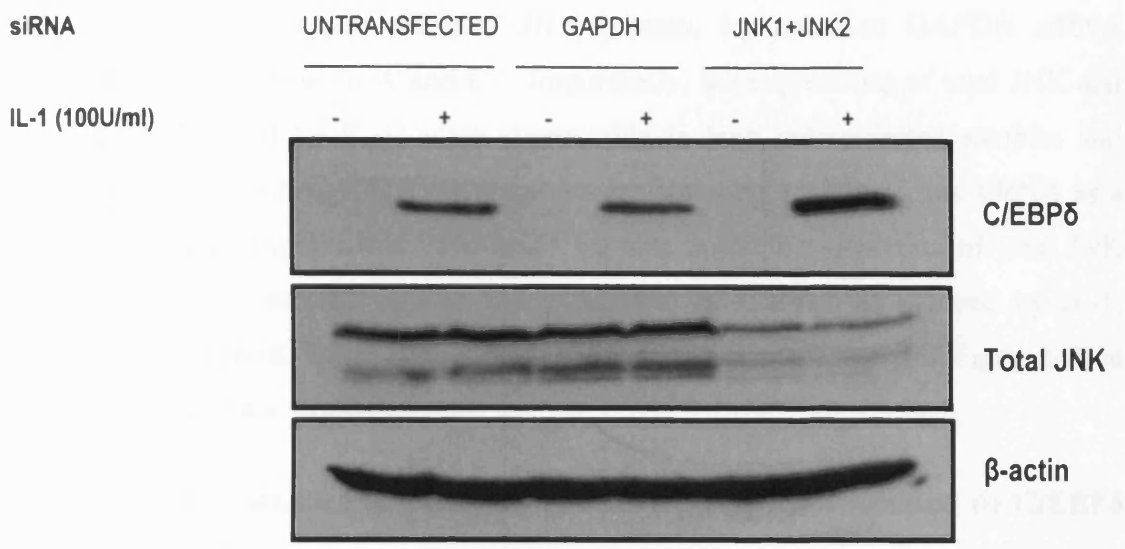
### 6.2.2 EFFECT OF SILENCING JNK1 AND JNK2 ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ PROTEIN LEVELS

To further validate the use of GAPDH siRNA as a control, and to determine the effects of silencing JNK1 and JNK2 on C/EBPδ expression by IL-1 on the protein level, western blot analysis was carried out.

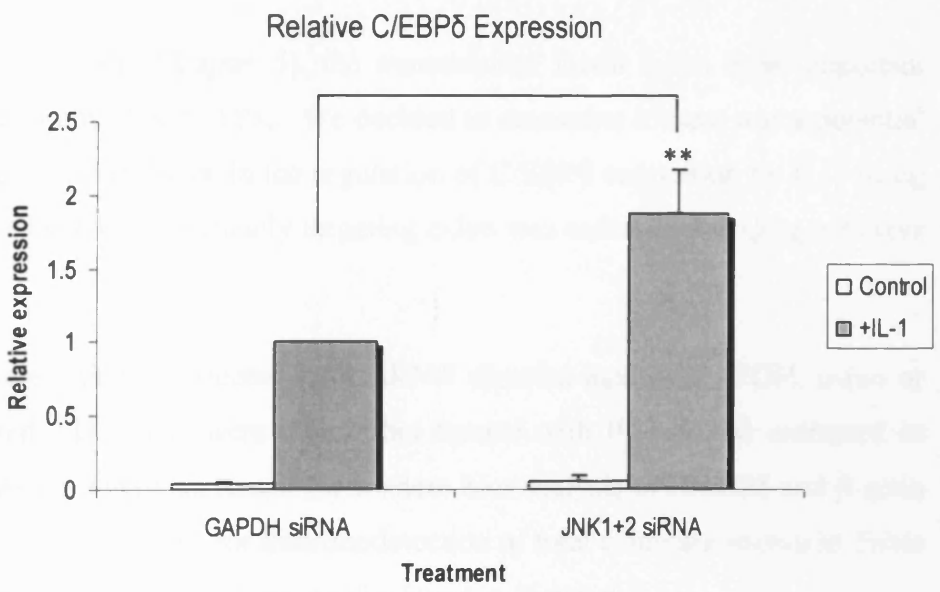
Hep3B cells were either transfected with siRNA directed against GAPDH, co-transfected with siRNA against JNK1 and JNK2 or alternatively left untransfected (included for comparative purposes). Post IL-1 treatment, samples were harvested for protein extraction (Section 2.7.1) and used for western blot analysis (Sections 2.7.7 and 2.7.8). Immunodetection of C/EBPδ, total JNK and β-actin was carried out as described previously. The results of these experiments are displayed in Figure 6.6.

**FIGURE 6.6. Effect of simultaneously silencing JNK1 and JNK2 on IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (12.5nM), co-transfected with siRNA against both JNK1 and JNK2 (6.25nM of each siRNA) or left untransfected as described before. Post transfection, the cells were either treated with IL-1 (100U/ml) or left untreated for 3h exactly as before. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using antibodies against C/EBPδ, total JNK and β-actin for immunodetection. For β-actin detection, the western blot used to detect C/EBPδ was re-probed with the anti-β-actin antibody, Panel A. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratios of total JNK:β-actin, were determined for the specified samples and the % reduction in expression of total JNK was calculated by normalising its expression in GAPDH siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio ±SD from three independent experimental series. **P<0.01, ***P<0.001.

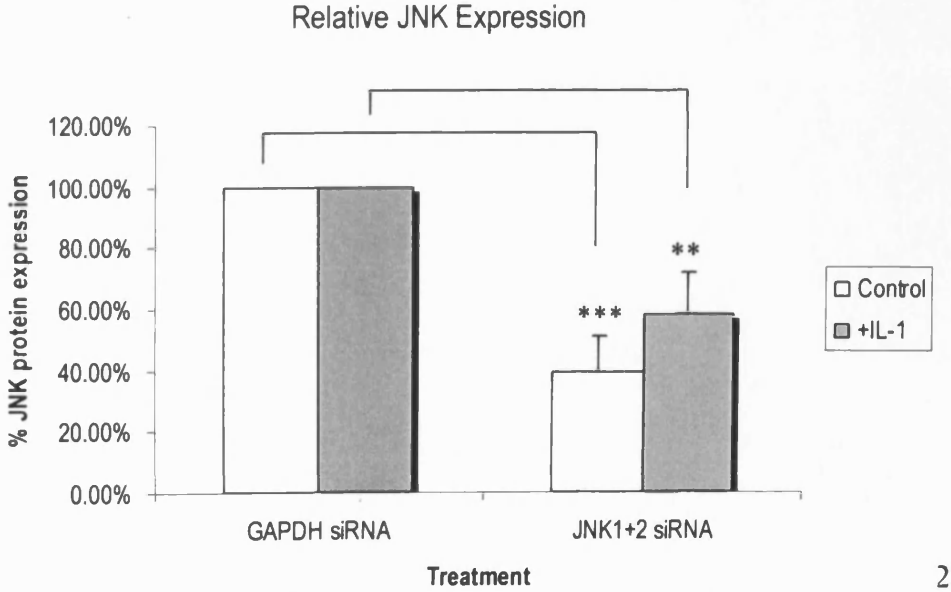
**A.**


siRNA
UNTRANSFECTED
GAPDH
JNK1+JNK2
IL-1 (100U/ml)
- + - + - +
C/EBPδ
Total JNK
β-actin

**B.**


Relative C/EBPδ Expression
Relative expression
2.5
2
1.5
1
0.5
0
**
Control
+IL-1
GAPDH siRNA
JNK1+2 siRNA
Treatment

**C.**


Relative JNK Expression
% JNK protein expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
***
**
Control
+IL-1
GAPDH siRNA
JNK1+2 siRNA
Treatment

As expected, siRNA directed against both JNK1 and JNK2 produced a significant decrease in the expression of total JNK protein, compared to GAPDH siRNA transfected controls (Panels A and C). Importantly, the expressions of total JNK and C/EBPδ (as induced by IL-1) were comparable in both untransfected samples and those transfected with GAPDH siRNA, further validating the use of this siRNA as a control for future experiments. However we also noted that depletion of total JNK produced a significant increase in the expression of C/EBPδ as induced by IL-1, compared to controls. These data support the RT-PCR data presented in Figure 6.4, in which we observed a similar effect.

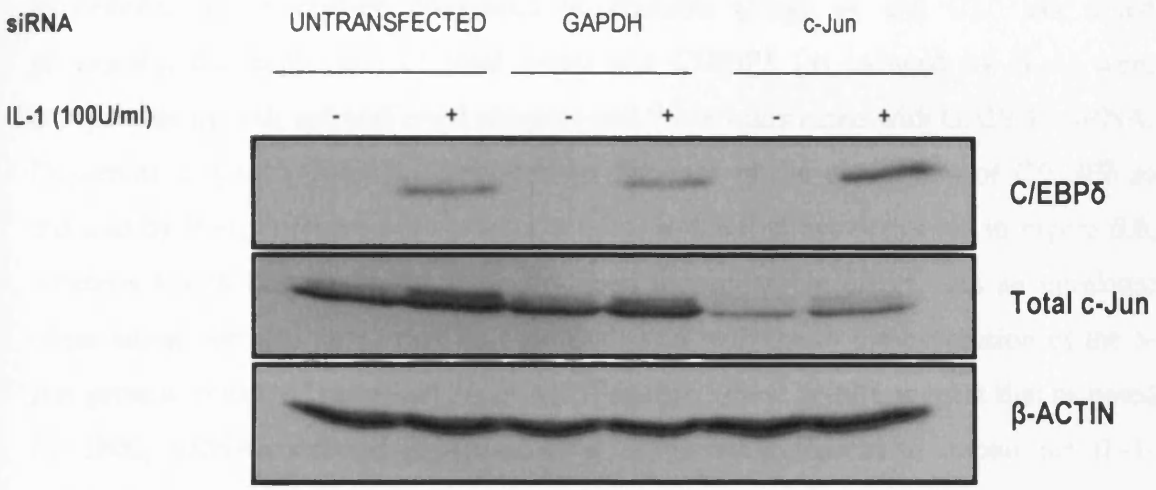
### 6.2.3 EFFECT OF SILENCING C-JUN ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ PROTEIN LEVELS

As discussed previously (Chapter 5), the transcription factor c-Jun is an important target for activation by JNK MAPK. We decided to determine if there was a potential role for this transcription factor in the regulation of C/EBPδ expression by IL-1 using RNAi. Validated siRNA specifically targeting c-Jun was ordered from Qiagen (Table 2.1).

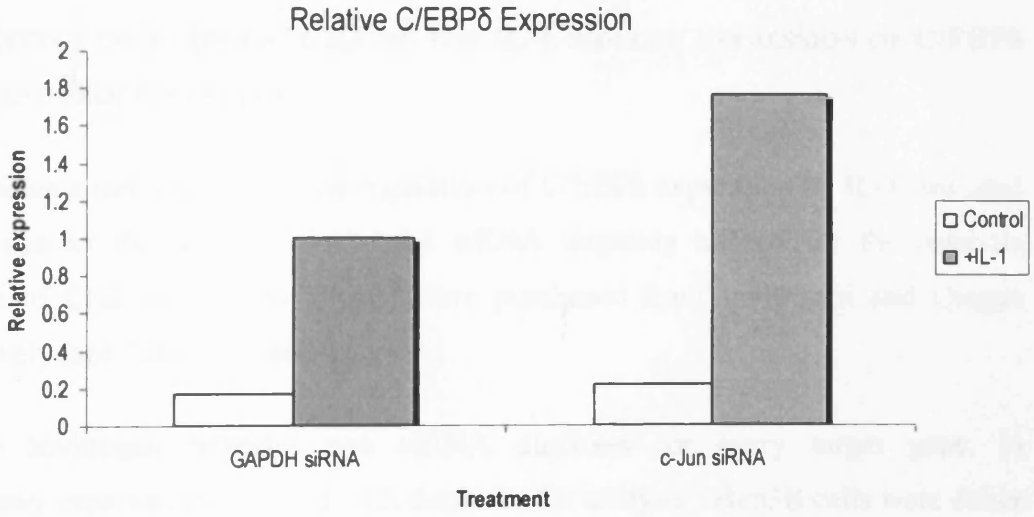
Hep3B cells were either transfected with siRNA directed against GAPDH, c-Jun or left untransfected. The cells were then either treated with IL-1 or left untreated as before. Samples were then harvested for western blot analysis of C/EBPδ and β-actin as above, or for c-Jun. Details for immunodetection of total c-Jun are shown in Table 2.9. The results of these experiments are displayed in Figure 6.7.

**FIGURE 6.7. Effect of silencing c-Jun on IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were either transiently transfected with siRNA directed against GAPDH (6.25nM), c-Jun (6.25nM) or left untransfected as described above. The cells were either treated with IL-1 or left untreated for 3h exactly as before. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using antibodies against C/EBPδ, total c-Jun and β-actin for immunodetection. Western blot analysis was carried out by SDS-PAGE using 12.5% (w/v) gels, for subsequent immunoblotting using the anti-c-Jun antibody and western blot analysis of C/EBPδ was carried out as described previously. For β-actin detection, the western blot used to detect C/EBPδ was re-probed with the anti-β-actin antibody. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratios of total c-Jun:β-actin, were determined for the specified samples and the % reduction in expression of total c-Jun was calculated by normalising its expression in GAPDH siRNA transfected samples to 100%. Results are representative of two independent experiments.

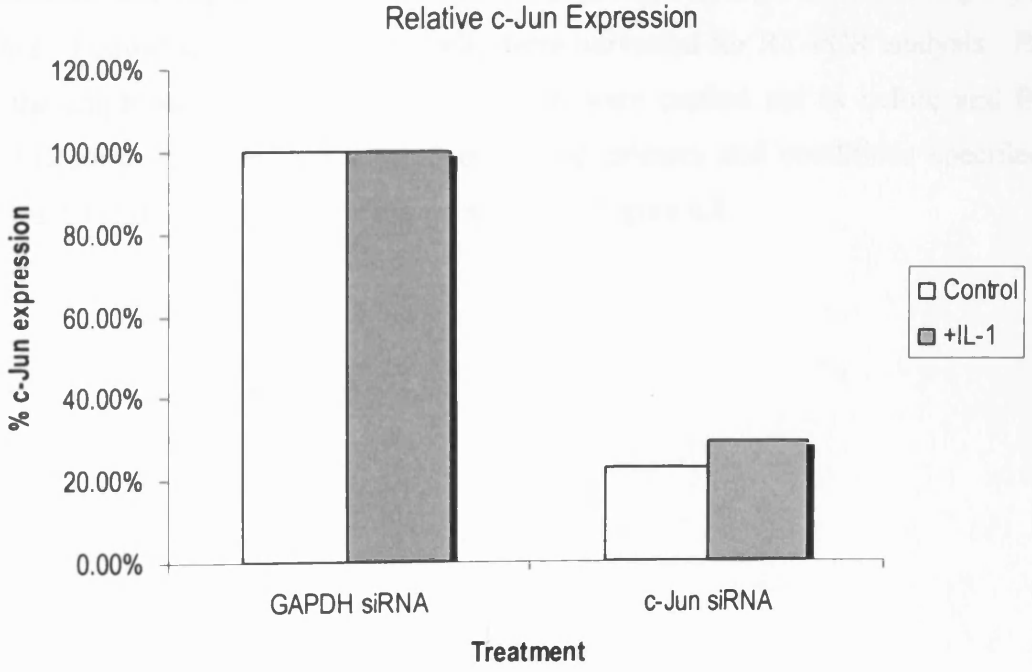
**A.**


siRNA
UNTRANSFECTED
GAPDH
c-Jun
IL-1 (100U/ml)
- + - + - +
C/EBPδ
Total c-Jun
β-ACTIN

**B.**


Relative C/EBPδ Expression
Relative expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GAPDH siRNA
c-Jun siRNA
Treatment
Control
+IL-1

**C.**


Relative c-Jun Expression
% c-Jun expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
GAPDH siRNA
c-Jun siRNA
Treatment
Control
+IL-1

siRNA targeting the transcription factor c-Jun caused a 70-80% decrease in its expression, as anticipated compared to controls (Panel A and C). As noted previously, the expression of total c-Jun and C/EBPδ (as induced by IL-1) were comparable in both untransfected samples and those transfected with GAPDH siRNA. Depletion of total c-Jun also produced an increase in the expression of C/EBPδ as induced by IL-1, compared to controls, similar to the findings presented in Figure 6.6, whereby knock-down of total JNK produced a comparable effect. As an unrelated observation, we also noted that IL-1 produced an increase in the expression of the c-Jun protein in treated samples (Panel A). Together, these results suggest that as noted for JNK, siRNA-mediated depletion of c-Jun is not sufficient to impair the IL-1-mediated induction of C/EBPδ expression in transfected cells.

### 6.2.4 EFFECT OF SILENCING CK2 ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ MRNA AND PROTEIN LEVELS

To elucidate a role for CK2 in the regulation of C/EBPδ expression by IL-1, we used siRNA against this kinase. Validated siRNA targeting mRNA for the catalytic subunits of CK2, CK2α and CK2α' were purchased from Invitrogen and Qiagen respectively (see Tables 2.1 and 2.2).

Because Invitrogen provides two siRNA duplexes for every target gene, in preliminary experiments we used both duplexes for analysis. Hep3B cells were either transfected with negative siRNA or siRNA targeting CK2α (both siRNA duplexes) as before. Following IL-1 treatment, cells were harvested for RT-PCR analysis. PCRs for the amplification of C/EBPδ and β-actin were carried out as before and PCR-amplification of CK2α was carried out using primers and conditions specified in Tables 2.4 and 2.5. The results are presented in Figure 6.8.

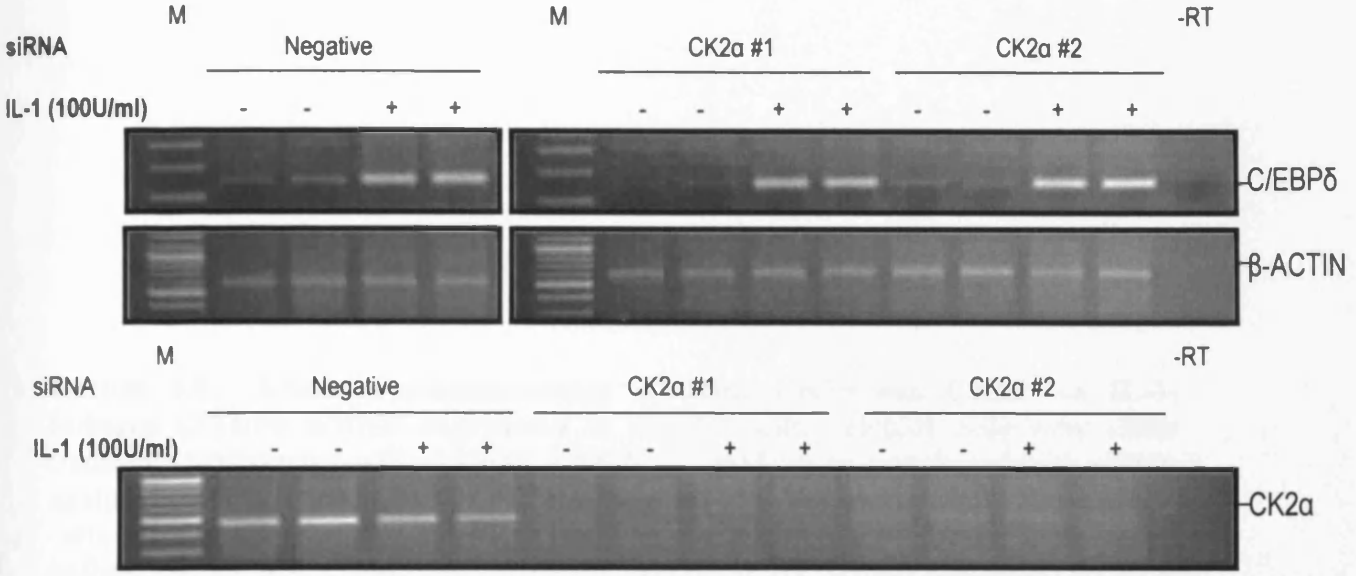
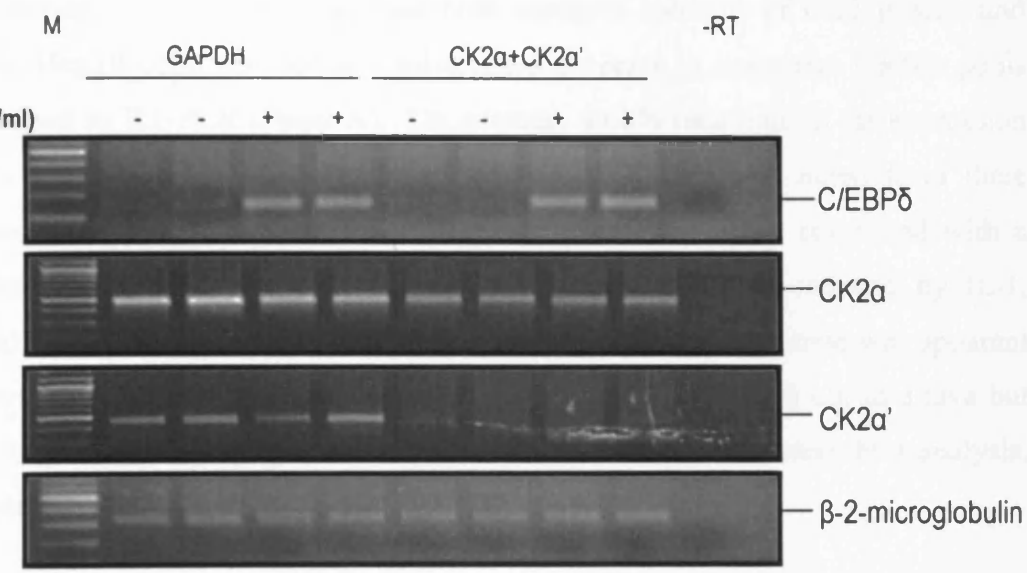



**FIGURE 6.8. Effect of silencing CK2α on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with negative siRNA (6.25nM) or with two siRNA duplexes (duplex #1 and duplex #2) designed against CK2α (6.25nM) as described before, with each transfection carried out in duplicate. The cells were either treated with IL-1 (100U/ml) or left untreated for 3h as before. Total RNA was extracted from cells and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, β-actin and CK2α were resolved by electrophoresis. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using negative siRNA transfected, -IL-1 RNA). Results are representative of two independent experiments.

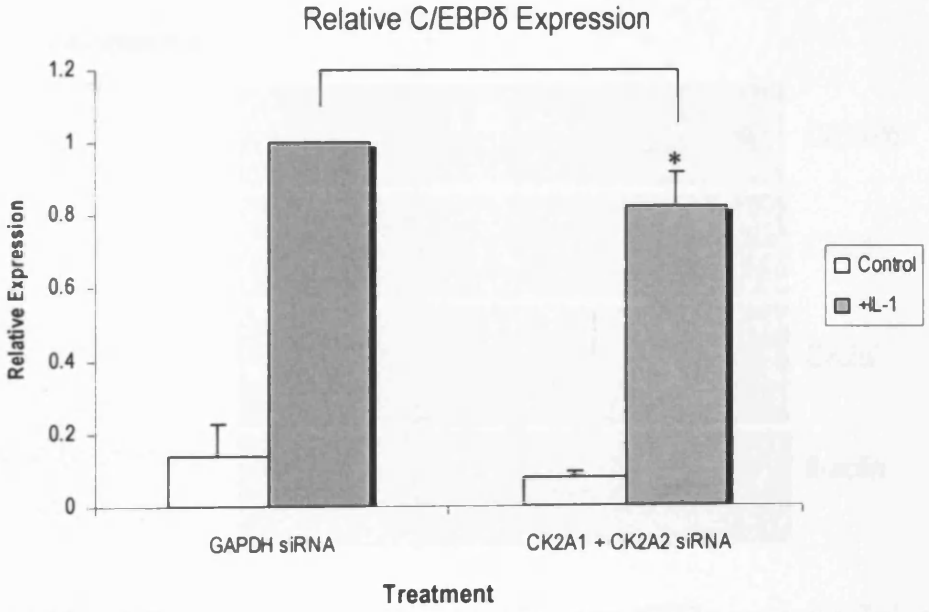
Both siRNA duplexes targeting CK2α, appeared to be equally efficient at producing a decrease in the expression of the CK2α transcript in transfected cells, compared to controls. Neither siRNA duplex appeared to affect the expression of the β-actin control, supporting the specificity of siRNA action. Furthermore, the IL-1-induced expression of C/EBPδ did not appear to be affected in the near absence of the CK2α transcript in cells transfected with corresponding siRNA, compared to controls. Therefore, we next examined the effect of knocking-down the expression of both CK2α and CK2α' simultaneously, on the IL-1-mediated induction of C/EBPδ mRNA expression. For this, Hep3B cells were either transfected with siRNA targeting GAPDH or co-transfected with siRNA against CK2α and CK2α' using the same procedure as described before. Following IL-1 treatment, the cells were harvested for RT-PCR analysis for the expression of C/EBPδ, CK2α, CK2α' and β-2-microglobulin. Primers and conditions for the PCR-amplification of CK2α' are presented in Tables 2.4 and 2.5 respectively. Results are presented in Figure 6.9.

**FIGURE 6.9. Effect of simultaneously silencing CK2α and CK2α' on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (12.5nM) or co-transfected with siRNA against both CK2α and CK2α' (6.25nM of each siRNA), as described above. The cells were either treated with IL-1 (100U/ml) or left untreated for 3h as described before. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. PCR amplification products for C/EBPδ, CK2α (CK2A1), CK2α'(CK2A2) and β-2-microglobulin were resolved by electrophoresis. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected, -IL-1 RNA), Panel A. Panel B displays the ratios of C/EBPδ:β-2-microglobulin, averaged for duplicate samples, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratios of CK2α:β-2-microglobulin and CK2α':β-2-microglobulin, were determined for the specified samples and the % reduction in expression for both CK2α and CK2α' was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio $\pm$SD from three independent experimental series. *P<0.05, **P<0.01, ***P<0.001.

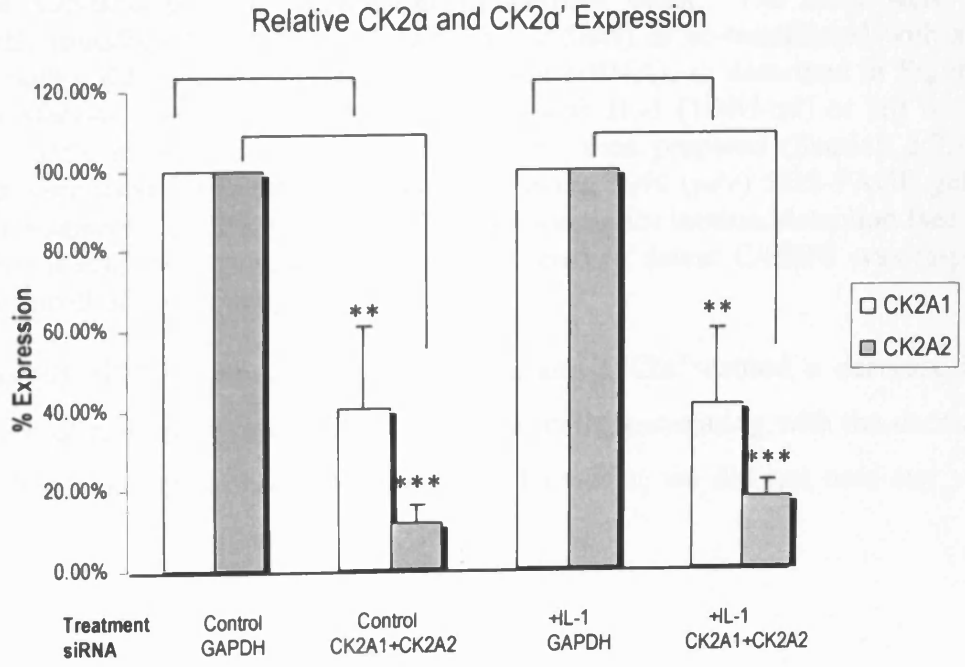
**A.**


M
-RT
siRNA
GAPDH
CK2α+CK2α'
IL-1 (100U/ml)
- - + + - - + +
C/EBPδ
CK2α
CK2α'
β-2-microglobulin

**B.**


Relative C/EBPδ Expression
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
+IL-1
GAPDH siRNA
CK2A1 + CK2A2 siRNA
Treatment

**C.**


Relative CK2α and CK2α' Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
**
***
**
***
CK2A1
CK2A2
Treatment
siRNA
Control
GAPDH
Control
CK2A1+CK2A2
+IL-1
GAPDH
+IL-1
CK2A1+CK2A2

Co-transfecting siRNA directed against both catalytic subunits of CK2 (CK2α and CK2α') in Hep3B cells, resulted in a subsequent decrease in respective mRNA pools as determined by RT-PCR (Panel A). On average, a 60% reduction in the expression of CK2α and a 90% decrease in the expression of CK2α' was noted from three experiments (Panel C). Knock-down in the expression of CK2 correlated with a minor but significant decrease in the expression of C/EBPδ as induced by IL-1, compared to controls (Panel B). In order to assess whether this decrease was apparent at the level of protein expression, the same experiment was carried out as above but samples were harvested for protein extraction and subsequent western blot analysis. The results are shown in Figure 6.10.

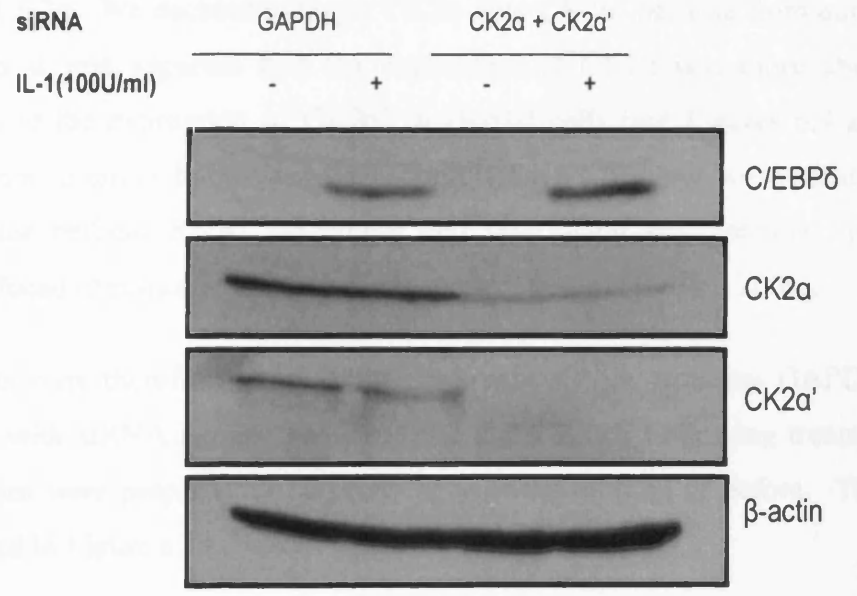



**FIGURE 6.10. Effect of simultaneously silencing CK2α and CK2α' on IL-1-induced C/EBPδ protein expression in Hep3B cells.** The cells were either transiently transfected with GAPDH siRNA (12.5nM) or co-transfected with siRNA against both CK2α and CK2α' (6.25nM of each siRNA), as described in Figure 6.9. Post transfection, the cells were either treated with IL-1 (100U/ml) or left untreated for 3h exactly as before. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using 10% (w/v) SDS-PAGE gels and antibodies against C/EBPδ, CK2α, CK2α' and β-actin for immunodetection (see Table 2.9). For β-actin detection, the western blot used to detect C/EBPδ was re-probed with the anti-β-actin antibody.

As expected, siRNAs directed against CK2α and CK2α' caused a decrease in the expression of respective proteins in transfected cells, correlating with the decrease in mRNA levels as noted above (Figure 6.9). However, we did not note any visible

decrease in the expression of C/EBPδ by IL-1 in this experiment, compared to the control.

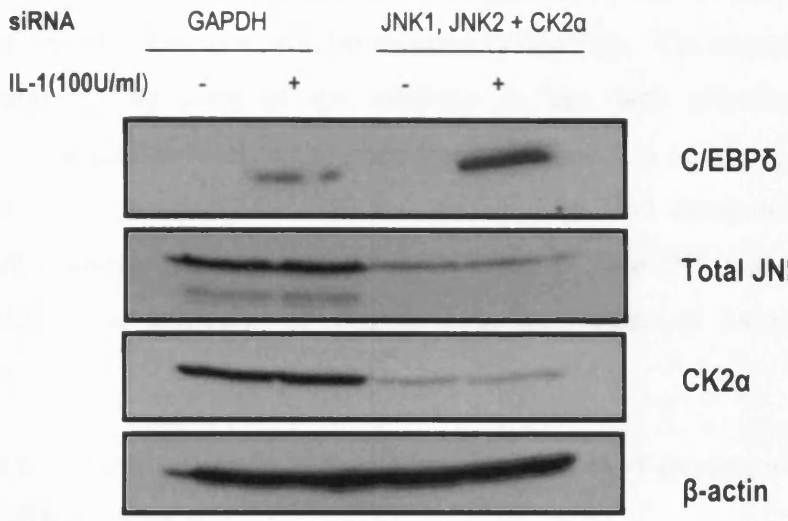
### 6.2.5 EFFECT OF SILENCING JNK MAPK AND CK2 SIMULTANEOUSLY ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ PROTEIN LEVELS

In light of the findings presented in the previous chapter and so far in this chapter, we wanted to determine if there could be some functional redundancy between JNK MAPK and CK2 in relation to the regulation of the IL-1-mediated induction of C/EBPδ expression. Therefore, we targeted both these signalling mediators in our next RNAi experiment. Three genes were selected for knock-down by siRNA, JNK1, JNK2 and CK2α. We decided to target CK2α over CK2α' because from our previous experiments it was apparent that the expression of CK2α was more abundant in comparison to the expression of CK2α' in Hep3B cells (see Figures 6.9 and 6.10). We chose not to target both catalytic subunits of CK2 because we wanted to avoid saturating the cellular RNAi machinery and thus avoid any potential 'off-target' effects produced in response to high siRNA concentrations (Cullen 2006).

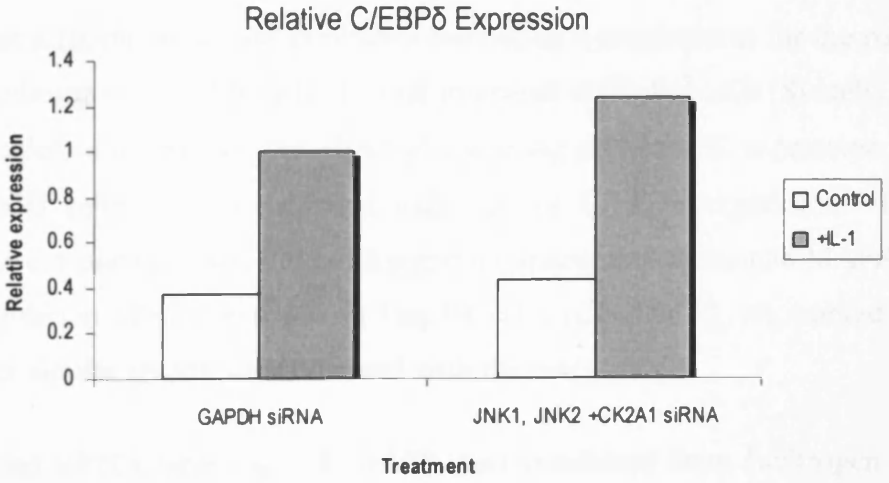
Hep3B cells were therefore either transfected with siRNA targeting GAPDH or co-transfected with siRNA against JNK1, JNK2 and CK2α. Following treatment with IL-1, samples were prepared for analysis by western blotting as before. The results are presented in Figure 6.11.

**FIGURE 6.11. Effect of simultaneously silencing JNK1, JNK2 and CK2α on IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (18.75nM), co-transfected with siRNA against JNK1, JNK2 and CK2α (CK2A1) (6.25nM of each siRNA) as described above. Post transfection, the cells were either treated with IL-1 (100U/ml) or left untreated for 3h exactly as before. Samples were used for western blot analysis as described previously, using antibodies against C/EBPδ, total JNK, CK2α and β-actin. For β-actin detection, the western blot used to detect C/EBPδ was re-probed with the anti-β-actin antibody, Panel A. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratios of total JNK:β-actin and CK2α:β-actin, were determined for the specified samples and the % reduction in expression of total JNK and CK2α was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. The results shown are representative of two independent experiments.

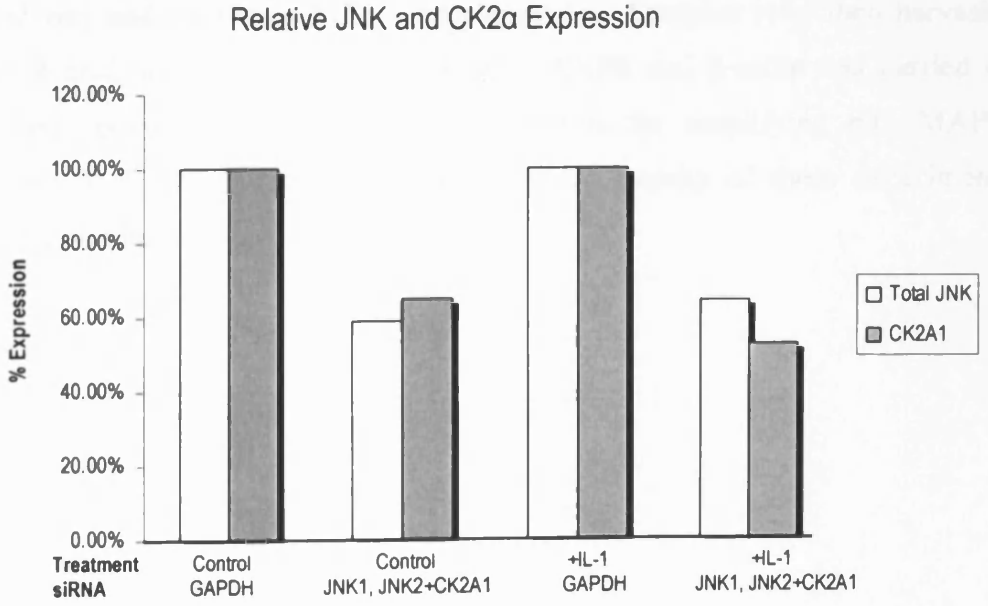
**A.**


siRNA
GAPDH
JNK1, JNK2 + CK2α
IL-1(100U/ml)
- + - +
C/EBPδ
Total JNK
CK2α
β-actin

**B.**


Relative C/EBPδ Expression
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
+IL-1
GAPDH siRNA
JNK1, JNK2 +CK2A1 siRNA
Treatment

**C.**


Relative JNK and CK2α Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Total JNK
CK2A1
Treatment
siRNA
Control
GAPDH
Control
JNK1, JNK2+CK2A1
+IL-1
GAPDH
+IL-1
JNK1, JNK2+CK2A1

Consistent with our previous results, siRNA against JNK MAPK and CK2α depleted the expressions of respective proteins relative to controls. The constant expression of β-actin suggests that none of the siRNAs in use were affecting cellular gene expression on a global level at the concentration used. In addition, we observed an increase in the expression of C/EBPδ as induced by IL-1 compared to the control. These results indicate that reducing the expressions of total JNK and CK2α proteins in Hep3B cells is not sufficient to attenuate the IL-1-mediated induction of C/EBPδ expression.

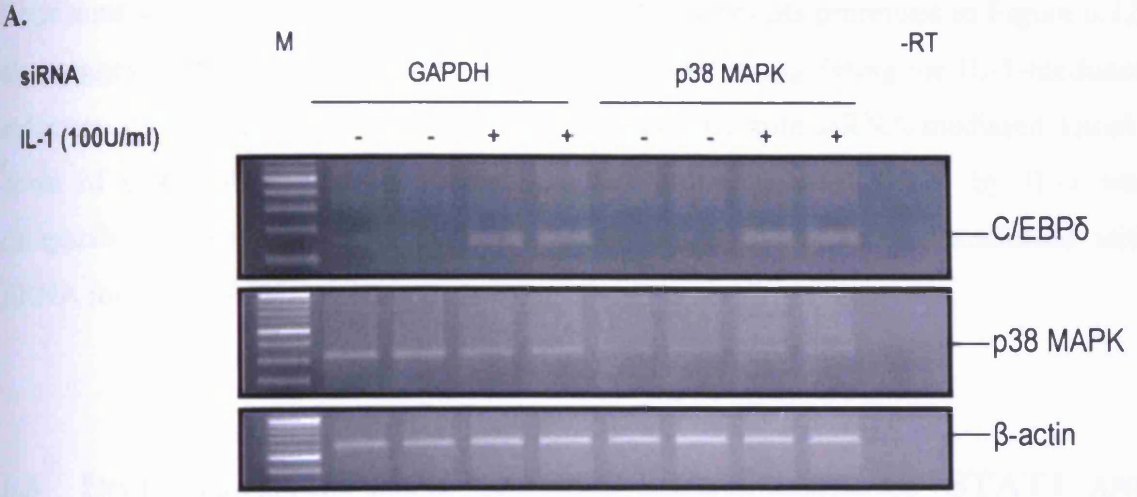
### 6.2.6 EFFECT OF SILENCING P38 MAPK ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ MRNA LEVELS

Because a recent paper was published indicating a requirement for the p38 MAPK in the regulation of C/EBPδ by IL-1 in rat intestinal epithelial cells (Svotelis *et al.* 2005), we decided to investigate the effect of depleting p38 MAPK expression with siRNA in Hep3B cells on IL-1-mediated induction of C/EBPδ expression. Furthermore, because our previous data did not suggest a requirement of the p38 MAPK pathway in the regulation of this response in Hep3B cells (Chapter 4), we wanted to ascertain whether similar results were obtained with the use of RNAi.

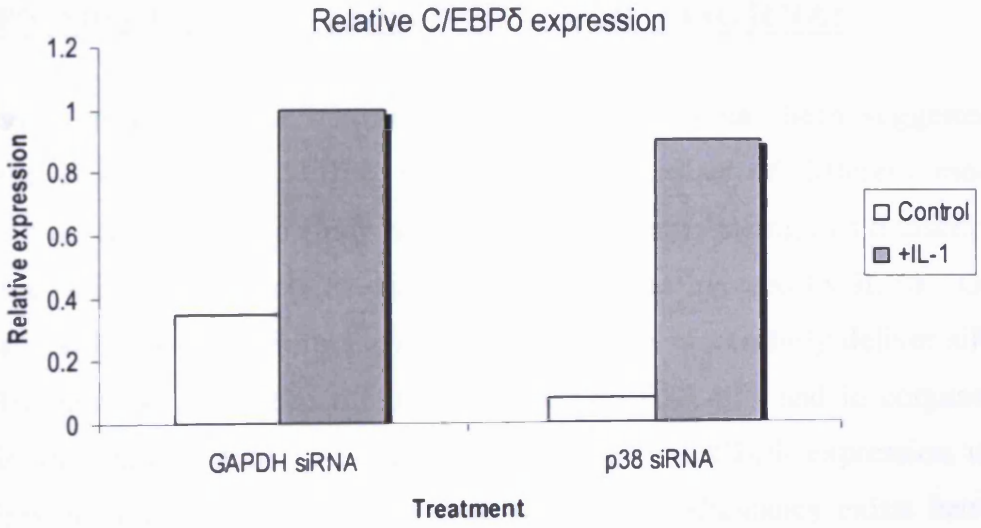
Validated siRNA targeting p38 MAPK was purchased from Invitrogen (Table 2.2). Hep3B cells were transfected with siRNA against GAPDH or p38 MAPK in the normal way and treated with IL-1 appropriately. Samples were then harvested for RT-PCR analysis. PCR-amplification of C/EBPδ and β-actin was carried out as described above. The primers and conditions for amplifying p38 MAPK are presented in Tables 2.4 and 2.5 respectively. Results of these experiments are presented in Figure 6.12.

**FIGURE 6.12. Effect of silencing p38 MAPK on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (6.25nM) or siRNA directed against p38 MAPK (6.25nM), as before. The cells were then either treated with IL-1 (100U/ml) or left untreated for 3h as described above. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. PCR amplification products for C/EBPδ, p38 MAPK and β-actin were resolved by electrophoresis. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected, -IL-1 RNA), Panel A. Panel B displays the ratios of C/EBPδ:β-actin, averaged for duplicate samples, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratio of p38 MAPK:β-actin was determined for the specified samples and the % reduction in p38 MAPK expression was calculated by normalising its expression in GAPDH siRNA transfected samples to 100%. Results are representative of two independent experiments.

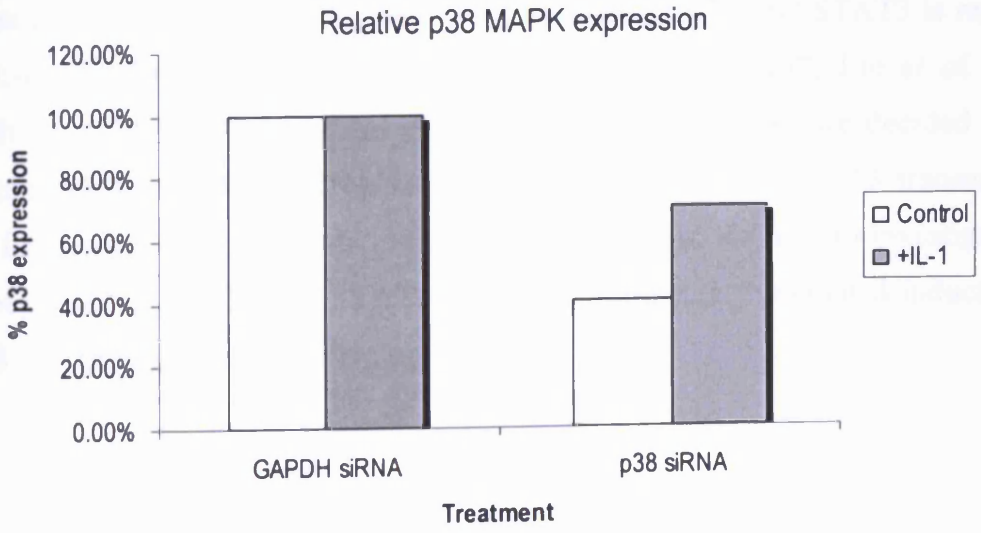
A.


M
-RT
siRNA
GAPDH
p38 MAPK
IL-1 (100U/ml)
- - + + - - + +
C/EBPδ
p38 MAPK
β-actin

B.


Relative C/EBPδ expression
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
GAPDH siRNA
p38 siRNA
Treatment
Control
+IL-1

C.


Relative p38 MAPK expression
% p38 expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
GAPDH siRNA
p38 siRNA
Treatment
Control
+IL-1

Consistent with our previous data from chapter 4, the results presented in Figure 6.12, also suggest that the p38 MAPK may not be involved in regulating the IL-1-mediated induction of C/EBPδ expression in Hep3B cells. Despite siRNA-mediated knock-down of p38 MAPK (Panels A and C), the induction of C/EBPδ by IL-1 was comparable between GAPDH siRNA transfected cells and those transfected with siRNA for p38 MAPK.

## 6.3 INVESTIGATIONS INTO DETERMINING A ROLE OF STAT1 AND STAT3 TRANSCRIPTION FACTORS IN THE REGULATION OF IL-6-MEDIATED INDUCTION OF C/EBPδ EXPRESSION USING RNAi

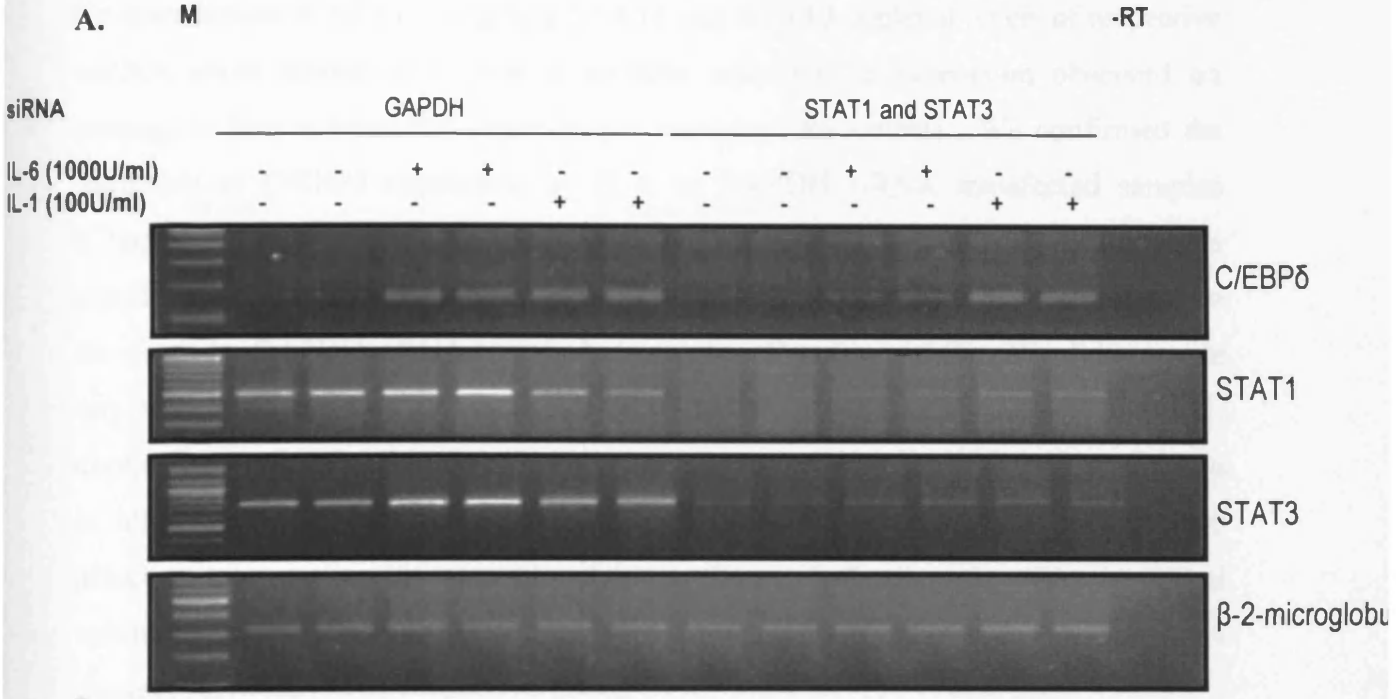
As previously mentioned, the transcription factor STAT3 has been suggested to regulate the expression of C/EBPδ by IL-6, in a number of different models. However, to our knowledge no study has provided evidence linking this transcription factor to the regulation of endogenous C/EBPδ levels (as induced by IL-6). Given that we had established a working transfection system to successfully deliver siRNA into Hep3B cells, we wanted to investigate the role of STAT3 and in conjunction STAT1, in the regulation of IL-6-mediated increase in C/EBPδ expression using RNAi. Previously published reports suggest that some redundancy exists between STAT1 and STAT3 in relation to regulation of gene expression in fibroblasts (Ramana *et al.* 2005). In addition, activation of both STAT1 and STAT3 is reported upon IL-6 stimulation of various cell types (Chung *et al.* 1997; Liu *et al.* 1998; Heinrich *et al.* 2003). Taking this evidence into consideration, we decided to co-transfect Hep3B cells with siRNA targeting both STAT1 and STAT3 transcription factors for our RNAi experiments. In association, we also decided to investigate the effect of depleting STAT1 and STAT3 by RNAi, on the IL-1-mediated induction of C/EBPδ expression for comparative purposes.

### 6.3.1 EFFECT OF SILENCING STAT1 AND STAT3 ON THE IL-6- AND IL-1-MEDIATED EXPRESSION OF C/EBPδ MRNA LEVELS

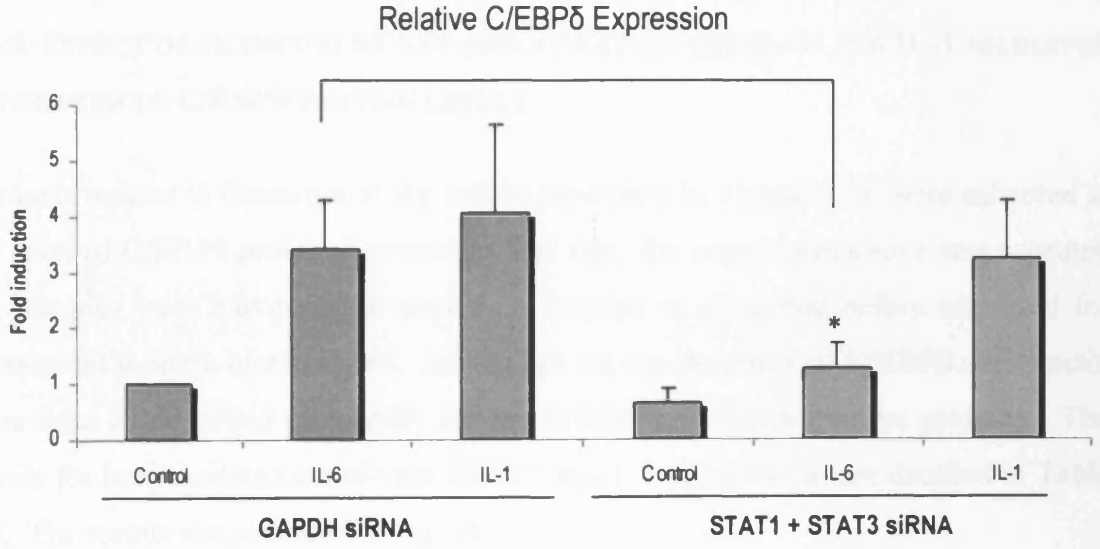
Validated siRNA targeting both STAT1 and STAT3 was purchased from Invitrogen (Table 2.2). Hep3B cells were transfected with GAPDH siRNA or co-transfected with siRNA against STAT1 and STAT3 in the normal way. The cells were then treated with IL-1 or IL-6 appropriately and harvested for RT-PCR analysis. PCR-amplification of C/EBPδ and β-2-microglobulin was carried out as outlined previously and details for the PCR-amplification of STAT1 and STAT3 are presented in Tables 2.4 and 2.5. The results of these experiments are presented in Figure 6.13.

**FIGURE 6.13. Effect of simultaneously silencing STAT1 and STAT3 on IL-6- and IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (12.5nM) or co-transfected with siRNA against both STAT1 and STAT3 (6.25nM of each siRNA) as described previously, with each transfection carried out in duplicate. Post transfection, the cells were either treated with IL-6 (1000U/ml), IL-1 (100U/ml) or left untreated for 3h as described before. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, β-2-microglobulin, STAT1 and STAT3 were resolved by electrophoresis as before. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected, untreated RNA), Panel A. Panel B displays the ratios of C/EBPδ:β-actin, averaged for duplicate samples, normalised to the untreated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratios of STAT1:β-2-microglobulin and STAT3:β-2-microglobulin, were determined for the specified samples and the % reduction in expression for both STAT1 and STAT3 was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio ±SD from four independent experimental series. *$P<0.05$, ***$P<0.001$.

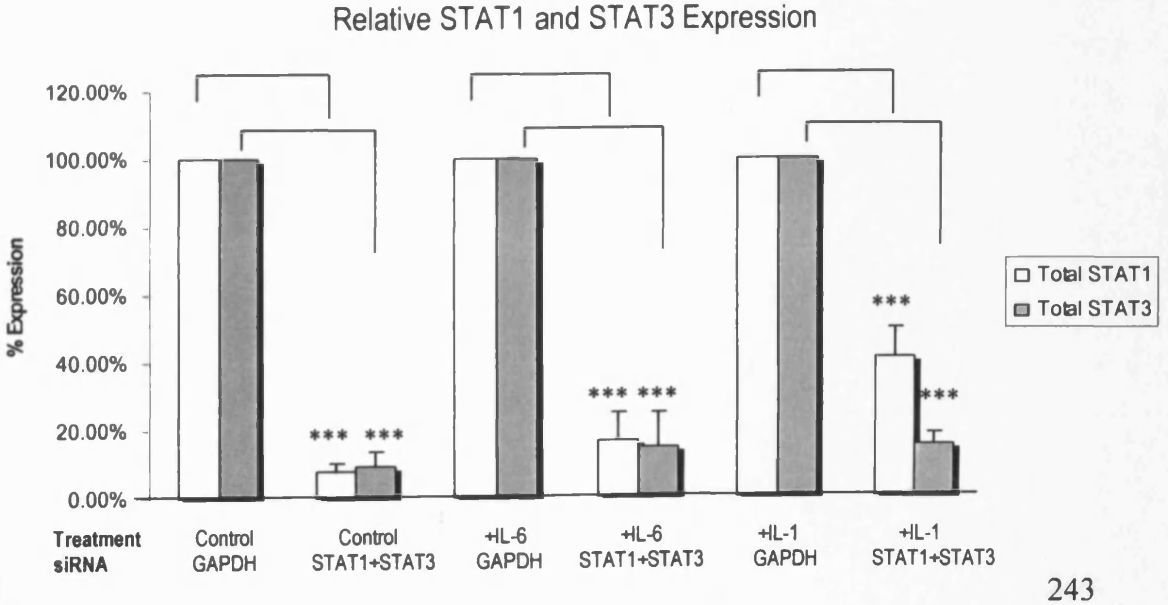
**A.**


M
-RT
siRNA
GAPDH
STAT1 and STAT3
IL-6 (1000U/ml)
IL-1 (100U/ml)
C/EBPδ
STAT1
STAT3
β-2-microglobu

**B.**


Relative C/EBPδ Expression
Fold induction
0
1
2
3
4
5
6
*
Control
IL-6
IL-1
Control
IL-6
IL-1
GAPDH siRNA
STAT1 + STAT3 siRNA

**C.**


Relative STAT1 and STAT3 Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
*** ***
*** ***
***
***
Total STAT1
Total STAT3
Treatment
siRNA
Control
GAPDH
Control
STAT1+STAT3
+IL-6
GAPDH
+IL-6
STAT1+STAT3
+IL-1
GAPDH
+IL-1
STAT1+STAT3

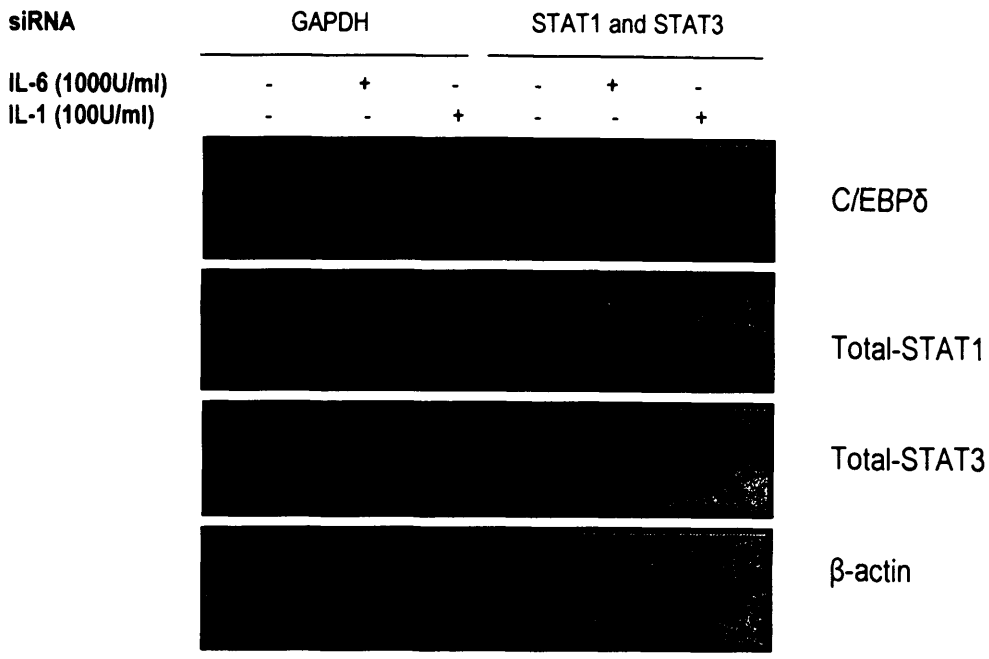
Co-transfection of siRNAs targeting STAT1 and STAT3 depleted levels of respective mRNA pools dramatically, with a 60-90% reduction in expression observed on average in four independent experiments, compared to controls. We confirmed the induction of C/EBPδ expression by IL-6 in GAPDH siRNA transfected samples (Chapter 3). However, we also found that depletion of STAT1 and STAT3 caused a significant decrease in the expression of C/EBPδ as mediated by IL-6, compared to the controls, GAPDH siRNA transfected samples (Panel A and B). We did not note any significant changes in the levels of C/EBPδ expression as induced by IL-1, compared to controls (Panel A and B). The constant expression of β-2-microglobulin in all samples suggests that none of the siRNAs used in these transfections were affecting gene expression at a global level, further indicating the aforementioned results were specific. These results were confirmed with the use of both siRNA duplexes provided by Invitrogen targeting STAT1 and STAT3 (Table 2.2).

### 6.3.2 EFFECT OF SILENCING STAT1 AND STAT3 ON THE IL-6- AND IL-1-MEDIATED EXPRESSION OF C/EBPδ PROTEIN LEVELS

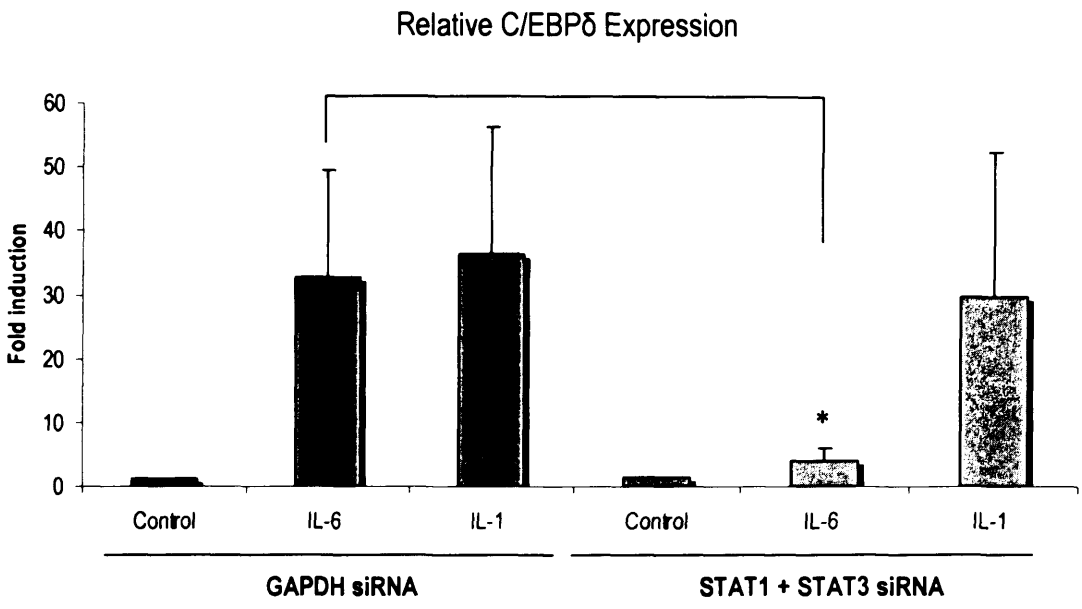
We next wanted to determine if the results presented in Figure 6.13, were mirrored at the level of C/EBPδ protein expression. For this, the experiment above was repeated but samples were harvested for protein extraction as described before and used for subsequent western blot analysis. Antibodies for the detection of C/EBPδ and β-actin were used as described previously for the detection of the respective proteins. The details for immunodetection of total STAT1 and STAT3 proteins are detailed in Table 2.9. The results are presented in Figure 6.14.

**FIGURE 6.14. Effect of simultaneously silencing STAT1 and STAT3 on IL-6-and IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were either transiently transfected with siRNA directed against GAPDH (12.5nM) or co-transfected with siRNA directed against STAT1 and STAT3 (6.25nM of each siRNA) as described in Figure 6.13. The cells were either treated with IL-6, IL-1 or left untreated for 3h exactly as described above. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using antibodies against C/EBPδ, total STAT1, total STAT3 and β-actin for immunodetection. Western blot analysis was carried out by SDS-PAGE using 7.5% (w/v) gels, for subsequent immunoblotting using antibodies specific for STAT1 and STAT3, and for C/EBPδ, as described previously. For β-actin detection, the western blot used to detect C/EBPδ was re-probed with the anti-β-actin antibody. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the untreated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratios of total STAT1:β-actin and total STAT3:β-actin, were determined for the specified samples and the % reduction in their expression was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. Results in Panel B are displayed as the mean ratio $\pm$SD from three independent experiments (*$P<0.05$). From two of these three independent experiments, the same samples were also used for the immunodetection of STAT1 and STAT3. Therefore, data in Panel C are shown as the mean ratios of two independent experiments.

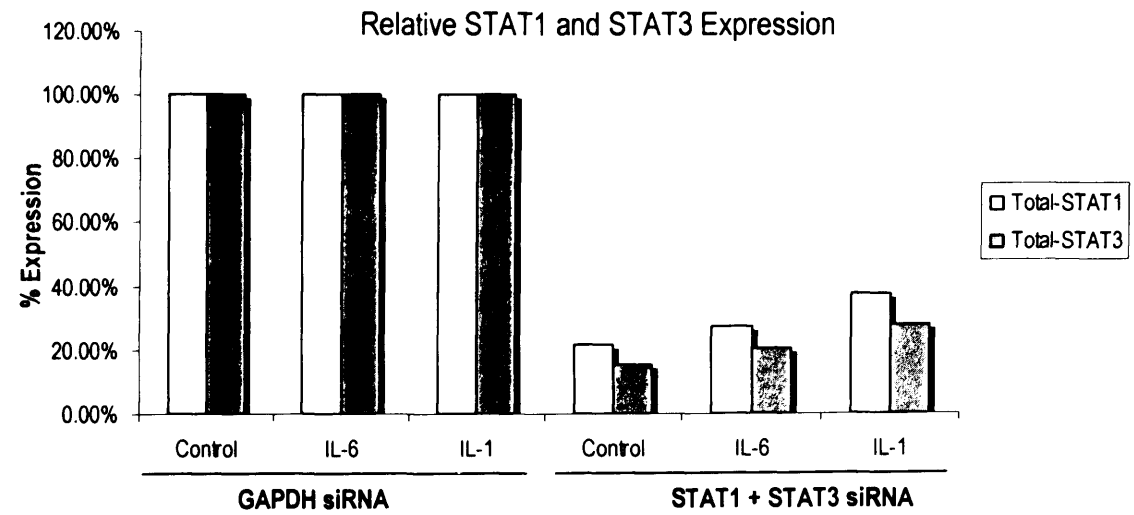
A.


siRNA
GAPDH
STAT1 and STAT3
IL-6 (1000U/ml) - + - - + -
IL-1 (100U/ml) - - + - - +
C/EBPδ
Total-STAT1
Total-STAT3
β-actin

B.


Relative C/EBPδ Expression
Fold induction
60
50
40
30
20
10
0
*
Control
IL-6
IL-1
Control
IL-6
IL-1
GAPDH siRNA
STAT1 + STAT3 siRNA

C.

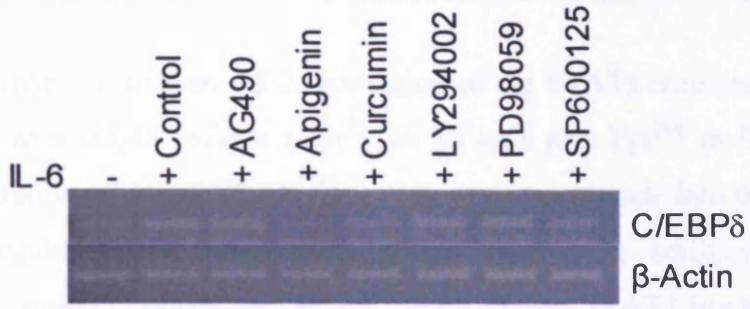

Relative STAT1 and STAT3 Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Total-STAT1
Total-STAT3
Control
IL-6
IL-1
Control
IL-6
IL-1
GAPDH siRNA
STAT1 + STAT3 siRNA

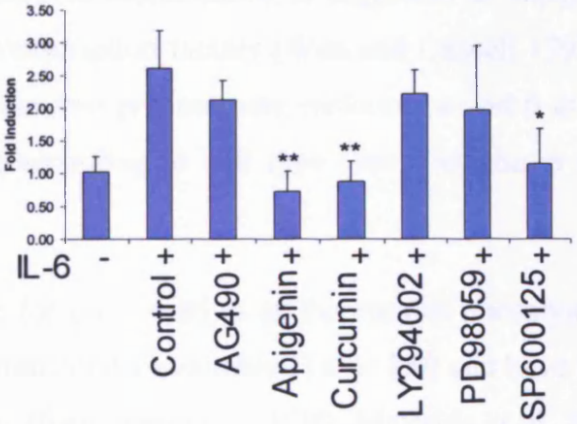
The data presented in Figure 6.14 are consistent with the RT-PCR results presented in Figure 6.13. In essence, siRNA-mediated depletion of STAT1 and STAT3 proteins in Hep3B cells significantly attenuates the IL-6-induced expression of C/EBPδ protein compared to controls. As before, the IL-1 response was unaffected by STAT1 and STAT3 depletion. Our results strongly suggest that the IL-6-mediated expression of C/EBPδ is regulated by the STAT transcription factors in Hep3B cells.

Previous data (see Figure 6.15) from our laboratory, suggested a role of CK2 and JNK MAPK in the regulation of IL-6-mediated induction of C/EBPδ, as the pharmacological inhibitors apigenin, curcumin and SP600125 significantly impaired this response. Therefore, in light of the findings presented in Figures 6.13 and 6.14, we hypothesised that CK2 and JNK MAPK may be regulating STAT activation in response to IL-6. A series of experiments were conducted to test our hypothesis.

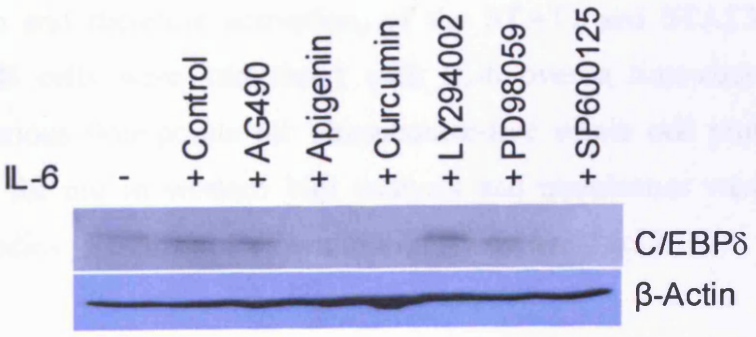
**FIGURE 6.15. Effect of pharmacological inhibitors on the induction of C/EBPδ expression as mediated by IL-6.** ***Data and figure courtesy of Dr S. A. Rogers.*** Hep3B cells were pre-treated with pharmacological inhibitors [AG490 (100μM), Apigenin (50μM), Curcumin (30μM), LY294002 (20 μM), PD98059 (50 μM) and SP600125 (50 μM)] or with DMSO as a vehicle control for 1h. They were then either treated with IL-6 (1000U/ml) or left untreated for 3h. Total RNA was extracted and used for RT-PCR analysis (A). Alternatively, cells were harvested for protein extraction and samples were used for western blot analysis (B). Experiments were carried out at least three times. *P<0.05, **P<0.005.

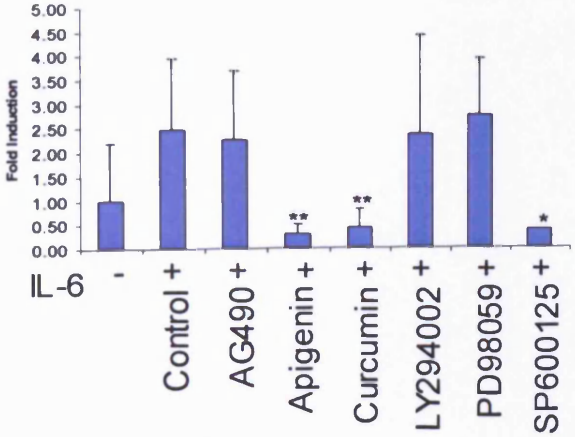
(A)


IL-6
-
+ Control
+ AG490
+ Apigenin
+ Curcumin
+ LY294002
+ PD98059
+ SP600125
C/EBPδ
β-Actin


Fold Induction
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
**
*
IL-6
-
Control +
AG490 +
Apigenin +
Curcumin +
LY294002 +
PD98059 +
SP600125 +

(B)


IL-6
-
+ Control
+ AG490
+ Apigenin
+ Curcumin
+ LY294002
+ PD98059
+ SP600125
C/EBPδ
β-Actin


Fold Induction
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
**
**
*
IL-6
-
Control +
AG490 +
Apigenin +
Curcumin +
LY294002 +
PD98059 +
SP600125 +

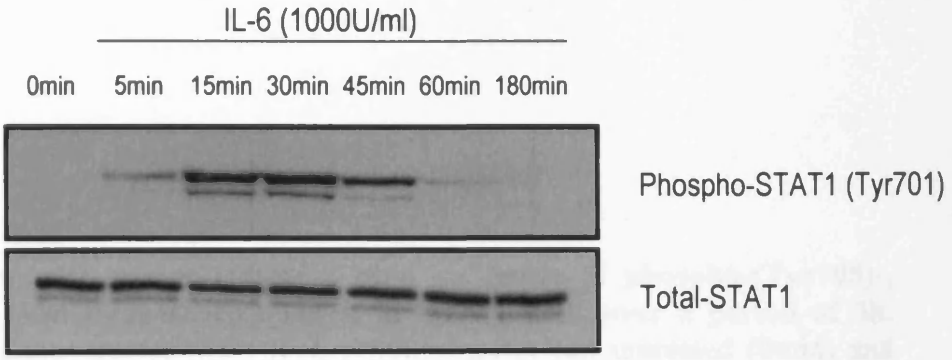
### 6.3.3 EFFECT OF IL-6 ON STAT1 AND STAT3 PHOSPHORYLATION IN HEP3B CELLS

As discussed in detail in section 1.5.2, activation of the STATs requires their tyrosine phosphorylation at a single residue ($Tyr^{701}$ in STAT1 and $Tyr^{705}$ in STAT3). This leads to the formation of active STAT dimers that can translocate into the nucleus and subsequently regulate gene expression accordingly. In addition to tyrosine phosphorylation, $serine^{727}$ phosphorylation of STAT1 and STAT3 has been described and this post-translational modification is suggested to maximise the *trans*-activation potential of these transcription factors (Wen and Darnell 1997; Heinrich *et al.* 2003). STAT1 and 3 exist as two predominate isoforms, α and β in cells, with ratios of α:β expression varying according to cell type (see Biethahn *et al.* 1999 and references therein ).

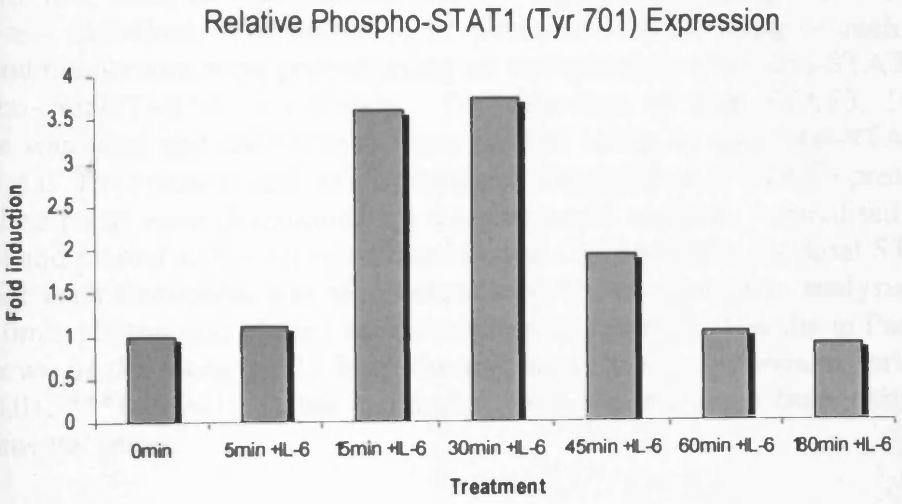
Antibodies specific for the detection of the various phosphorylated forms of STAT1 and STAT3 are commercially available (Table 2.0) and have been used extensively in a number of studies (Bromberg *et al.* 1999; McBride *et al.* 2000; Mitsui *et al.* 2003) and in the Hep3B cell line (Sehgal *et al.* 2003). Therefore, initial experiments were conducted to determine whether IL-6 stimulation of Hep3B cells resulted in the phosphorylation and therefore activation, of the STAT1 and STAT3 over a time-course. Hep3B cells were stimulated with IL-6 over a time-course of 3h and harvested at various time-points for phosphatase-free whole cell protein extraction (Section 2.7.2) for use in western blot analysis and membranes were probed with indicated antibodies. Results are shown in Figures 6.16 and 6.17.

**FIGURE 6.16. Time dependent effect of IL-6 on levels of phospho-(Tyr701)- and total-STAT1 levels in Hep3B cells over a period of 3h.** Hep3B cells were either treated with IL-6 (1000U/ml) or left untreated (0min) and harvested for phosphatase-free protein extraction at each of the indicated time-points. Western blot analysis was carried out by SDS-PAGE using 7.5% (w/v) gels as previously described. For detection of phospho-STAT1, 40μg of each sample was used and membranes were probed with an anti-phospho-(Tyr701)-STAT1 antibody. For detection of total-STAT1, 10μg of each sample was used and membranes were probed using an anti-total-STAT1 antibody (Panel A). The tyrosine phosphorylated STAT1 protein level for each time-point was determined by densitometric analysis, normalised to the 0min control and plotted as a histogram (Panel B). Similarly, the total STAT1 protein level for each time-point was also determined by densitometric analysis, normalised to the 0min control and plotted as a histogram in Panel C. The results are representative of two independent experimental series.

A.


IL-6 (1000U/ml)
0min 5min 15min 30min 45min 60min 180min
Phospho-STAT1 (Tyr701)
Total-STAT1

B.


Relative Phospho-STAT1 (Tyr 701) Expression
Fold induction
4
3.5
3
2.5
2
1.5
1
0.5
0
0min
5min +IL-6
15min +IL-6
30min +IL-6
45min +IL-6
60min +IL-6
180min +IL-6
Treatment

C.

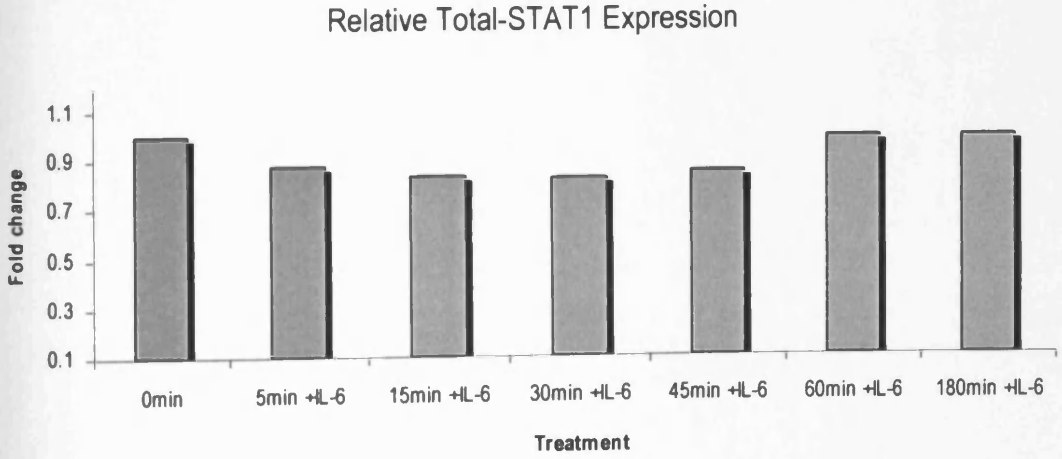
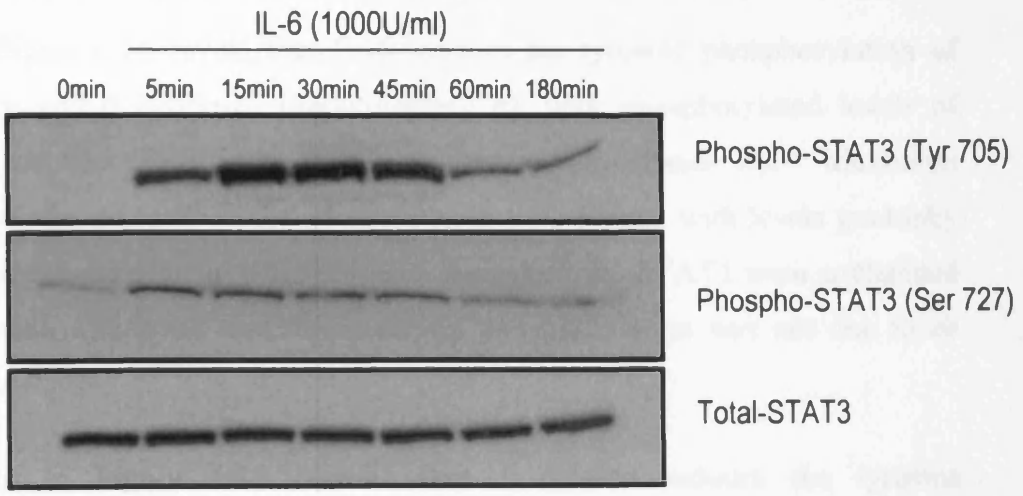
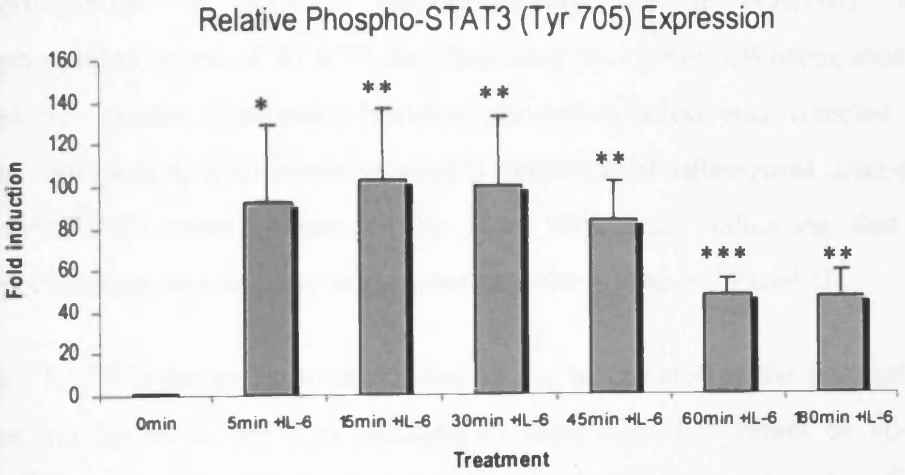

Relative Total-STAT1 Expression
Fold change
1.1
0.9
0.7
0.5
0.3
0.1
0min
5min +IL-6
15min +IL-6
30min +IL-6
45min +IL-6
60min +IL-6
180min +IL-6
Treatment

**FIGURE 6.17. Time dependent effect of IL-6 on levels of phospho-(Tyr705)-, phospho-(Ser727)- and total-STAT3 levels in Hep3B cells over a period of 3h.** Hep3B cells were either treated with IL-6 (1000U/ml) or left untreated (0min) and harvested for phosphatase-free protein extraction at each of the indicated time-points. Western blot analysis was carried out by SDS-PAGE using 7.5% (w/v) gels as previously described. For detection of phospho-STAT3, 40μg of each sample was used and membranes were probed using an anti-phospho-(Tyr705)-STAT3 or an anti-phospho-(Ser727)-STAT3 antibody. For detection of total-STAT3, 10μg of each sample was used and membranes were probed using an anti-total-STAT3 antibody (Panel A). The tyrosine and serine phosphorylated forms of STAT3 protein level, for each time-point were determined by densitometric analysis, normalised to the 0min control and plotted as histograms (Panel B and C). Similarly, the total STAT3 protein level for each time-point was also determined by densitometric analysis, normalised to the 0min control and plotted as a histogram in Panel D. Results in Panels B and D are shown as the mean ± SD from three independent experimental series (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Data in Panel C is the mean value from two independent experimental series.

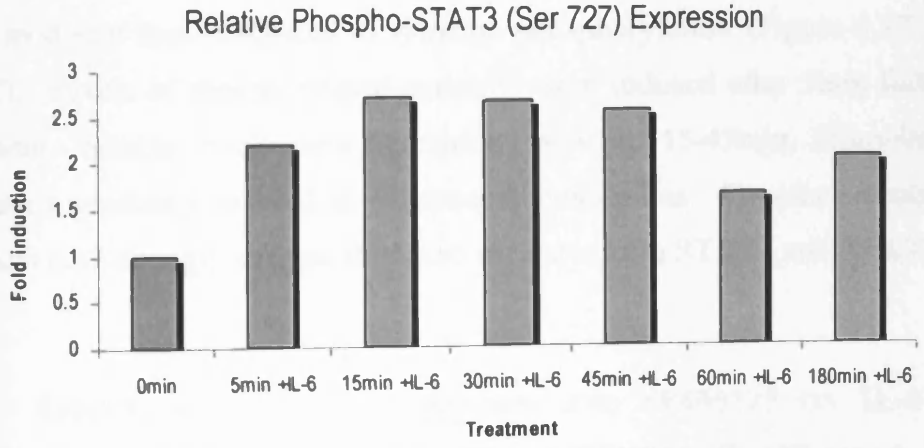
A.


IL-6 (1000U/ml)
0min 5min 15min 30min 45min 60min 180min
Phospho-STAT3 (Tyr 705)
Phospho-STAT3 (Ser 727)
Total-STAT3

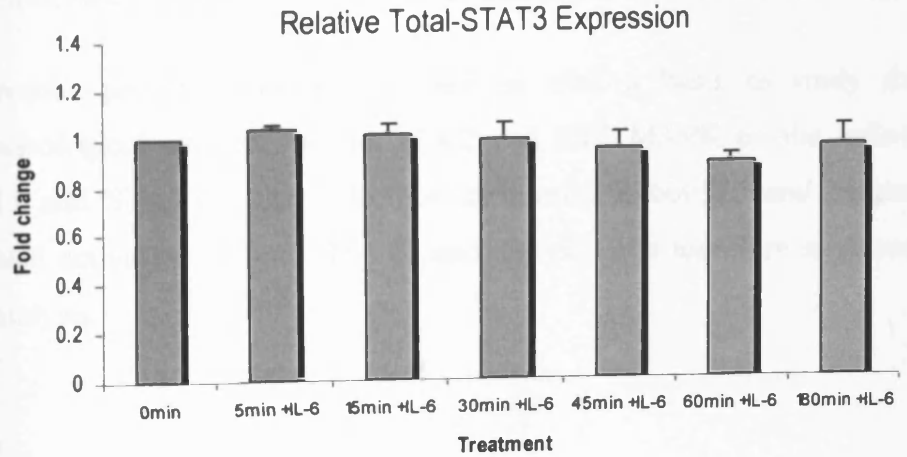
B.


Relative Phospho-STAT3 (Tyr 705) Expression
Fold induction
160
140
120
100
80
60
40
20
0
*
**
**
**
***
**
0min
5min +IL-6
15min +IL-6
30min +IL-6
45min +IL-6
60min +IL-6
180min +IL-6
Treatment

C.


Relative Phospho-STAT3 (Ser 727) Expression
Fold induction
3
2.5
2
1.5
1
0.5
0
0min
5min +IL-6
15min +IL-6
30min +IL-6
45min +IL-6
60min +IL-6
180min +IL-6
Treatment

D.


Relative Total-STAT3 Expression
Fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0min
5min +IL-6
15min +IL-6
30min +IL-6
45min +IL-6
60min +IL-6
180min +IL-6
Treatment

Results from Figure 6.16 reveal that IL-6 induces the tyrosine phosphorylation of STAT1 (both α and β isoforms, predominately α), with phosphorylated levels of STAT1 detected after just 5min following stimulation (Panel A). Maximum phosphorylation was detected at the 15 and 30min time-points, with levels gradually returning to basal at subsequent time-points. Levels of total-STAT1 were unchanged by IL-6 treatment, indicating that the observed phosphorylation was not due to *de novo* protein synthesis.

Similarly, data in Figure 6.17 reveals that IL-6 also induces the tyrosine phsophorylation of STAT3 (predominately the α isoform), with tyrosine phosphorylated levels of STAT3 detected after just 5min following stimulation (Panel A and B). Again, maximum tyrosine phosphorylation was detected at the 15 and 30min time-points, with levels gradually declining at subsequent time-points. Levels of total-STAT3 were unchanged by IL-6 treatment, indicating that the observed phosphorylation was not due to *de novo* protein synthesis (Panel D).

Since STAT3 is the major transcription factor implicated in the regulation of C/EBPδ expression by IL-6, we also decided to determine the effect of IL-6 on STAT3 serine$^{727}$ phosphorylation. IL-6 also induced STAT3 serine phosphorylation, although more modestly than compared to tyrosine phosphorylation (Figure 6.17 Panel A, B and C). Levels of phosphorylated serine$^{727}$ were induced after 5min following IL-6 treatment. Similar levels were maintained between 15-45min, following treatment and levels gradually reduced at subsequent time-points. Together results in Figure 6.16 and 6.17 strongly suggest that IL-6 activates both STAT1 and STAT3 in Hep3B cells.

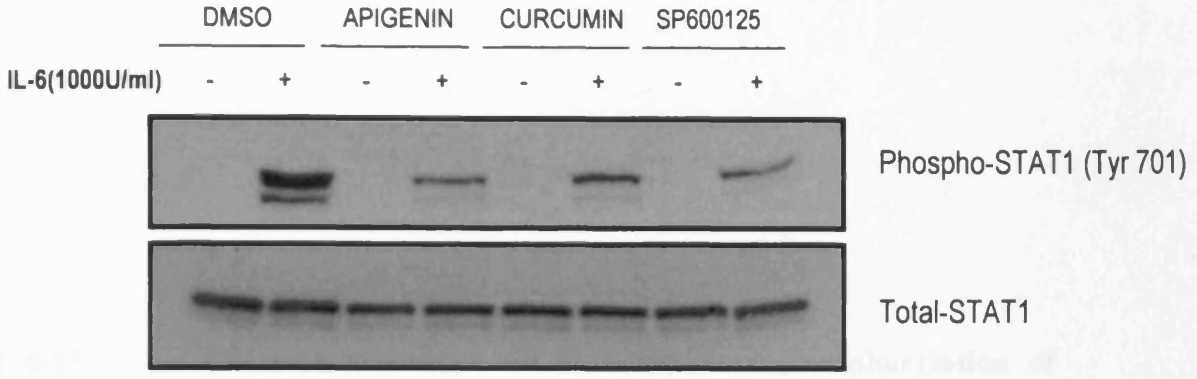
### 6.3.4 EFFECT OF APIGENIN, CURCUMIN AND SP600125 ON IL-6-MEDIATED TYROSINE PHOSPHORYLATION OF STAT1 AND STAT3 IN HEP3B CELLS

The results presented above provided us with a basis to study the effects of pharmacological inhibitors against CK2 and JNK MAPK on the activation of both STAT1 and STAT3. The effect of curcumin, SP600125 and apigenin on IL-6-mediated activation of both STAT1 and STAT3 was therefore analysed by western blot analysis.

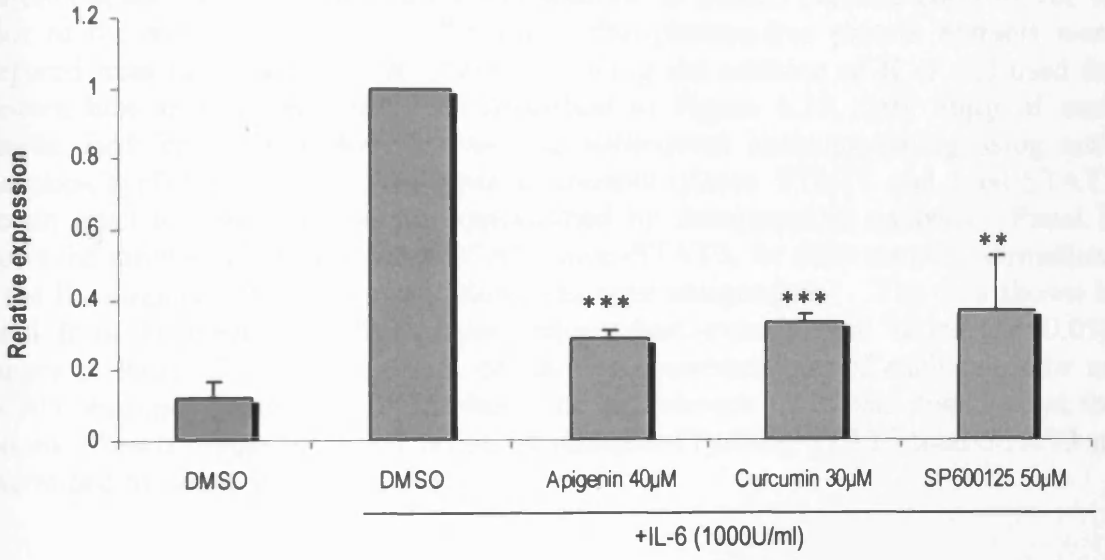
Hep3B cells were pre-treated with inhibitors at various concentrations or vehicle for 1h at which point they were treated with IL-6 appropriately and harvested for phosphatase-free protein extraction 15min following stimulation. The cells were harvested at this time-point because it is one of the time-points at which IL-6-mediated phosphorylation of STAT1 and STAT3 is maximal. Samples were used for western blot analysis as described in the above sections, using antibodies to detect tyrosine phosphorylated forms of STAT1 and STAT3 and total levels of these proteins. Results are presented in Figures 6.18 and 6.19.

**FIGURE 6.18. The effect of inhibitors on IL-6-mediated phosphorylation of STAT1 (Tyr701) in Hep3B cells.** Hep3B cells were treated with the indicated inhibitor at the specified concentration in addition to DMSO (vehicle-control), for 1h prior to the addition of IL-6 (1000U/ml). Phosphatase-free protein extracts were prepared from cells harvested at 15min following the addition of IL-6 and used for western blot analysis as described in Figure 6.16 (Panels A and C). The tyrosine-phosphorylated STAT1 and total-STAT1 protein level for each sample was determined by densitometric analysis. Panel B shows the ratios of phosphorylated-STAT1:total-STAT1, for each sample, normalised to the IL-6-treated DMSO control, which has been assigned as 1. The data shown in Panel B is the mean $\pm$SD from three independent experimental series (**P<0.01, ***P<0.001). Images in Panel C show the effect of various concentrations of each inhibitor on Tyr701 phosphorylation of STAT1, from one experiment.

**A.**


DMSO
APIGENIN
CURCUMIN
SP600125
IL-6(1000U/ml) - + - + - + - +
Phospho-STAT1 (Tyr 701)
Total-STAT1

**B.**


Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
***
***
**
DMSO
DMSO
Apigenin 40μM
Curcumin 30μM
SP600125 50μM
+IL-6 (1000U/ml)

**C.**

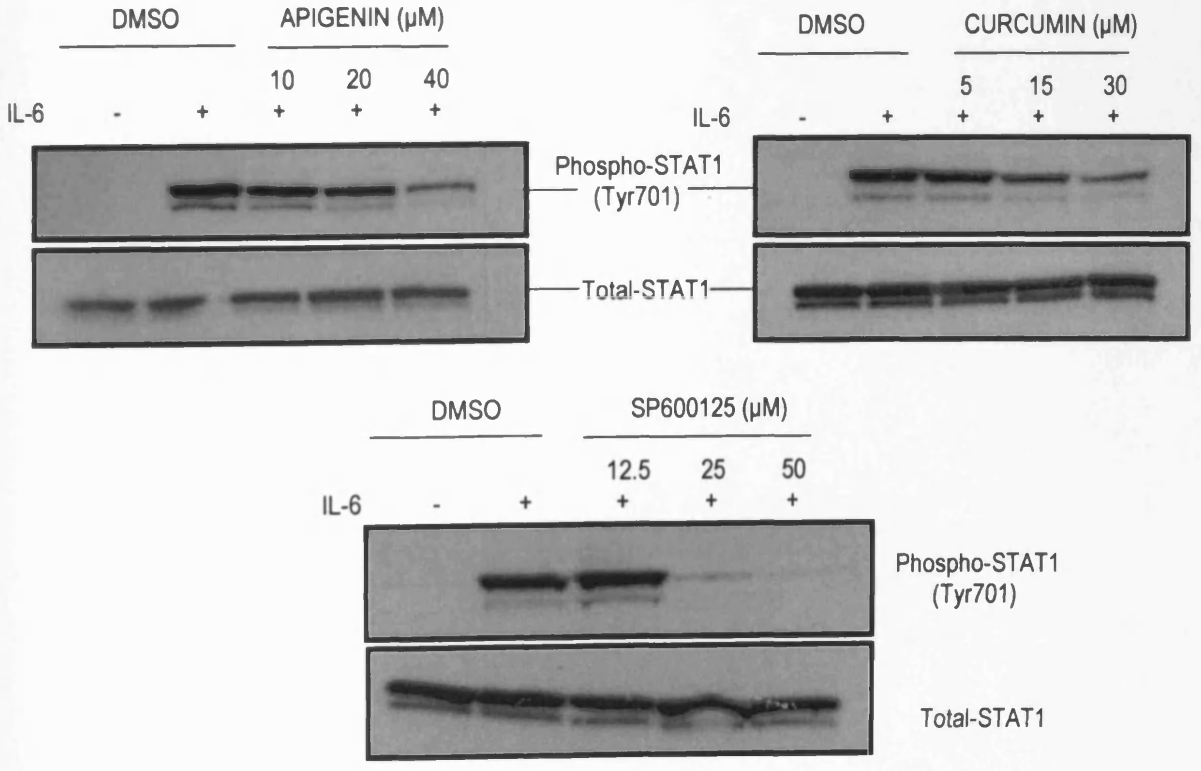
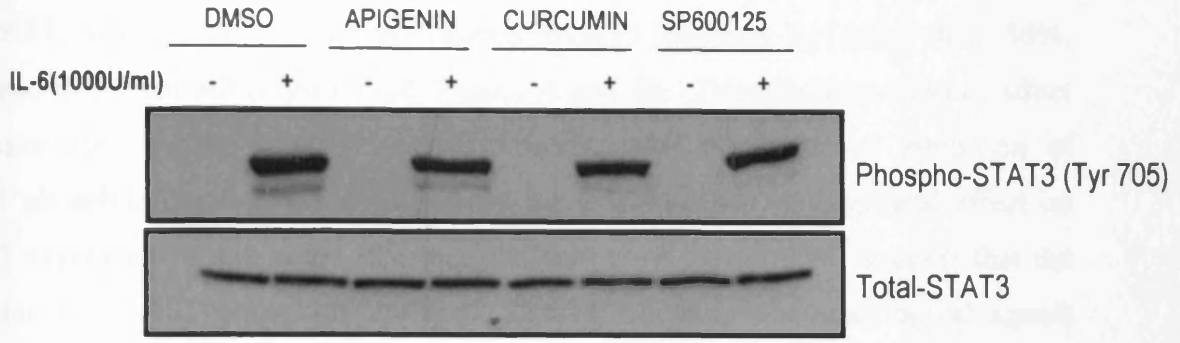
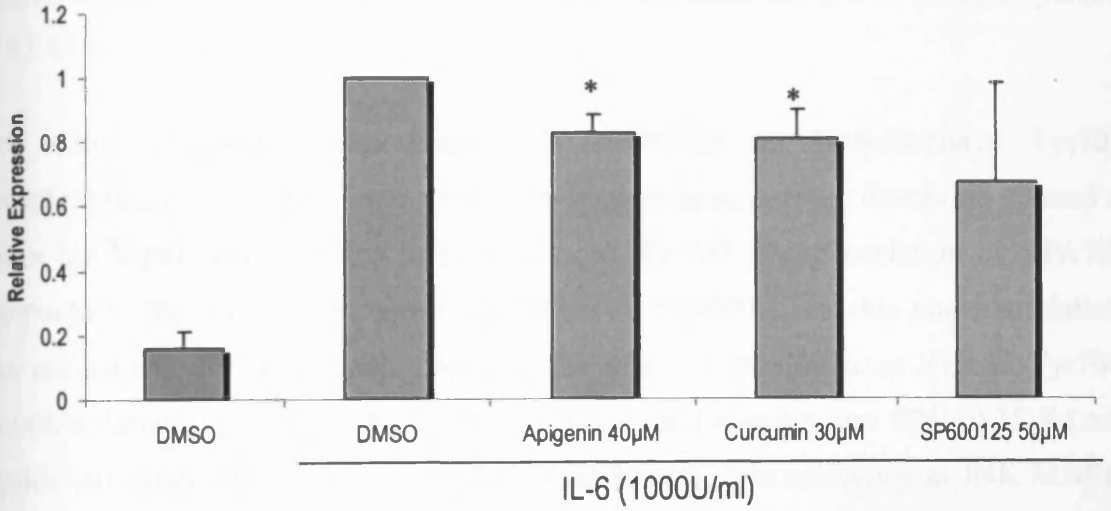

DMSO
APIGENIN (μM)
10 20 40
IL-6 - + + + +
DMSO
CURCUMIN (μM)
5 15 30
IL-6 - + + + +
Phospho-STAT1 (Tyr701)
Total-STAT1
DMSO
SP600125 (μM)
12.5 25 50
IL-6 - + + + +
Phospho-STAT1 (Tyr701)
Total-STAT1

**FIGURE 6.19. The effect of inhibitors on IL-6-mediated phosphorylation of STAT3 (Tyr705) in Hep3B cells.** Hep3B cells were treated with the indicated inhibitor at the specified concentration in addition to DMSO (vehicle-control), for 1h prior to the addition of IL-6 (1000U/ml). Phosphatase-free protein extracts were prepared from cells harvested at 15min following the addition of IL-6 and used for western blot analysis essentially as described in Figure 6.17, with 20μg of each sample used for western blot analysis and subsequent immunoblotting using anti-phospho-(Tyr705)-STAT3. The tyrosine-phosphorylated STAT3 and total-STAT3 protein level for each sample was determined by densitometric analysis. Panel B shows the ratios of phosphorylated-STAT3:total-STAT3, for each sample, normalised to the IL-6-treated DMSO control, which has been assigned as 1. The data shown in Panel B is the mean ±SD from three independent experimental series (*$P<0.05$). Images in Panel C show the effect of various concentrations of each inhibitor on Tyr705 phosphorylation of STAT3, from one experiment. Numbers displayed at the bottom of each image represent ratios of phospho-(Tyr705)-STAT3:total-STAT3 as determined by densitometric analysis.

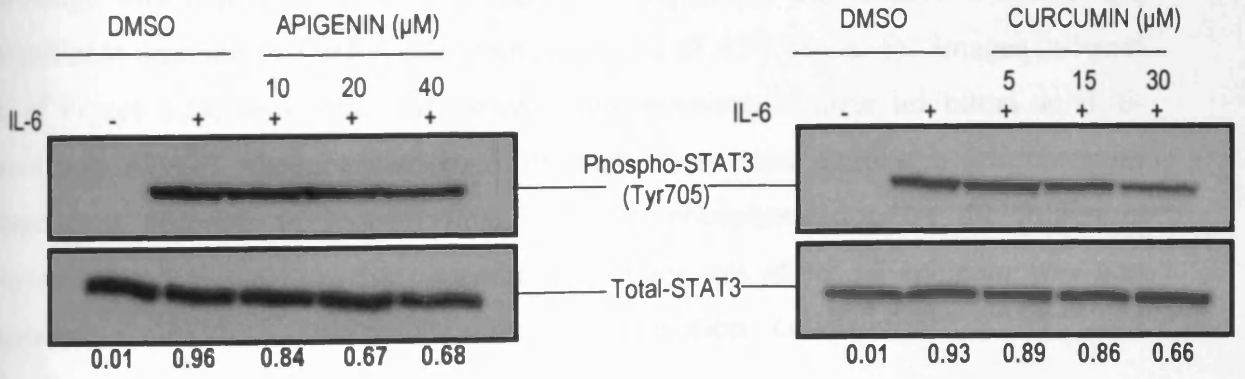
A.

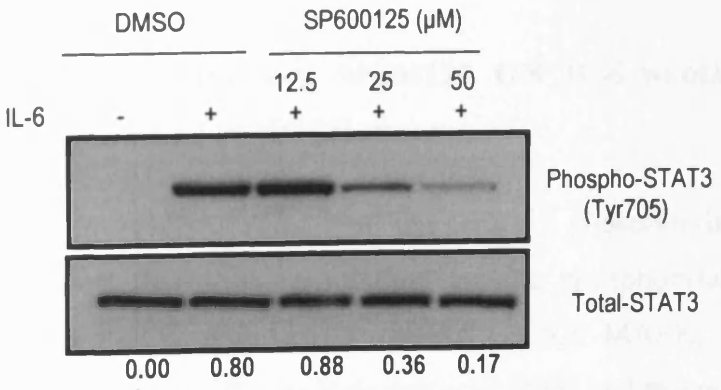

DMSO
APIGENIN
CURCUMIN
SP600125
IL-6(1000U/ml) - + - + - + - +
Phospho-STAT3 (Tyr 705)
Total-STAT3

B.


Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
DMSO
DMSO
Apigenin 40μM
Curcumin 30μM
SP600125 50μM
IL-6 (1000U/ml)

C.


DMSO
APIGENIN (μM)
10 20 40
IL-6 - + + + +
Phospho-STAT3 (Tyr705)
Total-STAT3
0.01 0.96 0.84 0.67 0.68
DMSO
CURCUMIN (μM)
5 15 30
IL-6 - + + + +
0.01 0.93 0.89 0.86 0.66


DMSO
SP600125 (μM)
12.5 25 50
IL-6 - + + + +
Phospho-STAT3 (Tyr705)
Total-STAT3
0.00 0.80 0.88 0.36 0.17

All three inhibitors significantly inhibited the IL-6-mediated Tyr701 phosphorylation of STAT1, with levels of phosphorylated STAT1 reduced by more than 50%, compared to the control (Figure 6.18, Panels A and B). The inhibitors did not affect the expression of total-STAT1 levels, indicating that the observed inhibition of Tyr701 phosphorylation was not caused by the inhibitors having a general effect on STAT1 expression in the cells. Images in Panel C of Figure 6.18, suggest that the inhibition of Tyr701 phosphorylation of STAT1 by these pharmacological agents occurs in a concentration dependent manner. Results presented here potentially suggest a role for CK2 and JNK MAPK in the regulation of Tyr701 phosphorylation of STAT1 (by IL-6) in Hep3B cells.

The effect of apigenin, curcumin and SP600125 on IL-6-mediated Tyr705 phosphorylation of STAT3 was next examined (Figure 6.19). Curcumin caused a minor but significant decrease in IL-6-induced Tyr705 phosphorylation of STAT3, compared to the control. However, the effect of SP600125 on this phosphorylation was not statistically significant. Because the effect of curcumin on STAT3-Tyr705 phosphorylation (as mediated by IL-6) was minor, and also because SP600125 did not significantly attenuate this phosphorylation (at 50μM), it is unlikely that JNK MAPK plays a major role in regulating STAT3-Tyr705 phosphorylation as induced by IL-6, although this requires further investigation. Apigenin also caused a minor and significant decrease in Tyr705 phosphorylation of STAT3 (by IL-6). Images in Panel C of Figure 6.19, show effect of various concentrations of these inhibitors on IL-6-mediated STAT3 phosphorylation. Although these data suggest a concentration dependent decrease in IL-6-mediated Tyr705 phosphorylation by the inhibitors curcumin and SP600125, the concentration-dependent effect of apigenin was less apparent, therefore all these results require confirmation. Levels of total-STAT3 were unaffected in the presence of all three inhibitors.

### 6.3.5 EFFECT OF APIGENIN, CURCUMIN AND SP600125 ON IL-6-MEDIATED SERINE727 PHOSPHORYLATION OF STAT3 IN HEP3B CELLS

The mechanisms and/or kinases involved in regulating the serine$^{727}$ phosphorylation of STATs are less well understood than those regulating tyrosine phosphorylation. Evidence for the involvement of PKCδ, p38 MAPK, MEKK1, ERK MAPK, JNK MAPK, Ca2+/calmodulin dependent kinase II (see Heinrich *et al.* 2003 and references

therein) and CK2 (Timofeeva *et al.* 2006) have been reported. IL-6-mediated STAT3 serine$^{727}$ phosphorylation and *trans*-activation is dependent on the sequential activation of Rac-1, MKK4/SEK1 and PKCδ in HepG2 cells (Schuringa *et al.* 2001).

Because some studies have suggested that serine$^{727}$ phosphorylation of STAT3 may positively regulate its *trans*-activation potential (Wen and Darnell 1997; Schuringa *et al.* 2001) and because a number of different kinases, including JNK MAPK (Lim and Cao 1999) and CK2 (Timofeeva *et al.* 2006) have been suggested to regulate serine$^{727}$ phosphorylation of STAT3 and STAT1 respectively, we sought to determine a potential role for these kinases in the regulation of serine$^{727}$ phosphorylation of STAT3 in Hep3B cells. We have already shown that IL-6 induces the phosphorylation of STAT3 at serine$^{727}$ (Figure 6.17) in these cells. Therefore, protein extracts used for experiments presented in Figure 6.19, were also used to assess the effect of apigenin, curcumin and SP600125 on this phosphorylation. Results are presented in Figure 6.20 below.

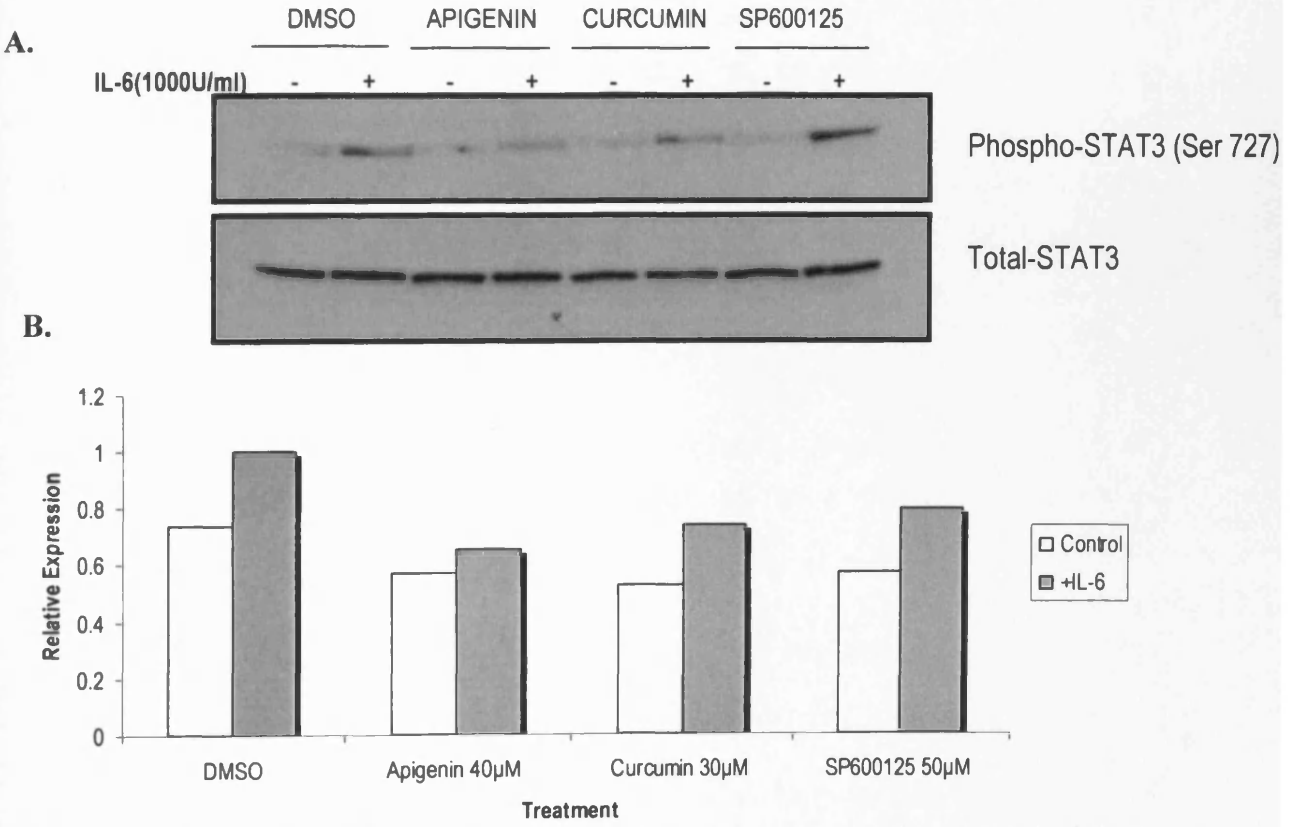



**FIGURE 6.20. The effect of inhibitors on IL-6-mediated phosphorylation of STAT3 (Ser727) in Hep3B cells.** Phosphatase-free protein extracts used for experiments presented in Figure 6.19, were used for western blot analysis and subsequent immunoblotting using the anti-phospho-(Ser727)-STAT3 antibody. Total-STAT3 levels from experiments presented in Figure 6.19 (Panel A) were used as controls. The serine-phosphorylated STAT3 and total-STAT3 protein level for each sample was determined by densitometric analysis. Panel B shows the ratios of serine-phosphorylated-STAT3:total-STAT3, for each sample, normalised to the IL-6-treated DMSO control, which has been assigned as 1. Results are representative of two independent experiments.

A modest induction of serine$^{727}$ phosphorylation as mediated by IL-6 was observed, consistent with previously presented data (Figure 6.17). This induction was, at least in part, inhibited by all the pharmacological agents examined, although the inhibition was less apparent with the inhibitor SP600125. Nevertheless, densitometric analysis of the two datasets, suggested that all three inhibitors may inhibit this phosphorylation. These preliminary data suggest that CK2 and JNK MAPK may contribute, to some extent, to the serine$^{727}$ phosphorylation of STAT3, although these results require further confirmation. These results together with all the data presented in this chapter are discussed in detail in section 6.4.

## 6.4 DISCUSSION

RNAi mediated knock-down of gene expression has been successfully applied as a tool for pathway dissection in an ever growing list of studies and as mentioned in section 6.1 has allowed researchers to overcome barriers associated with genetic redundancy. In some cases its application has also helped resolve controversial issues in a particular area of research. For example, insulin signalling is reported to be principally regulated through activation of the PI3K and subsequent activation of its downstream effector, Akt/PKB (see Jiang *et al.* 2003 and references therein). However, the role of Akt in insulin signalling is debatable. Mice lacking the Akt1 isoform have normal reactions to insulin (Cho *et al.* 2001a) and skeletal muscle from mice deficient in Akt2 (the predominate isoform expressed in muscle and fat), is only marginally less sensitive to low levels of the hormone, compared to controls. Furthermore, insulin sensitivity is restored in these cells exposed to higher dose of the hormone (Cho *et al.* 2001b). Genetic knock-out of both Akt1 and Akt2 genes in mice is lethal. However, RNAi-mediated knock-down of Akt1 and/or Akt2 gene expression in cultured adipocytes helped elucidate a role for both Akt isoforms during insulin signalling. In addition, the same investigation revealed that some functional redundancy existed between the two genes, results that were only revealed with the simultaneous siRNA-mediated knock-down of Akt1 and Akt2 gene expression (Jiang *et al.* 2003). The results from this study also provided evidence to potentially explain previously reported contradictory data.

Given the success of this application in scientific research we decided to use RNAi as a technique to investigate potential pathways by which the cytokines IL-1 and IL-6 regulate C/EBPδ expression.

### 6.4.1 CK2 AND JNK MAPK PATHWAY IN THE REGULATION OF C/EBPδ EXPRESSION BY IL-1

Results presented in the previous chapter suggest a role for the JNK MAPK during IL-1 signalling in Hep3B cells, owing to the activation of this kinase in response to this cytokine. In relation to the expression of C/EBPδ by IL-1, dominant-negative mutants of JNK and c-Jun (a downstream effector of this pathway), transfected into Hep3B cells did not impair this response. Similarly, transfecting Hep3B cells with a

dominant negative mutant of CK2 also did not abrogate the response. We hypothesised that these results may suggest the existence of some functional redundancy between JNK and CK2 in the regulation of this response. With the use of siRNA we tested this hypothesis.

Initial experiments were carried out with the aim of investigating a role for either JNK MAPK or CK2 in the regulation of C/EBPδ expression by IL-1. siRNA targeting JNK was used to deplete levels of this kinase in Hep3B cells. In all our experiments (Figures 6.3-6.6), siRNA against JNK caused a dramatic decrease in the expression of this kinase. However, depleting levels of JNK did not result in a concomitant decrease of C/EBPδ expression as induced by IL-1 at the mRNA or protein level. In fact, results in Figures 6.4 and 6.6 suggest that depleting JNK actually enhanced the response, compared to controls. We next decided to target a more downstream component of the JNK pathway using siRNA. The expression of the transcription factor c-Jun was depleted using siRNA and the effect on the IL-1-induced expression of C/EBPδ was examined by western blot analysis (Figure 6.7). siRNA against c-Jun caused a dramatic decrease in its expression as expected. Despite the knock-down in its expression, levels of C/EBPδ induced by IL-1 were not decreased. As observed previously, with siRNA-mediated knock-down of JNK, the response was in fact enhanced slightly. These results could suggest that by interrupting the JNK MAPK pathway in Hep3B cells, other signalling pathway(s) are engaged to compensate for its loss and in turn these pathway(s) are able to restore the expression of C/EBPδ by IL-1. Indeed, a similar concept has been described by Mestre *et al.* (2001), as described in section 5.3.

We next decided to analyse the effect of siRNA-mediated depletion of CK2 expression on the IL-1-mediated induction of C/EBPδ expression. Targeting CK2α alone did not attenuate this response as determined by RT-PCR analysis (Figure 6.8). However, our data suggested that siRNA-mediated depletion of both CK2α and CK2α' caused a minor but significant decrease (~15%) in the IL-1-mediated induction of C/EBPδ expression compared to controls, at the mRNA level (Figure 6.9). To further investigate this result, we decided to determine the effect of siRNA-mediated depletion of CK2α and CK2α' on IL-1-mediated induction of C/EBPδ protein levels. We did not note any change in the levels of C/EBPδ protein as induced by IL-1,

compared to the control in this experiment (Figure 6.10). Therefore, further experiments would have to be carried out to fully investigate these findings. Importantly, this is necessary so as results from at least three independent experiments can be assessed for convincing statistical significance and therefore results from RT-PCR experiments can be compared to data from western blotting experiments.

To address the issue of functional redundancy, we next decided to target both JNK MAPK and CK2α using siRNA and investigate the effect of this on IL-1-mediated induction of C/EBPδ at the protein level. However, knocking-down the expression of both CK2α and JNK caused no apparent decrease in the expression of C/EBPδ as induced by IL-1 compared to the control (Figure 6.11). Ideally, knocking-down both CK2α and CK2α' in conjunction with JNK MAPK would be more appropriate to determine the existence of any functional redundancy between the two pathways. However, in our current transfection system this was not feasible, given the high concentration of siRNA required for the transfection and the risk of potential off-target effects caused by this (Cullen 2006). Also, given that the expression of three CK2α (CK2α, CK2α' and CK2α'') subunits has been described in the human hepatoma cell line, HuH-7 (Shi *et al.* 2001), the existence of a third CK2α isoform in the human hepatoma cell line, Hep3B cannot be ruled out. It is therefore tempting to speculate that by depleting the expression of CK2α and/or CK2α' (with RNAi), CK2 activity could be compensated by the third catalytic isoform CK2α''. Further investigations would have to be initiated in order to determine the expression levels of CK2α'' in Hep3B cells. Therefore, although our current data does not suggest that any functional redundancy exists between CK2 and JNK MAPK, in relation to the regulation of C/EBPδ expression by IL-1, further experiments would have to be carried out in order to fully examine this.

Another potential explanation for our results discussed in this section was suggested by Professor D.R. Alessi (University of Dundee), a leading expert in protein kinases. In a seminar presented at our university, Prof. Alessi mentioned that siRNA-mediated knock-down of kinases used for the purposes of pathway dissection did not always lead to interruption of the signalling pathway under examination. Because siRNA-mediated depletion of a target protein only leads to a knock-down of its expression, any remaining protein, escaping the silencing process (especially in the case of

kinases), can still function to compensate for its reduced expression, and in the case of cellular signalling, still activate the pathway.

A similar concept is described for recessive traits acquired by a Mendelian mode of inheritance. As an example, the genetic inheritance of Duchenne Muscular Dystrophy (DMD) is described. DMD is a debilitating genetic disease characterised by progressive muscle weakness caused by the inability of muscle fibres to produce the protein dystrophin. The dystrophin gene is located on the X chromosome making DMD an X-linked recessive disorder, usually affecting only males (Hoffman *et al.* 1987; Koenig *et al.* 1987). Because females have two copies of the X chromosome, a woman who has one correct copy of the dystrophin gene and one mutated copy of the same gene (heterozygote or carrier), can nearly always produce enough dystrophin to have normal muscle function. This is despite the fact she can only produce 50% of the wild-type protein compared to a non carrier. In essence, due to random X-chromosome inactivation, 50% of her muscle fibres are able to produce the wild-type dystrophin protein, whereas the other 50% are not (they produce the mutated protein). The presence of only half the amount of wild-type protein produced by a carrier compared to a non carrier can compensate for the missing 50% of the protein (information regarding genetic inheritance of DMD retrieved from OMIM(TM), Online Mendelian Inheritance in Man at http://www.ncbi.nlm.nih.gov/omim and the Centre of Genetics Education at http://www.genetics.com.au).

Furthermore, some studies have shown that although in homozygous gene knock-out mouse models, disruption/deletion of a target gene results in the manifestation of an abnormal phenotype, the heterozygous gene knock-out counterparts have a normal phenotype. For example, this is the case for the mouse knock-out model for the insulin receptor gene (IR). IR-/- mice display major metabolic defects, such as severe diabetes and hepatic steatosis resulting from abnormally high levels of triglycerides and free-fatty acids. These mice die within one week of birth as a result of these metabolic defects. However, the IR+/- mice do not show any major metabolic abnormalities and have normal glucose tolerance compared to control mice (Kadowaki 2000). Similarly, whereas knocking out the regulatory β subunit of CK2 in mice leads to early embryonic lethality, heterozygous CK2β ($CK2\beta^{+/-}$) knockout mice do not show an obvious phenotype. Interestingly, $CK2\beta^{+/-}$ embryonic stem cells

(ES) express a considerably lower level of CK2β than wild-type ES, whereas the level of CK2β in organs from heterozygous adult mice does not significantly differ from those of wild-type mice (Buchou *et al.* 2003; Blond *et al.* 2005). The results of such studies suggests that although heterozygous mice can technically only produce 50% of the wild-type protein, compared to controls, this amount is enough to compensate for the missing 50% of the protein.

Another possible explanation for our results is that the pharmacological agents used to identify CK2 and JNK MAPK as players in the regulation C/EBPδ expression by IL-1 (Chapter 4) could be inhibiting more than just CK2 and JNK MAPK in Hep3B cells. Indeed, both curcumin and apigenin have been described to inhibit NF-κB, albeit in some cases inhibition of NF-κB is secondary to inhibition of JNK MAPK and CK2 respectively, by these agents (Singh and Aggarwal 1995; Xu *et al.* 1997; Liang *et al.* 1999; Romieu-Mourez *et al.* 2001; Shukla and Gupta 2004a; Shukla and Gupta 2004b; Cho *et al.* 2007; Shakibaei *et al.* 2007). Preliminary data presented in chapter 4 suggests a role for NF-κB in the regulation of C/EBPδ expression by IL-1. Therefore the role of NF-κB in the regulation of this response is explored in detail in the following chapter.

### 6.4.2 ROLE OF STAT1 AND STAT3 IN THE REGULATION OF C/EBPδ EXPRESSION BY IL-6

Several studies have suggested a role for STAT3 in the regulation of C/EBPδ expression as mediated by IL-6 (Yamada *et al.* 1997; Cantwell *et al.* 1998; Sanford and DeWille 2005). We expanded on the findings of these studies and with the use of RNAi, report a role for the STATs in the regulation of endogenous C/EBPδ expression by IL-6, a novel finding. Our RNAi experiments (Figures 6.13 and 6.14) consistently show that simultaneous siRNA-mediated depletion of STAT1 and STAT3 abrogates the expression of C/EBPδ by IL-6, whereas the IL-1 response is unaffected. Further RNAi experiments carried out by Dr S.A. Rogers' (personal communication), suggest a predominant role of STAT3 over STAT1 in the regulation IL-6-mediated expression of C/EBPδ, consistent with previous findings (Cantwell *et al.* 1998). In support of our findings, we also show that IL-6 activates both STAT1 and STAT3 in stimulated Hep3B cells (Figures 6.16 and 6.17 respectively). Also consistent with previous reports (Heinrich *et al.* 2003; Sehgal *et al.* 2003) we show

that IL-6 activates STAT3 more efficiently than STAT1 and with slightly different kinetics.

As a potential role for JNK MAPK and CK2 had previously been suggested in the regulation of C/EBPδ expression by IL-6 (Figure 6.15), we carried out experiments to assess a role for these kinases in the activation of STAT1 and STAT3 as mediated by IL-6, with the use of pharmacological agents, in an effort to determine a mechanism of regulation. As shown in Figure 6.18, the CK2 inhibitor apigenin, and the two JNK MAPK inhibitors SP600125 and curcumin all significantly attenuated the Tyr701 phosphorylation and therefore activation of STAT1, as mediated by IL-6, without affecting the total levels of this transcription factor. Thus, these results potentially suggest a role for CK2 and JNK MAPK in the regulation of this response.

Similarly, in a separate study presently being carried out in our laboratory, SP600125 has been shown to inhibit the IFN-γ-mediated Tyr701 phosphorylation of STAT1, without affecting total-STAT1 levels in THP-1 macrophages (Na Li, personal communication). Although the effect of curcumin on this response has presently not been examined, these results could suggest a potential role for JNK MAPK in the regulation of both IL-6 and IFN-γ-mediated activation of STAT1, although further experiments would have to be carried out to determine this. In accordance with our results, curcumin is also reported to inhibit the oncostatin M (OSM)-mediated Tyr701 phosphorylation of STAT1 in both bovine and human chondrocytes (Li *et al.* 2001b). Oncostatin M is a member of the IL-6 superfamily of cytokines, and in chondrocytes activates the JAK/STAT signalling pathway by phosphorylation of JAK1, JAK2, JAK3 and STAT1. In addition, this cytokine also induces the activation of all three MAPKs, ERK1/2, p38 and JNK in these cells. Curcumin suppresses OSM-stimulated Tyr701 phosphorylation of STAT1, DNA-binding activity of STAT1 and in accordance with its role as a JNK MAPK inhibitor, OSM-mediated JNK MAPK phosphorylation. The specificity of this compound towards the inhibition of these two responses was confirmed as curcumin failed to inhibit OSM-mediated phosphorylation of JAK1, JAK2, JAK3, ERK1/2 and p38 (Li *et al.* 2001b). The mechanism by which curcumin specifically inhibited OSM-mediated Tyr701 phosphorylation of STAT1 was not reported in this study and unfortunately the effect of SP600125 on this phosphorylation was not examined. As curcumin also inhibited

the activation of JNK MAPK as mediated by OSM, the potential involvement of JNK in the regulation of OSM-mediated Tyr701 phosphorylation of STAT1 is entirely plausible.

Our data also suggests a possible role for CK2 in the regulation of Tyr701 phsophorylation of STAT1 as mediated by IL-6 (Figure 6.18). Judging from the present literature, there may be an emerging role for CK2 in the regulation of STAT1 activation (Harvey 2006; Timofeeva *et al.* 2006; Harvey *et al.* 2007). The IFN-γ-mediated phosphorylation of STAT1 (Ser727) is inhibited by the CK2 inhibitor apigenin, in J774.2 murine macrophages (Harvey 2006). However, no effect of this inhibitor on IFN-γ-stimulated phosphorylation of Tyr701 of STAT1 was noted in the same cells. In the Timofeeva *et al.* (2006) study, constitutively phosphorylated STAT1-Ser727 was inhibited by apigenin and another CK2 inhibitor, TBB (4, 5, 6, 7-tetrabromobenzotriazole), in cells derived from patients with Wilms' tumours and the human kidney cell line, HEK293T. The effect of these inhibitors on Tyr701 phosphorylation of STAT1 was not examined. The effect of apigenin on IFN-γ-mediated Tyr701 phosphorylation of STAT1 has also been examined in fibroblasts. However, apigenin did not attenuate this response, indicating CK2 may not be required in the regulation of this phosphorylation in this cell type (Higashi *et al.* 2003).

We also examined the effect of curcumin, SP600125 and apigenin on IL-6-mediated phosphorylation of STAT3 (Tyr705 and Ser727). Tyrosine phosphorylation of STAT3 (as induced by IL-6) was not significantly inhibited by SP600125 and only marginally inhibited by curcumin, indicating that JNK may not regulate this response in Hep3B cells (Figure 6.19). Apigenin caused a marginal but significant decrease in the phosphorylation of STAT3 (Tyr705) by IL-6, without affecting total levels of STAT3. However, the concentration-dependent effect of apigenin on this response requires confirmation. If the action of apigenin on this response is confirmed to act in concentration-dependent manner, the results could potentially indicate that CK2 may be required, at least in part, to regulate the response. In support of this possibility, the CK2 inhibitors K27 and TBB have been reported to inhibit both the Tyr and Ser phosphorylation of STAT3 as mediated by IL-6, in OPM2 cells, a multiple myeloma cell line. In addition, Tyr phosphorylation of STAT3 as mediated by IL-6, is also

inhibited in primary CD138+ cells extracted from multiple myeloma patients (Piazza *et al.* 2006). The possibility that these inhibitors were acting by inhibiting the activity of JAK tyrosine kinases was excluded by these authors because neither inhibitor is reported to affect the activation of a panel of tyrosine kinases tested (see Piazza *et al.* 2006 and references therein). Together, these findings support a novel role for CK2 in the regulation of STAT activation.

Because the activation of the STATs is best characterised by their tyrosine phosphorylation as mediated by the JAKs (JAK1-3 and Tyk2) during IFN-γ and IL-6 signalling (Leonard and O'Shea 1998; Heinrich *et al.* 2003), the role of other kinases, like JNK and CK2, in the regulation of this process is less well documented. Despite this, judging from the aforementioned publications, a role of JNK and CK2 in STAT activation is emerging and clearly warrants further investigation. In further support of this, a role for JNK in the regulation of EGF-stimulated Tyr705 phosphorylation of STAT3 has been reported (Lim and Cao 1999).

Given that STAT3 was the major transcription factor implicated in the regulation of C/EBPδ expression as induced by IL-6, we also decided to assess whether the pharmacological inhibitors apigenin, curcumin and SP600125 attenuated the IL-6-mediated Ser727 phosphorylation of STAT3. IL-6 induces the phosphorylation of this residue and this is believed to maximise the *trans*-activation potential of STAT3 in stimulated HepG2 cells (Schuringa *et al.* 2000; Schuringa *et al.* 2001). Components of the JNK MAPK pathway have been implicated in the regulation of STAT3 Ser727 phosphorylation (Lim and Cao 1999; Schuringa *et al.* 2000; Schuringa *et al.* 2001). Also, results from the Piazza *et al.* (2006) study suggest a role for CK2 in the regulation of IL-6-induced Ser727 phosphorylation of STAT3 in myeloma cells.

IL-6 induces the Ser727 phosphorylation of STAT3, in stimulated Hep3B cells (Figure 6.17). Our preliminary data suggests that there could be a potential role for CK2 in the regulation of Ser727 phosphorylation by IL-6, because apigenin attenuated this phosphorylation compared to controls (Figure 6.20). The effects of SP600125 and curcumin on this phosphorylation are more difficult to interpret and require further investigation.

The results presented in the aforementioned studies, including our own, are mainly generated with the use of pharmacological inhibitors. And although data generated with the use of pharmacological agents provides a good basis for study, further experiments are always required to back-up preliminary findings. Our results suggest that activation of STAT1 and in part, activation of STAT3 by IL-6 may be regulated by CK2 and JNK MAPK in Hep3B cells. These results warrant further investigation with alternative experimental approaches. Results generated from such studies could identify a potentially novel role for CK2 and JNK in STAT signalling, in relation to the IL-6-mediated induction of C/EBPδ expression.

In summary, RNAi data presented in this chapter suggests that CK2 and components of the JNK MAPK pathway may not be principle regulators modulating IL-1-induced expression of C/EBPδ in Hep3B cells. As the pharmacological agents apigenin and curcumin have also been demonstrated to act as inhibitors of NF-κB, and because preliminary data presented in chapter 4 suggests a role for NF-κB in the regulation of IL-1-mediated induction of C/EBPδ expression, we investigated this in more detail in the following chapter. In conjunction, we also investigated a role for JNK and CK2 in the regulation of NF-κB activation.

Additionally, with the use of RNAi we have shown, for the first time that STATs regulate endogenous expression of C/EBPδ as induced by IL-6 in Hep3B cells. Consistent with their role in IL-6 signalling, we also show both STAT1 and STAT3 are activated by this cytokine in Hep3B cells. Our data also suggests that STAT activation by IL-6 may be at least in part, regulated by CK2 and JNK MAPK, although these results require further investigation.

# CHAPTER SEVEN: AN INVESTIGATION INTO THE ROLE OF NF-κB IN THE REGULATION OF C/EBPδ EXPRESSION BY IL-1

## 7.1 INTRODUCTION

As previously mentioned in chapters 1 and 4 the activation of NF-κB by IL-1 is a well documented phenomenon. The signalling events resulting in the activation of NF-κB by IL-1 are discussed in detail in section 1.4 of this thesis. A brief summary of these processes is given below.

IL-1-mediated signalling is initiated when IL-1 binds to the receptor complex, consisting of IL-1RI and IL-1RAcP and the subsequent recruitment of MyD88. MyD88 is an adapter protein and functions to recruit the kinases IRAK1 and IRAK4 to the activated receptor. At the receptor complex, IRAK1 auto- and/or cross-phosphorylates itself, a process likely to be triggered by IRAK4 activation. Hyperphosphorylated IRAK1 then associates with the ubiquitin protein ligase, TRAF6 (Martin and Wesche 2002).

The MAPKKK, transforming growth factor-β-activated kinase-1 or TAK-1 and its associated adapter proteins TAB1-3 have also been implicated in IL-1 signalling (see Yao *et al.* 2007 and references therein). IRAK1 mediates the activation of TAK1 by introducing TRAF6 to the TAK1-TAB1-TAB2-TAB3 complex. Protein ubiquitinylation is thought to be essential for TRAF6-mediated TAK1 activation. TRAF6 itself is a target for ubiquitinylation and by an unknown mechanism polyubiquitinylated TRAF6 facilitates the complete activation of TAK1, which activates itself through auto-phosphorylation (Kishimoto *et al.* 2000). TAK-1 can then activate two kinase cascades, one which involves the MAPKs and the other leads to the activation of the IKK (Wang *et al.* 2001). IKK subsequently phosphorylates IκB and causes it to release NF-κB (typically p50:p65) which translocates into the nucleus to regulate gene transcription (see Figure 1.7 for details).

### 7.1.1 EXPERIMENTAL STRATEGY

Preliminary data from pharmacological inhibitor studies presented in chapter 4 indicates a potential requirement for NF-κB in the regulation of C/EBPδ expression by IL-1 and additionally a role for JNK MAPK and CK2 was also suggested. Despite this, experiments involving DN mutants (Chapter 5) and RNAi experiments presented in the previous chapter (Section 6.2) could not confirm the potential role of CK2 and JNK MAPK in the regulation of this response. However, both JNK MAPK and CK2

can act as regulators of NF-κB (McElhinny *et al.* 1996; Schulze-Osthoff *et al.* 1997 also see section 4.3). Furthermore, because several studies have shown that the activity of NF-κB can be inhibited by treatment of cells with apigenin or curcumin (Gerritsen *et al.* 1995; Singh and Aggarwal 1995; Xu *et al.* 1997; Jobin *et al.* 1999; Choi *et al.* 2004; Schulze-Tanzil *et al.* 2004; Shukla and Gupta 2004a; Shukla and Gupta 2004b; Cao *et al.* 2005; Moon *et al.* 2005; Moon *et al.* 2006; Cho *et al.* 2007), we investigated a role for NF-κB in the regulation of C/EBPδ expression by IL-1 in more detail. This was initially achieved with the use of siRNA technology, by targeting NF-κB. The effect of siRNA-mediated knock-down of NF-κB on the IL-1-mediated induction of C/EBPδ expression was examined. The same approach was also used to determine the effect of siRNA-mediated knock-down of TAK1 on the response, given that TAK1 is a key component implicated in IL-1 signalling.

We have previously demonstrated that IL-1 stimulates *trans*-activation by NF-κB in Hep3B cells, using a luciferase-reporter construct driven by four NF-κB enhancer elements in a transfection based assay (Chapter 3). Using the same system we also determined the effect of apigenin and curcumin on this activation. Additionally, a role for CK2 and JNK MAPK in the regulation of this response was also investigated with the use of DN mutants. The effect of IL-1 on NF-κB DNA binding was also investigated by EMSAs and in conjunction, the effect of apigenin and curcumin on IL-1-mediated NF-κB DNA binding was also examined.

Figure 7.1 summarises our overall experimental approach that was designed to achieve the outlined experimental objectives.

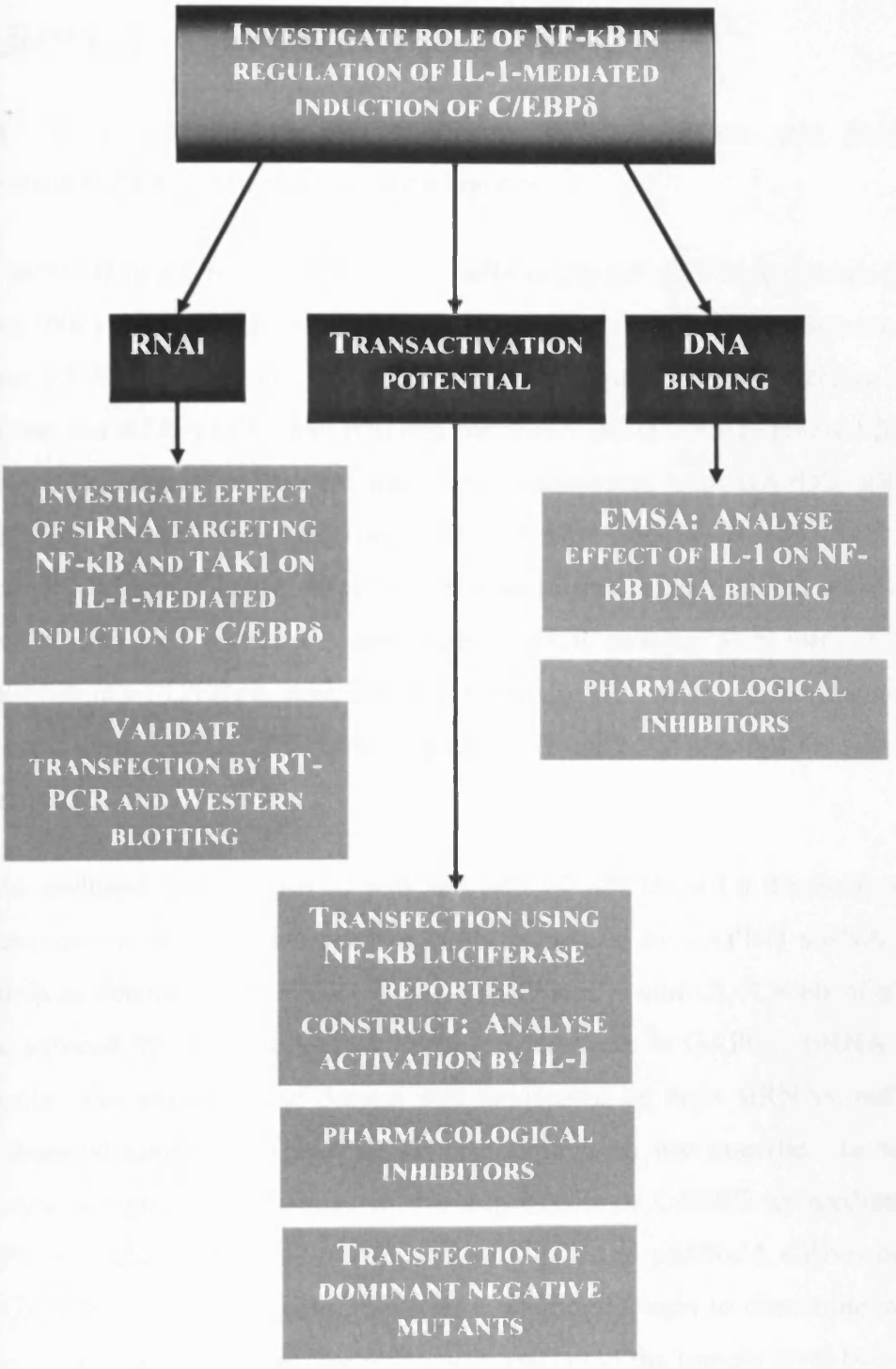



**FIGURE 7.1 Experimental strategy.** Potential roles of NF-κB and TAK1 in the regulation of C/EBPδ expression by IL-1 were assessed with the use of siRNA technology. *Trans*-activation by NF-κB as mediated by IL-1 was investigated in a transient transfection assay. The effect of apigenin and curcumin on this *trans*-activation was also examined. In conjunction, the effect of DN CK2 and JNK mutants on *trans*-activation was investigated in an effort to determine a possible mechanism of activation. Finally, the effect of IL-1 on NF-κB DNA binding was examined by EMSA analysis and in association the effect of apigenin and curcumin on DNA binding was also determined.

## 7.2 RESULTS

### 7.2.1 EFFECT OF SILENCING P50 AND P65 NF-κB ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ MRNA AND PROTEIN

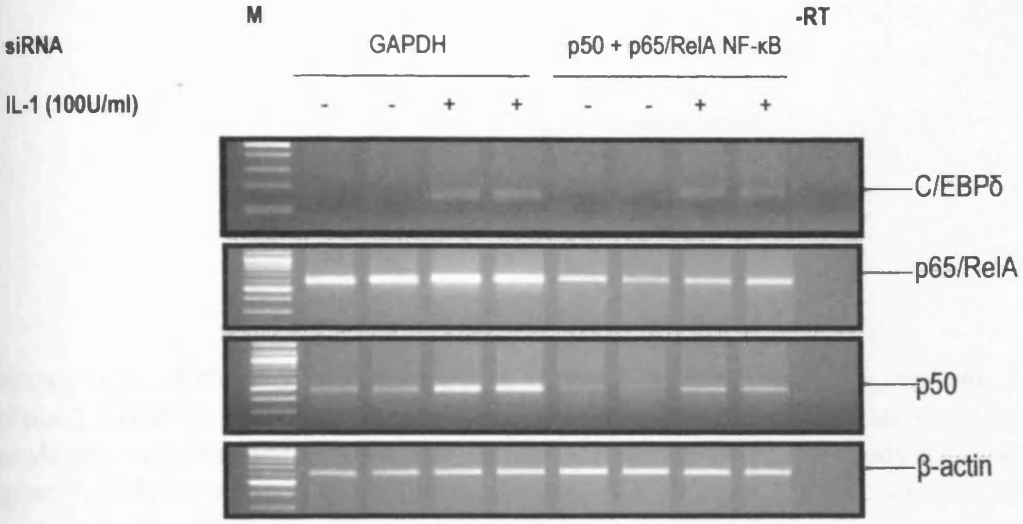
The most abundant form of NF-κB in cells is the p50:p65 heterodimer (Ghosh and Karin 2002) and therefore we decided to target this for siRNA-mediated knock-down in our RNAi experiments. Commercially available validated siRNAs specifically targeting the mRNA of human p50 and p65/RelA genes were purchased from Qiagen (Table 2.1). Hep3B cells were transiently transfected with GAPDH siRNA or co-transfected with siRNA targeting p50 and p65 NF-κB as described previously (Section 2.5.1 and Chapter 6). The cells were then either treated with IL-1 or left untreated and used for semi-quantitative RT-PCR analysis as before. PCRs for the amplification of C/EBPδ, p50, p65/RelA and β-actin were carried out with primers and conditions detailed in Tables 2.4 and 2.5. Figure 7.2 displays the results of these experiments.

RNAi-mediated knock-down of p50 and p65 NF-κB caused a dramatic depletion in the expression of respective mRNA pools compared to GAPDH siRNA transfected controls as determined by RT-PCR analysis (Panel A and C). Levels of p50 and p65 were reduced by ~80% compared to their expression in GAPDH siRNA transfected controls. The expression of β-actin was unaffected by these siRNAs, indicating that the observed knock-down in p50 and p65 expression was specific. In addition, we observed a significant decrease in the expression of C/EBPδ as mediated by IL-1 (~40% $\pm$ 10.6%) in samples transfected with p50 and p65/RelA siRNA compared to the GAPDH siRNA transfected controls. We next sought to determine whether this reduction in C/EBPδ expression was also apparent at the protein level by western blot analysis.

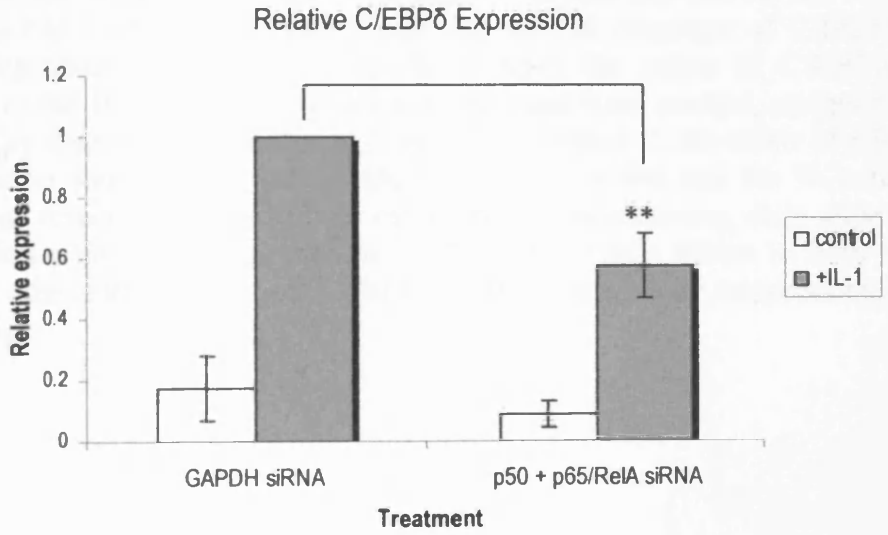
For this, the transfection outlined in Figure 7.2 was repeated, samples harvested for protein extraction (Section 7.2.1) and used for western blot analysis as described previously (Sections 2.7.7 and 2.7.8). Immunodetection of C/EBPδ and β-actin was carried out as described before (Section 2.7.9) and details for the detection of p50 and p65 NF-κB are presented in Table 2.9. The results of these experiments are displayed in Figure 7.3.

**FIGURE 7.2. Effect of silencing p50 and p65/RelA NF-κB on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (12.5nM) or co-transfected with siRNA against both p50 and p65/RelA (6.25nM of each siRNA) as described in section 2.5.1, with each transfection carried out in duplicate. Post transfection, the cells were either treated with IL-1 (100U/ml) or left untreated for 3h. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, p50, p65/RelA and β-actin were resolved by electrophoresis as before. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected -IL-1 RNA), Panel A. Panel B displays the ratios of C/EBPδ:β-actin, averaged for duplicate samples, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratios of p50:β-actin and p65:β-actin, were determined for the specified samples and the % reduction in expression for both p50 and p65 was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio ±SD from three independent experimental series. **P<0.01, ***P<0.001.

**A.**


M
-RT
siRNA
GAPDH
p50 + p65/RelA NF-κB
IL-1 (100U/ml)
- - + + - - + +
C/EBPδ
p65/RelA
p50
β-actin

**B.**


Relative C/EBPδ Expression
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
**
control
+IL-1
GAPDH siRNA
p50 + p65/RelA siRNA
Treatment

**C.**

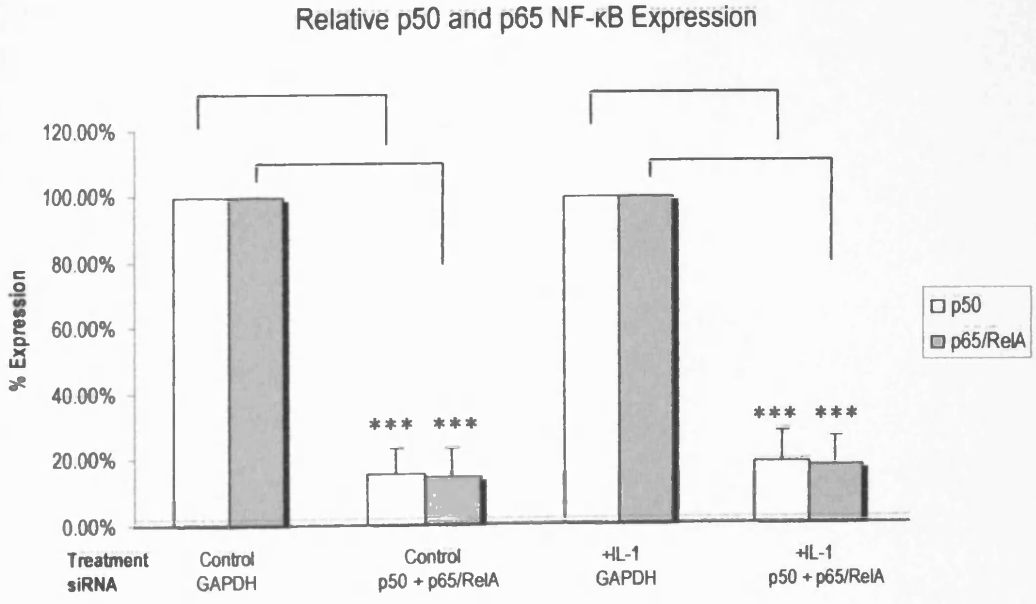
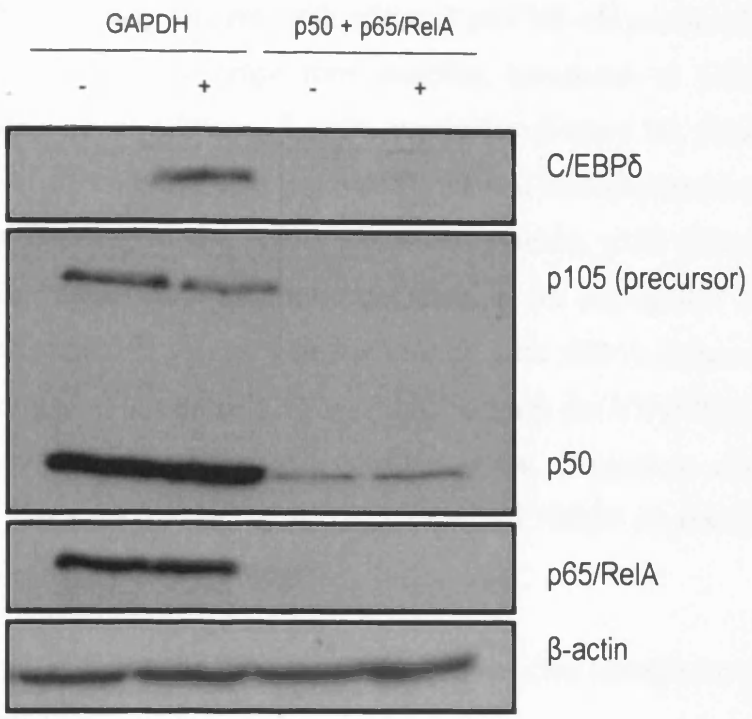
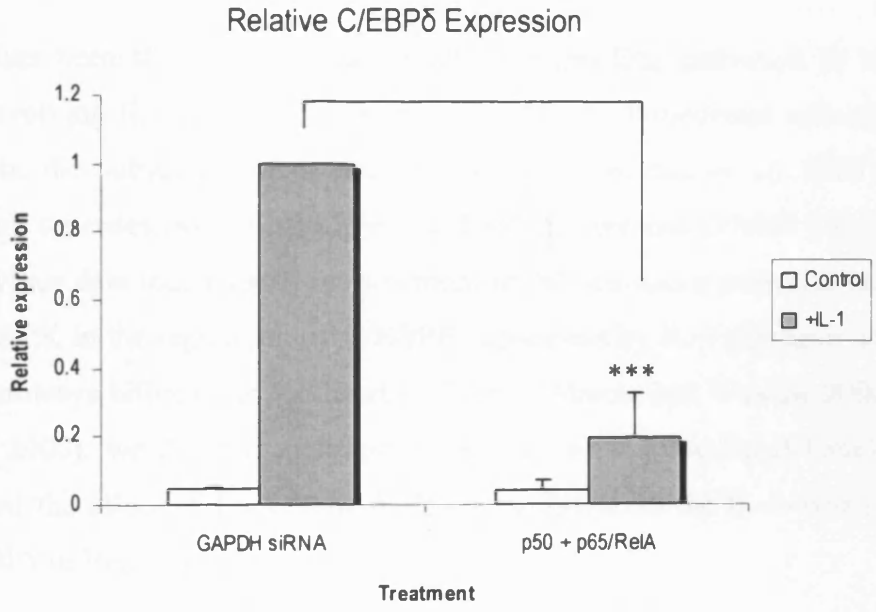

Relative p50 and p65 NF-κB Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
*** ***
*** ***
p50
p65/RelA
Treatment
siRNA
Control
GAPDH
Control
p50 + p65/RelA
+IL-1
GAPDH
+IL-1
p50 + p65/RelA

**FIGURE 7.3. Effect of simultaneously silencing p50 and p65 NF-κB on IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were transiently transfected with the indicated siRNAs and treated with IL-1 exactly as described in Figure 7.2. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using antibodies against C/EBPδ, p50, p65 and β-actin for immunodetection, Panel A. Western blot analysis of p50 and p65 NF-κB was carried out by SDS-PAGE using 7.5% (w/v) gels and for the detection of C/EBPδ and β-actin, as described previously. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratios of p50:β-actin and p65:β-actin were determined for the specified samples and the % reduction in expression of respective proteins was calculated by normalising their expression in GAPDH siRNA transfected samples to 100%. The results shown in both Panels B and C are the mean ratio ±SD from three independent experimental series. ***P<0.001.

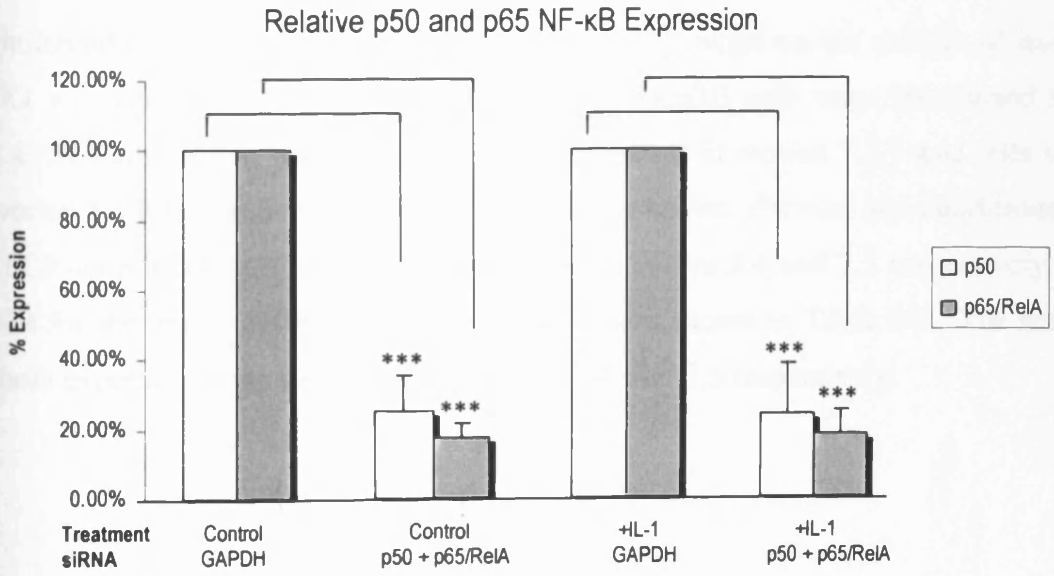
A.


siRNA
GAPDH
p50 + p65/RelA
IL-1 (100U/ml)
- + - +
C/EBPδ
p105 (precursor)
p50
p65/RelA
β-actin

B.


Relative C/EBPδ Expression
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
***
Control
+IL-1
GAPDH siRNA
p50 + p65/RelA
Treatment

C.


Relative p50 and p65 NF-κB Expression
% Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
***
***
***
***
p50
p65/RelA
Treatment
siRNA
Control
GAPDH
Control
p50 + p65/RelA
+IL-1
GAPDH
+IL-1
p50 + p65/RelA

As expected, siRNA directed against both p50 and p65 NF-κB produced a significant decrease in the expression of respective proteins, compared to GAPDH siRNA transfected controls without affecting β-actin expression (Figure 7.3, Panels A and C). Immunodetection of p50 NF-κB also produced a second immunoreactive band on the western blot corresponding to the precursor of this protein, p105 (Ghosh and Karin 2002) as shown in Panel A. A dramatic decrease in the expression of C/EBPδ as induced by IL-1 was noted in samples co-transfected with siRNA directed against p50 and p65 NF-κB compared to controls. These data support the RT-PCR data presented in Figure 7.2, in which we observed a similar effect. Together, our RNAi data strongly suggests a role for NF-κB in the regulation of C/EBPδ expression as induced by IL-1 in Hep3B cells, a novel finding.

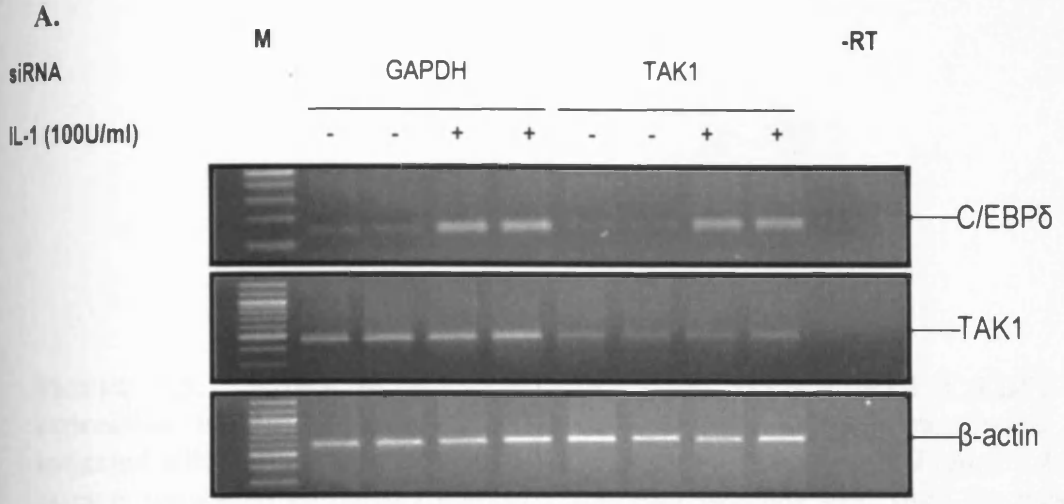
### 7.2.2 EFFECT OF SILENCING TAK1 ON THE IL-1-INDUCED EXPRESSION OF C/EBPδ MRNA AND PROTEIN

TAK1 has been shown to regulate multiple signalling pathways *in vivo* including those involving IL-1 action (Shim *et al.* 2005). IL-1-mediated activation of TAK1 results in the subsequent activation of NF-κB (Takaesu *et al.* 2003) and MAPK signalling cascades (Martin and Wesche 2002; Dunne and O'Neill 2003). Given that our previous data indicates an involvement of NF-κB and a potential requirement for JNK MAPK in the regulation of C/EBPδ expression by IL-1 (Chapter 4) and because these pathways bifurcate at the level of TAK1 (Martin and Wesche 2002; Dunne and O'Neill 2003), we decided to target TAK1 for siRNA-mediated knock-down. We examined the effect of TAK1 knock-down by RNAi on the IL-1-mediated induction of C/EBPδ in Hep3B cells.

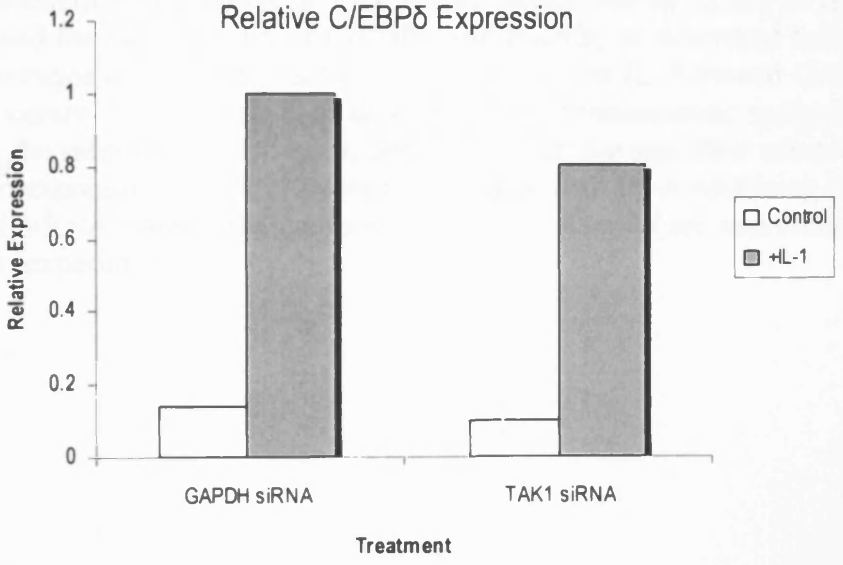
Commercially available validated siRNA specifically targeting the mRNA of human TAK1 was purchased from Qiagen (Table 2.1). Hep3B cells were transfected with this siRNA and treated with IL-1 exactly as described in section 7.2.1 and cells were harvested for RT-PCR or western blot analysis, as above. Primers and conditions for the PCR-amplification of TAK1 are displayed in Tables 2.4 and 2.5 respectively and details for the immunoblotting of TAK1 protein are shown in Table 2.9. The results of these experiments are displayed in Figures 7.4. and 7.5 respectively.

**FIGURE 7.4. Effect of silencing TAK1 on IL-1-induced C/EBPδ mRNA expression in Hep3B cells.** Hep3B cells were either transiently transfected with GAPDH siRNA (6.25nM) or with TAK1 siRNA (6.25nM) and treated with IL-1 as above with each transfection carried out in duplicate. Total RNA was then extracted and used for semi-quantitative RT-PCR analysis. The PCR amplification products for C/EBPδ, TAK1 and β-actin were resolved by electrophoresis as before. The size of each PCR-amplified product was determined by comparing it to standard DNA markers denoted as 'M' (New England Biolabs, 100bp DNA ladder. See Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using GAPDH siRNA transfected –IL-1 RNA), Panel A. Panel B displays the ratios of C/EBPδ:β-actin, averaged for duplicate samples, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the average ratio of TAK1:β-actin was determined for the specified samples and the % reduction in expression for TAK1 was calculated by normalising its expression in GAPDH siRNA transfected samples to 100%. Results are representative of two independent experiments.

A.


M
-RT
siRNA
GAPDH
TAK1
IL-1 (100U/ml)
- - + + - - + +
C/EBPδ
TAK1
β-actin

B.


Relative C/EBPδ Expression
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
GAPDH siRNA
TAK1 siRNA
Treatment
Control
+IL-1

C.

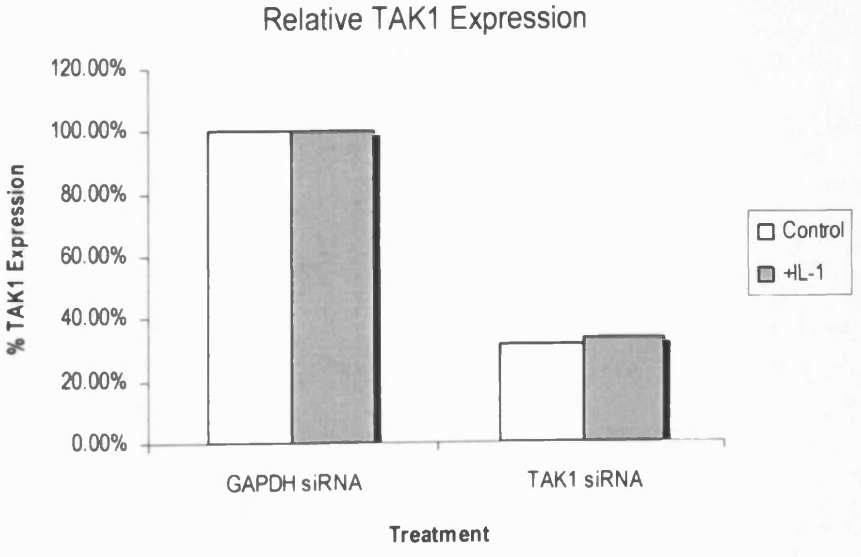
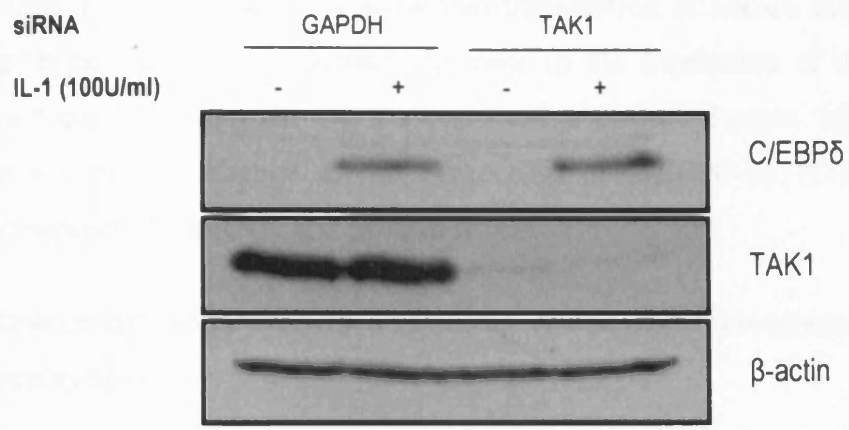
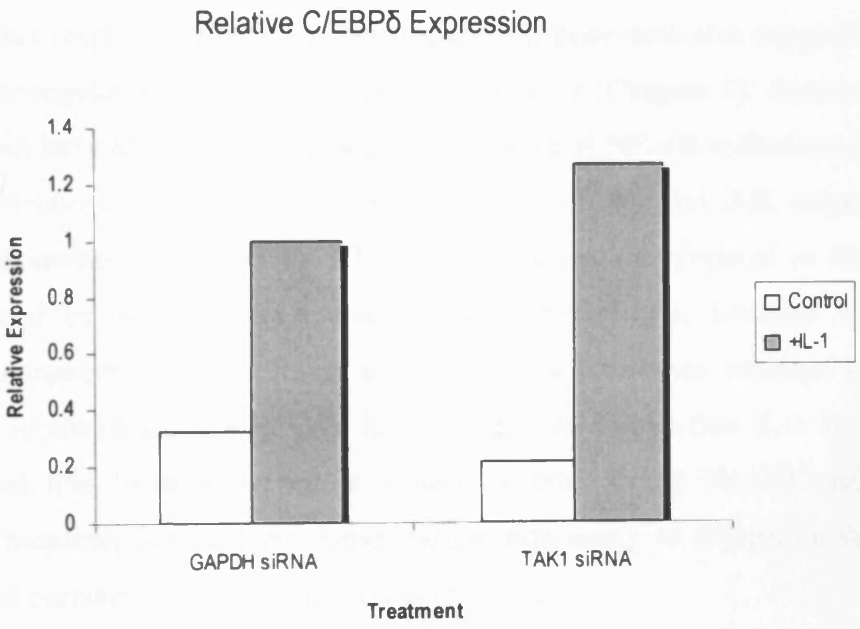

Relative TAK1 Expression
% TAK1 Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
GAPDH siRNA
TAK1 siRNA
Treatment
Control
+IL-1

**FIGURE 7.5. Effect of silencing TAK1 on IL-1-induced C/EBPδ protein expression in Hep3B cells.** Hep3B cells were transiently transfected with the indicated siRNAs and treated with IL-1 exactly as described in Figure 7.4. Protein extracts were then prepared (Section 2.7.1) and samples were used for western blot analysis, using antibodies against C/EBPδ, TAK1 and β-actin for immunodetection, Panel A. Western blot analysis of TAK1 was carried out by SDS-PAGE using 7.5% (w/v) gels and for the detection of C/EBPδ and β-actin, as described before. Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1-treated GAPDH siRNA transfected control, assigned as 1, as determined by densitometric analysis. Similarly, in Panel C, the ratio of TAK1:β-actin determined for the specified samples and the % reduction in expression of TAK1 protein was calculated by normalising its expression in GAPDH siRNA transfected samples to 100%. Results are representative of two independent experiments.

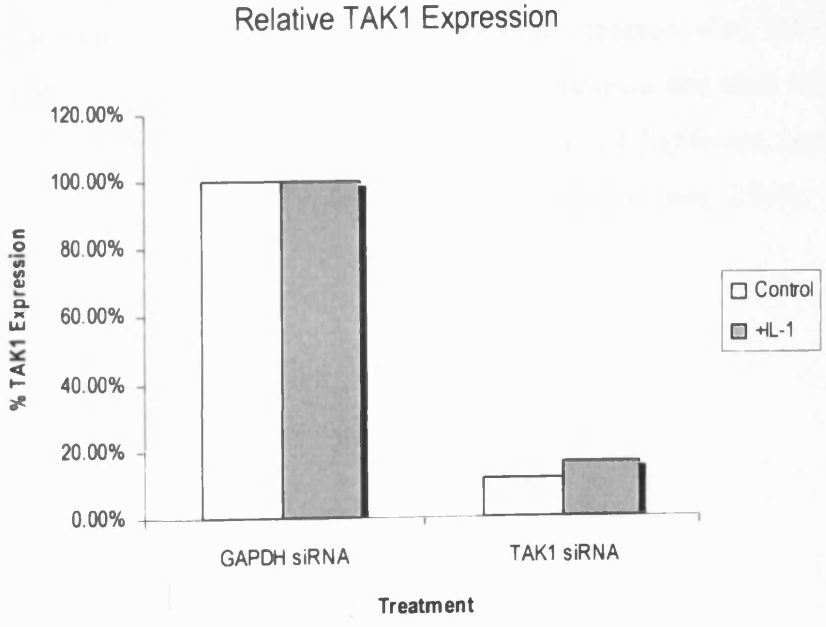
A.


siRNA
GAPDH
TAK1
IL-1 (100U/ml)
-
+
-
+
C/EBPδ
TAK1
β-actin

B.


Relative C/EBPδ Expression
Relative Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GAPDH siRNA
TAK1 siRNA
Treatment
Control
+IL-1

C.


Relative TAK1 Expression
% TAK1 Expression
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
GAPDH siRNA
TAK1 siRNA
Treatment
Control
+IL-1

Results from Figures 7.4 and 7.5 show that transfection of siRNA directed at TAK1 into Hep3B cells caused a dramatic decrease in the expression of this MAPKKK, without affecting the expression of the constitutive control, β-actin. However, we did not note any marked change in the expression of C/EBPδ by IL-1, compared to controls, between the mRNA and protein levels.

### 7.2.3 EFFECT OF APIGENIN AND CURCUMIN ON *TRANS*-ACTIVATION BY NF-κB AS MEDIATED BY IL-1

Thus far, our RNAi data suggest that NF-κB is required for the induction of C/EBPδ expression by IL-1 (Figures 7.2 and 7.3) and that TAK1 may not be involved in regulating this response. Our pharmacological inhibitor data also supports a role for NF-κB in the regulation of C/EBPδ expression by IL-1 (Chapter 4). Because apigenin and curcumin have also been described as inhibitors of NF-κB activation (see section 7.1.1 for references) in addition to direct inhibitors of CK2 and JNK respectively, we wanted to examine their effect on NF-κB *trans*-activation potential in Hep3B cells. As mentioned in section 2.4.4.4 the plasmid pNFκB-Luc, contains four NF-κB consensus enhancer elements fused upstream of a luciferase reporter gene in the pTAL-Luc vector (Appendix I). We have previously shown that IL-1 stimulates the activation of this luciferase-reporter system in transfected Hep3B cells (Section 3.3.1.2). Therefore, we used the same transfection assay to determine the effect of apigenin and curcumin on NF-κB activation by IL-1.

For this, Hep3B cells were transiently transfected (Section 2.4.1) with the pNFκB-Luc plasmid. Following transfection, the cells were pre-treated with DMSO (vehicle-control) or various concentrations of apigenin and curcumin and then stimulated with IL-1. They were then harvested for the determination of luciferase reporter activity (Sections 2.4.2 and 2.4.3) and protein concentration (Section 2.7.4). Results are shown in Figure 7.6.

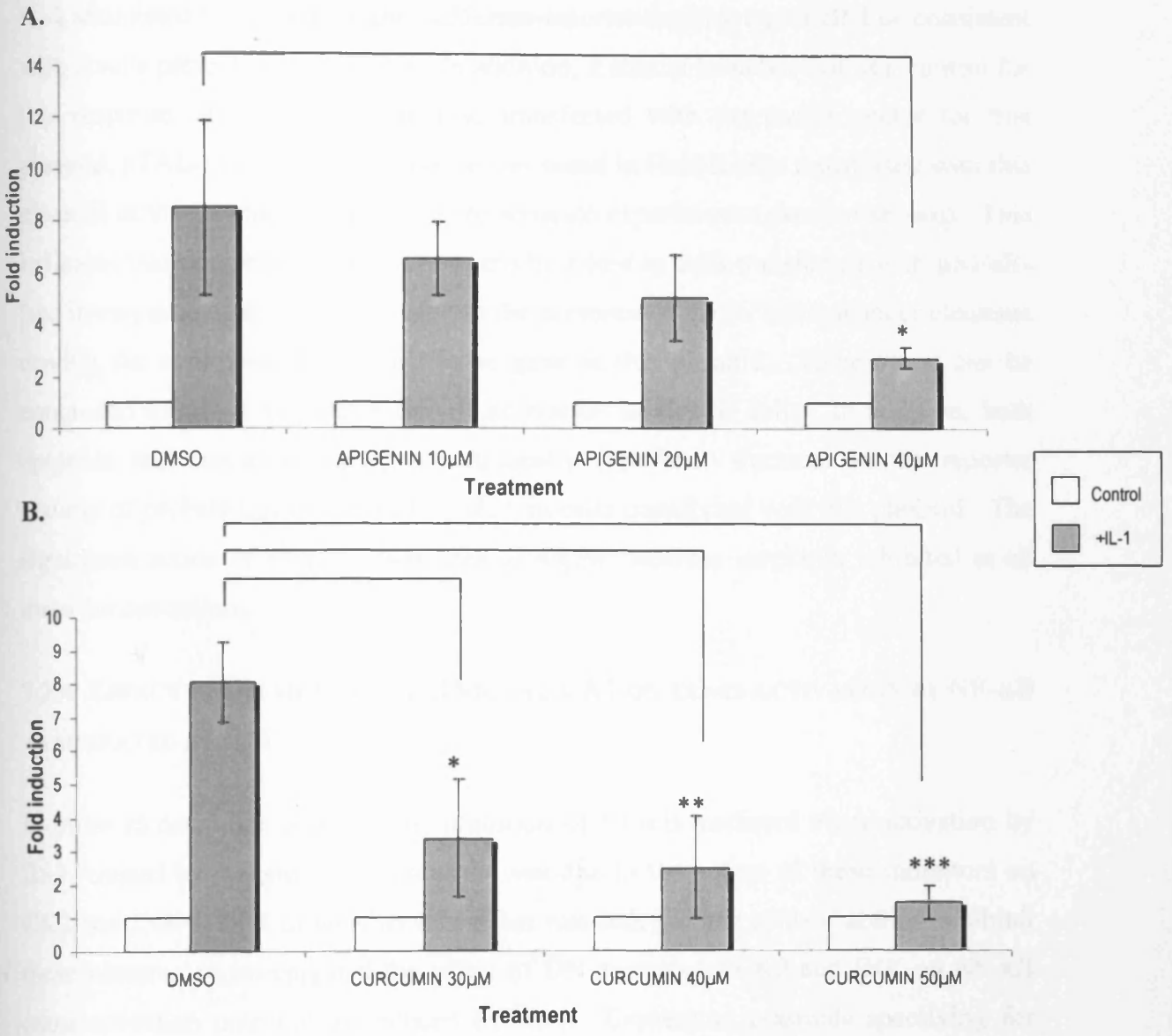



**FIGURE 7.6. Effect of apigenin and curcumin on the *trans*-activation potential of NF-κB stimulated by IL-1 in Hep3B cells.** The cells were transiently transfected with 2.5μg of pNFκB-Luc plasmid by the PEI method (Section 2.4.1). Post-transfection, the cells were washed with DMEM complete medium, and pre-treated with DMSO or the specified inhibitors at the indicated concentrations for 1h. The cells were then either stimulated with IL-1 (100U/ml) or left untreated for 3h, before being harvested for luciferase reporter activity and protein concentration determination. Luciferase activity was normalised to the protein concentration of each sample and is presented as a fold change in response to IL-1 relative to the untreated control for each treatment (assigned as 1). Results are presented as mean ±SD of three independent experiments, where all treatments were carried out in triplicate in each independent experimental series. *P<0.05, **P<0.01, ***P<0.001.

IL-1 stimulated the activity of the luciferase-reporter construct, pNFκB-Luc consistent with results presented in chapter 3. In addition, it should be noted that as a control for this response, Hep3B cells were also transfected with the parent vector for this plasmid, pTAL-Luc. No such response was noted in Hep3B cells transfected with this plasmid in the presence of IL-1 in three separate experiments (data not shown). This indicates that the increase in reporter activity noted in cells transfected with pNFκB-Luc in response to IL-1 was only due to the presence of the NF-κB enhancer elements driving the expression of the luciferase gene in this plasmid. Therefore it can be concluded that IL-1 stimulates NF-κB activation in Hep3B cells. In addition, both apigenin and curcumin caused a statistically significant decrease in the reporter activity of pNFκB-Luc as induced by IL-1 in cells transfected with this plasmid. The significant action of apigenin was seen at 40μM, whereas curcumin inhibited at all three concentrations.

### 7.2.4 EFFECT OF DN MUTANTS OF JNK AND CK2 ON *TRANS*-ACTIVATION BY NF-κB AS MEDIATED BY IL-1

In order to determine whether the inhibition of NF-κB-mediated *trans*-activation by IL-1, caused by apigenin and curcumin was due to the action of these inhibitors on CK2 and JNK MAPK or whether this effect was independent of their ability to inhibit these kinases, we investigated the effect of DN mutants of CK2 and JNK on NF-κB *trans*-activation potential as induced by IL-1. Expression plasmids specifying for these DN mutant proteins have previously been described in chapters 2 and 5. We additionally decided to examine the effect of an expression plasmid specifying for IκBα (IκB SR, section 2.4.4.8) on NF-κB activation. In resting cells, NF-κB is sequestered in the cytoplasm through its interaction with IκBα. Phosphorylation and subsequent ubiquitin-mediated degradation of IκBα permits NF-κB to translocate into the nucleus where it activates gene expression (Ghosh and Karin 2002). Over-expression of IκBα would therefore have an inhibitory effect on NF-κB activation. We therefore hypothesised that over expression of IκBα would inhibit the *trans*-activation potential of NF-κB as induced by IL-1 in our transfection based assay. We decided to include the expression plasmid IκB SR in our experiment, alongside the plasmids specifying for DN CK2 and DN JNK, as a positive control. That is, to ensure that the experimental conditions were appropriate for the expression of these plasmids. cDNAs specifying for IκBα and the DN mutant form of JNK are cloned in

to the expression plasmid pcDNA3 and cDNA specifying for the DN form of CK2 is cloned in the pSG5 plasmid vector (Appendix I). Therefore we used both the empty expression plasmids pcDNA3 and pSG5 as controls in our experiments. Hep3B cells were co-transfected with the pNFκB-Luc plasmid and plasmids specifying for DN CK2, DN JNK, or IκB SR as described in section 7.2.3. For control purposes, cells were also co-transfected with the pNFκB-Luc and the empty expression plasmids pcDNA3 or pSG5. Following IL-1 treatment, the cells were harvested for luciferase-reporter activity and for the determination of protein concentration, as before. The results of these experiments are shown in Figure 7.7 below.

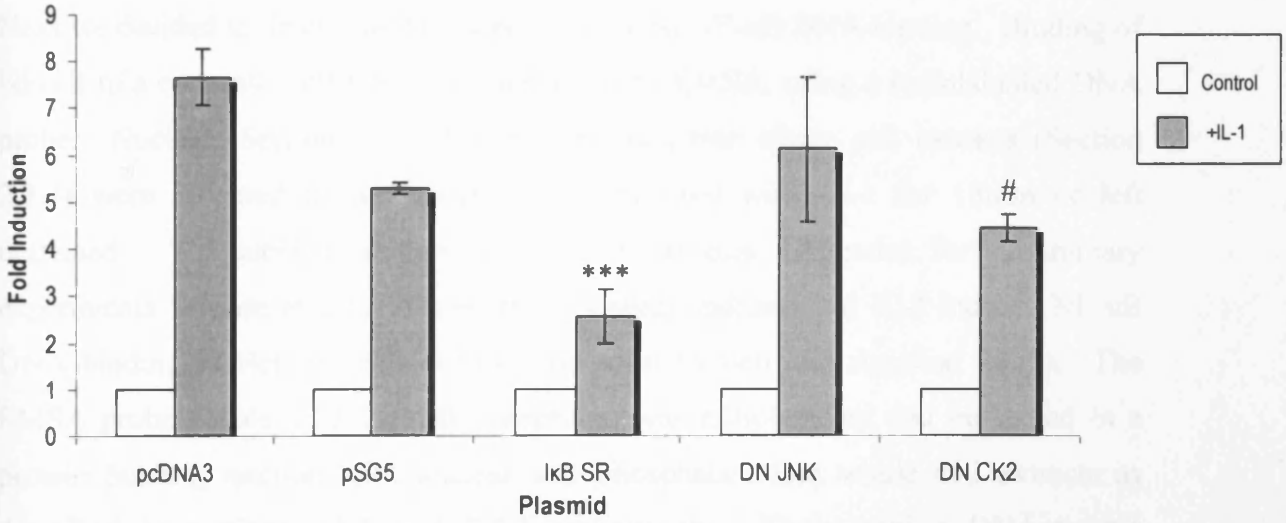



**FIGURE 7.7. Effect of IκB over-expression and DN mutants of CK2 and JNK MAPK on the *trans*-activation potential of NF-κB stimulated by IL-1 in Hep3B cells.** The cells were transiently co-transfected with 2μg of pNFκB-Luc plasmid and 4μg of the specified expression plasmids, as described in Figure 7.6. The cells were then either stimulated with IL-1 (100U/ml) or left untreated for 3h, before being harvested for luciferase reporter activity and protein concentration determination as before. Luciferase activity was normalised to the protein concentration of each sample and is presented as a fold change in response to IL-1 relative to the untreated control for each treatment (assigned as 1). Results are presented as mean ±SD of three independent experiments, where all treatments were carried out in triplicate in each independent experimental series. ***P<0.001, comparing the pcDNA3 IL-1-treated control to the IκB SR IL-1-treated data. #P<0.05, comparing the pSG5 IL-1-treated control to the DN CK2 IL-1-treated data.

As expected IL-1 treatment caused an increase in the reporter activity of the pNFκB-Luc plasmid, compared to controls. In addition, analysis of these data showed that the expression of IκBα, significantly inhibited the activation of NF-κB by IL-1, compared to the pcDNA3 transfected control, as we hypothesised, indicating that our experimental conditions were appropriate for the production of proteins specified by

the various expression plasmids used in our transfection system. However, expression of the DN mutant of JNK did not attenuate the activation of NF-κB as induced by IL-1, compared to the pcDNA3 transfected control. Furthermore, we also noted that the expression of DN CK2 caused a minor but significant decrease in the activation of NF-κB by IL-1, compared to the pSG5 transfected control. These results suggest that JNK may not be a regulator of NF-κB activation by IL-1 in Hep3B cells and that CK2 contributes marginally towards the regulation of this response.

### 7.2.5 EFFECT OF IL-1 ON PROTEIN BINDING TO AN NF-κB CONSENSUS DNA SEQUENCE

Next we decided to determine the effect of IL-1 on NF-κB DNA binding. Binding of NF-κB to a consensus κB site was determined by EMSA, using a radiolabelled DNA probe. Nuclear (Section 2.7.3) and phosphatase-free whole cell extracts (Section 2.7.2) were prepared from Hep3B cells stimulated with IL-1 for 15min or left untreated. We decided to harvest the cells at this time-point for preliminary experiments because results from a previous study indicate that IL-1 induces NF-κB DNA binding in Hep3B cells at this time-point (Voleti and Agrawal 2005). The EMSA probe (Table 2.10, NF-κB consensus) was radiolabelled and incubated in a protein binding reaction with nuclear and phosphatase-free whole cell extracts as described in sections 2.8.1 and 2.8.2 respectively. The resulting DNA-protein complexes were resolved on a non-denaturing polyacrylamide gel and visualised by exposure to X-ray film (Section 2.8.4). The results of this experiment are shown in Figure 7.8. IL-1 treatment of Hep3B cells resulted in the formation of five distinct DNA-protein complexes (**a-e**) in both whole cell and nuclear extracts. Four of these complexes (**a**, **c**, **d** and **e**) were also present in untreated samples (although signals for **c** and **d** were faint), but induced by varying degrees with IL-1 treatment. Complex **b** was entirely induced by IL-1 treatment. These results indicate that IL-1 rapidly induces NF-κB DNA binding in stimulated Hep3B cells.

The specificity of the DNA-binding reaction was next examined by competition- and antibody-supershift/interference-EMSA analysis (Section 2.8.3). The phosphatase-free whole cell extract sample prepared from IL-1 treated cells used in the experiment in Figure 7.8 was employed for further analysis. For competition-EMSA analysis, the extract (5μg) was pre-incubated with a 250 molar excess of unlabelled κB or AP-1

consensus probe (Table 2.10). Antibody supershift/interference-EMSA analysis was carried out using NF-κB specific antibodies (0.5μg of each) used previously (Figure 7.5, Section 2.8.3). Pre-incubation with non-immune serum (NIS) served as a control. Subsequent EMSA analysis was then carried out exactly as described previously. The results of these experiments are shown in Figure 7.9.

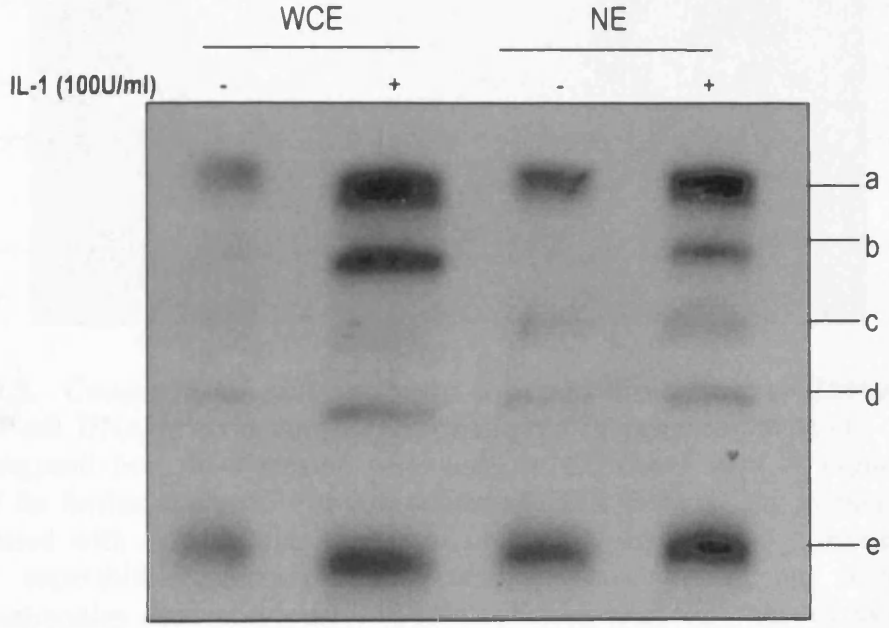



**FIGURE 7.8. Effect of IL-1 on NF-κB DNA binding to a consensus κB site. A comparison of DNA-protein complexes obtained from whole cell extracts (WCE) and nuclear extracts (NE).** Hep3B cells were either stimulated with IL-1 for 15min or left untreated before being harvested for the preparation of phosphatase-free whole cell and nuclear extracts. EMSA analysis was carried out for extracts (5μg) using a radiolabelled NF-κB consensus probe. DNA-protein complexes were resolved on 6% (w/v) non-denaturing polyacrylamide gels and visualised by autoradiography.

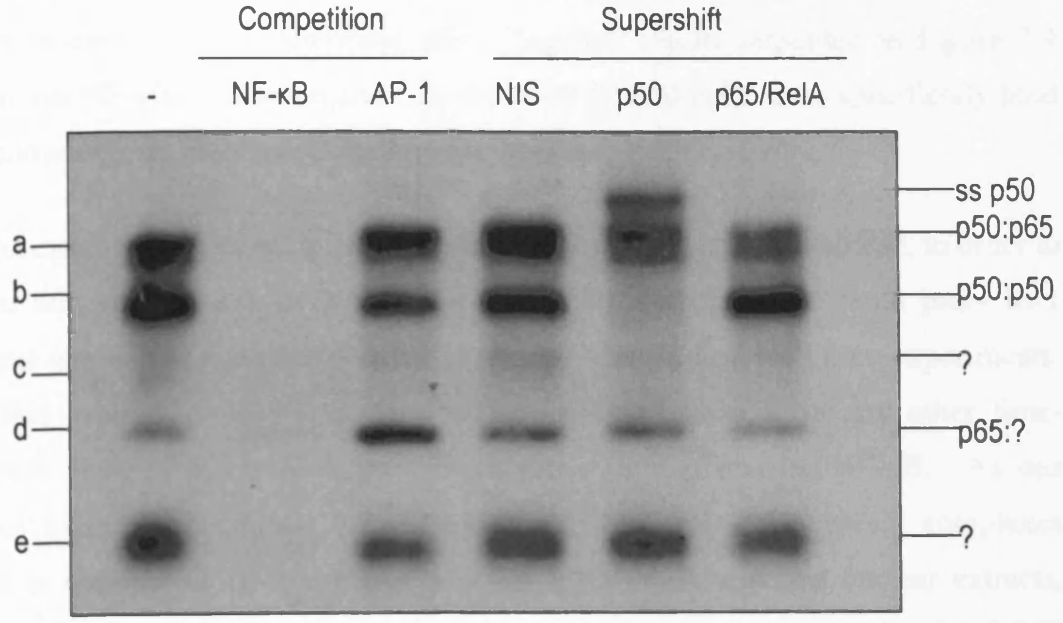



**FIGURE 7.9. Competition- and antibody supershift/interference-EMSA analysis of the NF-κB DNA-protein complexes produced in response to IL-1.** The WCE sample prepared from IL-1 treated cells used in the experiment in Figure 7.8 was employed for further analysis. For competition-EMSA analysis, the extract (5μg) was pre-incubated with a 250 molar excess of unlabelled κB or AP-1 consensus probe. Antibody supershift/interference-EMSA analysis was carried out using NF-κB specific antibodies used previously. Pre-incubation with NIS served as a control. EMSA analysis was then carried out using the radiolabelled κB consensus probe as above. Antibody supershift/interference studies are representative of two independent experiments. ? denotes that the identity of the protein involved in the DNA-protein interaction is unknown (see text for details). ss denotes supershift.

Protein binding was competed by an excess of the unlabelled κB probe, but not by a consensus sequence for AP-1 (Table 2.10, AP-1 consensus). Pre-incubation with the anti-p50 NF-κB antibody resulted in the formation of slower migrating antibody DNA-protein supershift complex (denoted ss p50 in Figure 7.9). Formation of complex **b** was completely inhibited in the presence of the anti-p50 NF-κB antibody but not with the anti-p65 NF-κB antibody, indicating this complex was formed by a p50:p50 homodimer. Formation of complex **a** was also partially inhibited, indicating that p50 was also necessary for the formation of this complex. Pre-incubation with anti-p65 NF-κB interfered with the formation of complexes **a** and **d**. Therefore, complex **a** could potentially be formed by a p50:p65 heterodimer, as it is also partially inhibited by pre-incubation with anti-p50 NF-κB. p65 NF-κB may also be a component of complex **d**. Complexes **c**, **e** and a component of complex **d** (flagged with ?) may potentially be formed by other NF-κB family members such as c-Rel,

RelB and p52, although further antibody supershift/interference experiments would have to be carried out to determine this. Together results presented in Figure 7.9 confirm that NF-κB, in extracts from IL-1-treated Hep3B cells, does specifically bind to the consensus κB probe used in our experiments.

We next examined the effect of IL-1 on NF-κB binding over a time-course, in order to confirm this binding and to ensure that harvesting Hep3B cells 15min post- IL-1 treatment was an optimal time-point to assess NF-κB binding for future experiments. Also, this experiment was important to determine if there were any other time-dependent changes in DNA-protein interactions as mediated by NF-κB. As our previous experiments (Figure 7.8) show that the NF-κB DNA-protein complexes formed in response to IL-1, are similar from both whole cell and nuclear extracts, cells were harvested for phosphatase-free whole cell protein extraction (Section 2.7.2) for subsequent experiments. Hep3B cells were treated with IL-1 and harvested for protein extraction over a time-course of 3h. Extracts were then used for EMSA analysis as before with the consensus κB radiolabelled probe. Results are presented in Figure 7.10.

As expected, IL-1 induced the formation of NF-κB DNA-protein complexes compared to the untreated control (0min), as determined by EMSA analysis. Complex **a** and **b** were most visibly induced. The induction was transient and apparent 5min post IL-1 treatment and decreased at 180min.

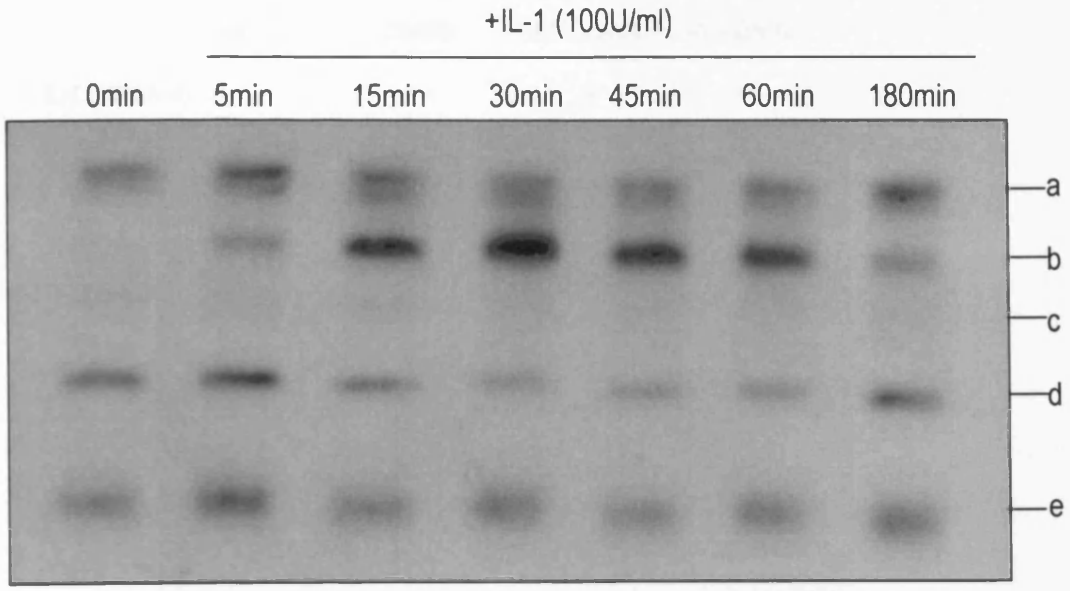



**FIGURE 7.10. Effect of IL-1 on NF-κB DNA-protein complex formation over a 3h time-course.** Hep3B cells were treated with IL-1 and harvested for phosphatase-free whole cell protein extraction at each of the indicated time-points over a 180min time-course. Protein extracts (5μg) were then used for EMSA analysis using the radiolabelled κB probe exactly as described in Figure 7.8. Results are representative of two independent experiments.

### 7.2.6 EFFECT OF APIGENIN AND CURCUMIN ON IL-1-INDUCED NF-κB DNA-PROTEIN BINDING

Our previous data suggests that the pharmacological inhibitors apigenin and curcumin prevent NF-κB-mediated *trans*-activation, as induced by IL-1 (Figure 7.6). Therefore, we next sought to determine if the action of these inhibitors prevented IL-1-induced NF-κB DNA binding in Hep3B cells. Phosphatase-free whole cell protein extracts were prepared from Hep3B cells pre-treated with these inhibitors or DMSO (vehicle) and stimulated with IL-1 for 15min. Extracts were then used for EMSA analysis using the radiolabelled consensus κB probe, as before. The results are presented in Figure 7.11.

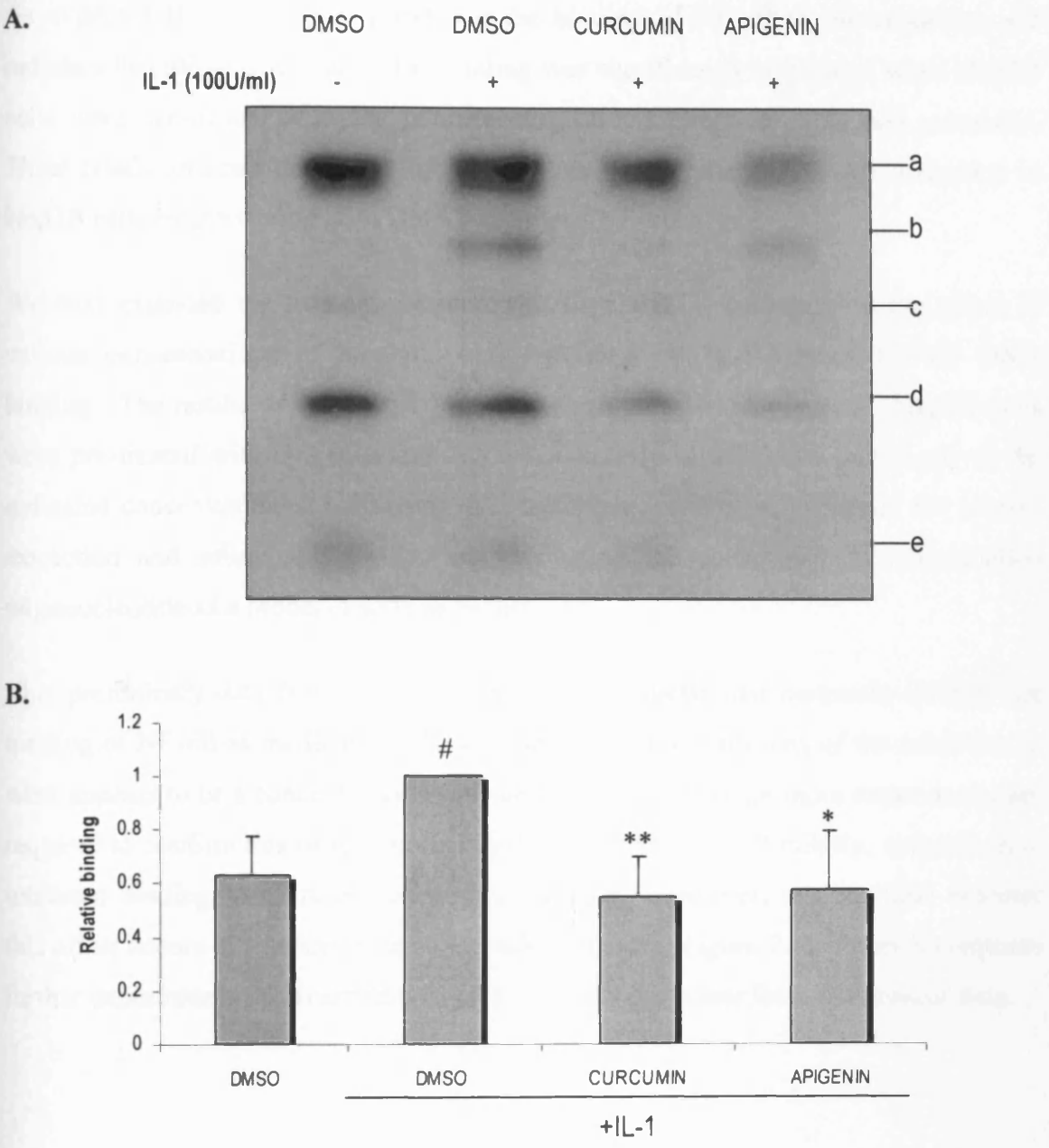



**FIGURE 7.11. Effect of apigenin and curcumin on IL-1-mediated DNA binding of NF-κB.** Hep3B cells were pre-treated with the inhibitors apigenin (40μM), curcumin (50μM) or DMSO (vehicle-control) for 1h. They were then either stimulated with IL-1 for 15min or left untreated, before being harvested for phosphatase-free whole cell protein extraction, as before. Extracts (5μg) were then used for EMSA analysis using the radiolabelled consensus κB probe, exactly as before (Panel A). Densitometric analysis (determined by measuring the intensity of each complex, in each sample and then calculating the sum value) of these data is presented in Panel B, normalised to the IL-1-treated DMSO control. The results shown in Panel B are the mean ratio ±SD from three independent experimental series. #P<0.05, compared to the DMSO-treated control. *P<0.05 and **P<0.01 compared to the IL-1-treated DMSO control.

As expected IL-1 significantly induced the binding of NF-κB to the consensus κB radiolabelled oligonucleotide. This binding was significantly attenuated when Hep3B cells were pre-treated with the pharmacological inhibitors apigenin and curcumin. These results indicate that these agents may act as inhibitors of NF-κB activation in Hep3B cells by interfering with DNA binding of NF-κB.

We next extended the findings presented in Figure 7.11, by assessing the effect of various concentrations of apigenin and curcumin on IL-1-induced NF-κB DNA binding. The results of these experiments are presented in Figure 7.12. Hep3B cells were pre-treated with apigenin and curcumin exactly as described previously at the indicated concentrations. Following IL-1 treatment, cells were harvested for protein extraction and subsequent EMSA analysis using the consensus κB radiolabelled oligonucleotide as a probe, exactly as before.

This preliminary data from a single experiment suggests that curcumin inhibits the binding of NF-κB as mediated by IL-1, at all three concentrations of the inhibitor, in what appears to be a concentration-dependent manner, although more experiments are required to confirm this observation (Figure 7.12, Panel A). Similarly, apigenin also inhibited binding, at all three concentrations used. However, to determine whether this effect occurs in a concentration-dependent manner (Figure 7.12, Panel B) requires further experiments to be carried out, as it is not entirely clear from the present data.

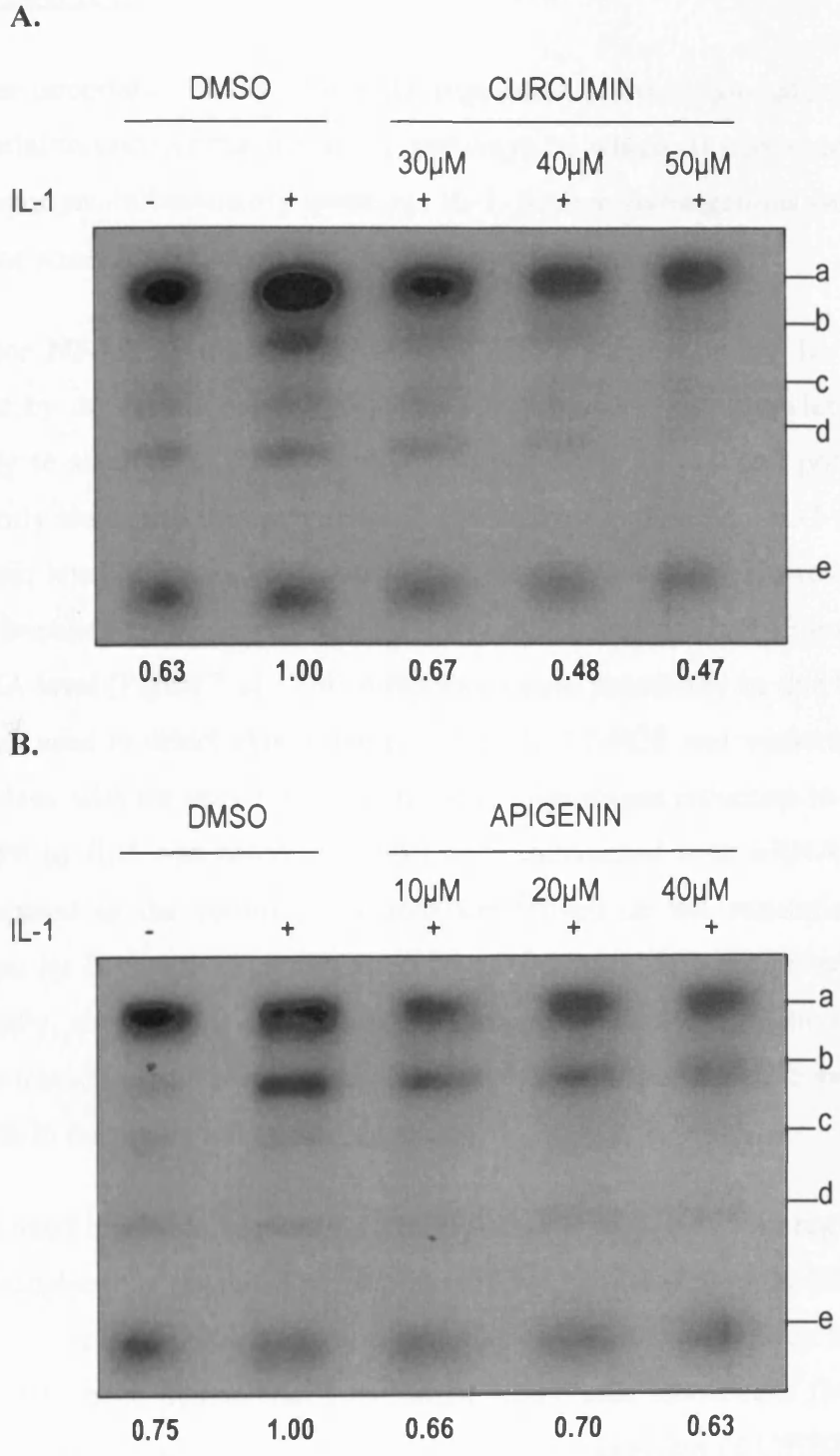



**FIGURE 7.12. Effect of various concentrations of curcumin and apigenin on IL-1-mediated DNA binding of NF-κB.** Hep3B cells were pre-treated with the inhibitors curcumin (A) and apigenin (B), at the indicated concentrations or DMSO (vehicle-control) for 1h. They were then either stimulated with IL-1 for 15min or left untreated, before being harvested for phosphatase-free whole cell protein extraction, as before. Extracts (5μg) were then used for EMSA analysis using the radiolabelled consensus κB probe, exactly as described previously. Densitometric analysis of these data was carried out as described in Figure 7.11 and is presented as ratio of the IL-1-treated DMSO control.

## 7.3 DISCUSSION

Due to the importance of C/EBPδ in the regulation of the inflammatory response and the potential to uncover the signalling pathways by which its expression is regulated by the major pro-inflammatory cytokine, IL-1, further investigations were undertaken to examine a role for NF-κB in the regulation of this response.

A role for NF-κB in the regulation of C/EBPδ expression by IL-1 was indeed suggested by the results presented in this chapter and to our knowledge; this is the first study to show this. RNAi-mediated knock-down of p50 and p65/RelA NF-κB significantly attenuated the expression of C/EBPδ as mediated by IL-1 at both mRNA and protein level, compared to controls (Figures 7.2 and 7.3). However, we noticed that this impaired response was more evident at the protein level (Figure 7.3) than at the mRNA level (Figure 7.2). This difference could potentially be due to the different techniques used to detect expression of C/EBPδ, RT-PCR and western blot analysis. Nevertheless, with the use of both techniques, a significant reduction in the expression of C/EBPδ by IL-1 was noted in Hep3B cells transfected with siRNA targeting NF-κB, compared to the controls. A role for NF-κB in the regulation of C/EBPδ expression by IL-1 was also suggested by preliminary data presented in chapter 4. Additionally, our previous data (Chapter 3), suggests that the regulation of C/EBPδ by IL-1 is a transcriptional response and in keeping with this we have suggested a role for NF-κB in the regulation of this response.

As mentioned briefly in section 4.3 the expression of C/EBPδ as regulated by LPS and peptidoglycan is regulated at least in part, by NF-κB (Liu *et al.* 2006b; Huang *et al.* 2007). A role for NF-κB in the regulation of C/EBPδ expression as mediated by LPS has also been demonstrated in mouse embryonic fibroblasts (MEFs). MEFs deficient in p65 NF-κB or IKKβ fail to induce the expression of C/EBPδ as mediated by LPS, compared to their wild-type counterparts (Kravchenko *et al.* 2003). Furthermore, also with the use of mutant MEFs deficient in IKKα, IKKβ or IKKγ, a role for NF-κB in the regulation of TNF-α-induced C/EBPδ expression has also been suggested, as determined by DNA microarray analysis (Li *et al.* 2002c). Although to our knowledge, no study has suggested a role for NF-κB in the regulation of C/EBPδ expression by IL-1, a requirement of this transcription factor in the regulation of this response is entirely plausible. As mentioned previously, NF-κB is a known regulator

of IL-1 dependent signalling events and NF-κB *cis*-acting elements have been identified in the promoter region of the rat, mouse (Yamada *et al.* 1997; Huang *et al.* 2007) and human C/EBPδ gene promoters (see Figure 7.13 below). Moreover, the fact that the aforementioned studies have suggested a role for NF-κB in the regulation LPS-mediated induction of C/EBPδ, is particularly intriguing. Receptors for IL-1 and LPS are members of the Toll/interleukin-1 receptor (TIR) family and therefore not surprisingly, utilise very similar intracellular signalling mechanisms to activate downstream targets, including NF-κB (Martin and Wesche 2002; Dunne and O'Neill 2003). Given this, it is possible that the signalling events governing the expression of C/EBPδ by IL-1 and LPS are similar.

-1830
ACTCTAAAGAAATATGTTTGTTCTTTAGGAAACCTATTAAATATCCCATAGTGCAACGTTAGAAATGAC
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CCCTTCCCCCGCGGCCCCGGGGCGCCCCCGCGGTGCCGGAGTCGGGGCGGGGCGTGCACGTCAGCCGGG
GCTAGAAAAGGCGGCGGGGCTGGGCCCAGCGAGGTG +1

**FIGURE 7.13. Putative NF-κB *cis*-acting elements in the promoter region of the human C/EBPδ gene promoter.** The promoter sequence was obtained from GenBank, nucleotide sequence of AC023991 (Homo sapiens chromosome 8, clone RP11-137L15, complete sequence). A putative p50 NF-κB binding site was identified and is blocked in yellow and the putative p65 NF-κB binding site is blocked in turquoise. Putative binding sites for c-Rel were also identified and are blocked in green. These are included because it has been reported that c-Rel can heterodimerise with either p50 or p65 NF-κB to activate target genes (Wietek and O'Neill 2007). The proposed TATAA box is highlighted in red for reference purposes. Binding sites for NF-κB transcription factors were identified using MatInspector transcription factor binding site search tool at http://www.genomatix.de/ and also TFSEARCH: Searching Transcription Factor Binding Sites (version 1.3) at http://www.cbrc.jp/research/db/TFSEARCH.html (see Appendix V for search results).

In an effort to initiate investigations into delineating the signalling pathway by which IL-1 induces the expression of C/EBPδ through NF-κB, we targeted the MAPKKK, TAK1 for RNAi-mediated knock-down. As mentioned in section 1.4 and 7.1, TAK1 is an upstream regulator of both MAPKs and NF-κB during IL-1-dependent signalling. RNAi-mediated knock-down of TAK1 expression resulted in a marginal decrease in the expression C/EBPδ as mediated by IL-1, at the mRNA level (Figure 7.4). However, the same observation was not noted by analysing the effect of siRNA-mediated TAK1 knock-down on the IL-1-mediated induction of C/EBPδ protein levels (Figure 7.5). Given that gene function is carried out by the protein that it encodes for, data generated relating to protein expression, is probably more conclusive than mRNA expression data and therefore taking this into consideration, we decided not to carry out any further experiments in relation to the role of TAK1 in the regulation of C/EBPδ expression by IL-1.

Interestingly, a recent study has demonstrated the existence of two independent pathways leading to the activation of NF-κB, as mediated by IL-1. One pathway is TAK1-dependent and the other MEKK3-dependent and both these pathways bifurcate at the level of IRAK1 (Yao *et al.* 2007). The possibility of an additional NF-κB activating mechanism, independent of TAK1 was first suggested through the study of TAK1 deficient cells (Sato *et al.* 2005; Shim *et al.* 2005). Although both studies reported that TAK1 deficiency results in defects in IL-1 signalling, IL-1-mediated NF-κB activation was only partially impaired in TAK1 deficient cells. As an extension of these studies, Yao and colleagues (2007) successfully identified two independent IL-1-regulated pathways leading to the activation of NF-κB. The first is TAK1-dependent and causes IKKα/β phosphorylation and IKKβ activation, leading to classical NF-κB activation through IκBα phosphorylation and degradation. The second pathway is TAK1-independent, MEKK3-dependent and induces IKKγ phosphorylation and IKKα activation, resulting in NF-κB activation through IκBα phosphorylation and its subsequent dissociation from NF-κB but without causing IκBα degradation. Furthermore, this novel MEKK3-dependent pathway of IL-1-mediated NF-κB activation was found to be conserved in primary intestinal epithelial cells, suggesting that the existence of this pathway is not just restricted to the mouse embryonic fibroblasts in which it was first identified.

Therefore, it is plausible that the regulation of IL-1-mediated C/EBPδ expression by NF-κB, in Hep3B cells, is not completely dependent on TAK1 activation (as suggested by our RNAi data) and could potentially be modulated through the activation of MEKK3, although further experiments would have to be carried out to elucidate this.

Results presented in chapter 4 show that both pharmacological inhibitors apigenin and curcumin significantly inhibit the IL-1-induced expression of C/EBPδ. Several studies have reported that these agents, apigenin and curcumin can also act as inhibitors of NF-κB (Gerritsen *et al.* 1995; Singh and Aggarwal 1995; Xu *et al.* 1997; Jobin *et al.* 1999; Liang *et al.* 1999; Plummer *et al.* 1999; Choi *et al.* 2004; Schulze-Tanzil *et al.* 2004; Shukla and Gupta 2004a; Shukla and Gupta 2004b; Cao *et al.* 2005; Moon *et al.* 2005; Moon *et al.* 2006; Cho *et al.* 2007; Shakibaei *et al.* 2007). In particular, with relation to the action of apigenin, inhibition of CK2 by this agent impairs NF-κB activation as CK2 is a known regulator of NF-κB (Romieu-Mourez *et al.* 2001; Romieu-Mourez *et al.* 2002; Cavin *et al.* 2003; Yu *et al.* 2006).

To determine whether apigenin and curcumin inhibited NF-κB activation as mediated by IL-1 in Hep3B cells, we carried out a transient transfection assay. The pNFκB-Luc plasmid contains four NF-κB enhancer sequence elements fused upstream of a luciferase-reporter gene. Transfection of this plasmid into Hep3B cells, coupled with IL-1 treatment, induces a dramatic increase in luciferase-reporter activity. However, pre-treatment with apigenin or curcumin significantly inhibits the increase in reporter activity as induced by IL-1, although with varying degrees of inhibition with each agent (Figure 7.6). Therefore, our results suggested that these agents can act to impair NF-κB activation in Hep3B cells. In related transfection based assays, several studies have reported similar findings (Gerritsen *et al.* 1995; Liang *et al.* 1999; Plummer *et al.* 1999; Cavin *et al.* 2003; Yu *et al.* 2006). It is also important to mention that, given that our pharmacological inhibitor data from chapter 4 also shows that the JNK MAPK specific-inhibitor, SP600125 significantly inhibits the IL-1-mediated increase in C/EBPδ expression, it is also necessary to establish the effects of this inhibitor on NF-κB activation. Unfortunately, due to time constraints, we were unable to carry out these experiments and therefore this is an area that requires future investigation.

In an attempt to determine a possible mechanism by which IL-1 action leads to an increase in the *trans*-activation potential of NF-κB, we investigated a role for CK2 and JNK MAPK in the regulation of this response. As previously mentioned (see Table 4.0); numerous studies have implicated apigenin and curcumin as inhibitors of CK2 and JNK MAPK respectively. Indeed, our results from chapter 5 (Figure 5.7) also suggest that curcumin is an effective inhibitor of JNK activity in Hep3B cells, consistent with previous studies (see chapter 5). Therefore, we wanted to determine if the inhibitory action of apigenin and curcumin on IL-1-mediated *trans*-activation by NF-κB was mediated through the action of these inhibitors on CK2 and JNK, (as mentioned in section 7.1.1, several studies have reported a role of JNK and CK2 in the regulation of NF-κB activation), or whether the effect was independent of their action on these kinases. For this, we used plasmids specifying for DN mutant forms of CK2 and JNK. We assessed the effect of these expression plasmids on NF-κB activation by IL-1 in co-transfection assays (Figure 7.7). Our results suggest that JNK may not contribute to the activation of NF-κB by IL-1, given that expression of a DN mutant of this kinase did not significantly attenuate this response, compared to the appropriate control. In contrast, we noted a minor but significant decrease in the IL-1-induced reporter activity, in cells co-transfected with a DN mutant form of CK2, compared to the control. Although, the decrease was minor, this result does suggest that CK2 may contribute to the activation of NF-κB by IL-1, in Hep3B cells to some extent. This is consistent with a role for CK2 as a regulator of NF-κB phosphorylation, as mediated by IL-1 in hepatocytes, suggested by Bird *et al.* (1997). In addition, a role for CK2 in the regulation of NF-κB is suggested in several other reports. For example, inhibition of CK2 blocks Her-2/neu (second member of the epidermal growth factor receptor family of tyrosine kinases) –mediated activation of NF-κB, in breast cancer cells (Romieu-Mourez *et al.* 2002). CK2 is also reported to regulate the phosphorylation of IKKβ and thus modulate activation of NF-κB in squamous carcinoma cells (Yu *et al.* 2006). In hepatocytes, TGF-β1-mediated inhibition of CK2 activity promotes IκBα stabilisation and therefore inhibits NF-κB activation (Cavin *et al.* 2003). CK2 is also known to regulate the *trans*-activation potential of the p65 NF-κB subunit by phosphorylation at serine$^{529}$ in response to TNF-α treatment (Wang *et al.* 2000). Interestingly, p65 NF-κB is also reported to be a phosphorylation target for CK2 in IL-1-treated HepG2 cells, and one of the putative

sites for phosphorylation was suggested to be a serine residue potentially located in the C-terminal *trans*-activation domain of this NF-κB subunit (Bird *et al.* 1997).

Although the results in Figure 7.7 do suggest a minimal requirement for CK2 in the regulation of NF-κB activation by IL-1, we at present, cannot rule out the possibility of potential functional redundancy between CK2 and JNK MAPK, as we have not analysed the effect of simultaneously inhibiting CK2 and JNK MAPK using DN mutants on NF-κB activation by IL-1 in our transient transfection assay. Thus, this suggested experiment may be necessary for future studies.

So, although our results from Figure 7.6 show that both apigenin and curcumin inhibit NF-κB activation by IL-1, it is unlikely that these agents mediate this effect through their action on CK2 or JNK MAPK exclusively, as suggested by our results in Figure 7.7. It is plausible that these agents may act to inhibit various components of the NF-κB activation pathway, independently of their ability to inhibit CK2 and JNK MAPK respectively. In support of this, several studies have suggested that curcumin acts as an inhibitor of NF-κB activation by blocking IκB phosphorylation and its subsequent ubiquitin-triggered degradation, in addition to its ability to inhibit the JNK-AP1 pathway (Singh and Aggarwal 1995; Xu *et al.* 1997; Jobin *et al.* 1999; Moon *et al.* 2005; Moon *et al.* 2006). In other studies, curcumin is suggested to prevent the nuclear translocation and DNA binding of NF-κB (Schulze-Tanzil *et al.* 2004; Cao *et al.* 2005; Shakibaei *et al.* 2007). The inhibitory action of apigenin on NF-κB activation is reported to act at multiple levels. Apigenin is reported to inhibit constitutive expression of both p50 and p65 NF-κB subunits in human prostate cancer cells, DU145 (Shukla and Gupta 2004b). Also, apigenin is reported to prevent NF-κB nuclear translocation, DNA binding (Choi *et al.* 2004), IκB degradation and IKK activity (Liang *et al.* 1999). However, it should also be noted is that a role for CK2 in the regulation of NF-κB activation was not investigated in any of the aforementioned studies. Therefore, it still remains to be determined whether the inhibitory effect of apigenin on NF-κB activation as documented in these studies, is through its inhibition of CK2 or whether its effect is independent of this.

We next examined the effect of IL-1 on NF-κB DNA binding. Using a radiolabelled oligonucleotide containing a consensus κB sequence, we show that IL-1 induces the DNA binding of NF-κB (Figures 7.8-7.10), consistent with our previous data

presented in this chapter supporting a role for this transcription factor in the regulation of C/EBPδ expression by IL-1. In an effort to initiate investigations into resolving the mechanism by which both curcumin and apigenin attenuate the activation of NF-κB by IL-1, we examined the effect of these inhibitors on NF-κB binding. Both these inhibitors significantly attenuated the DNA-binding of NF-κB as mediated by IL-1 (Figure 7.11). Preliminary data (Figure 7.12, Panel A) indicates that the DNA-binding of NF-κB, induced by IL-1 is inhibited by curcumin in a concentration-dependent manner. Although apigenin also does attenuate the DNA-binding of NF-κB (Figure 7.12, Panel B), consistent with our previous data, whether this effect is concentration-dependent is difficult to assess from the current data. Therefore, further experiments would have to be carried out in order to confirm these findings. Together, our data suggests that both apigenin and curcumin inhibit NF-κB activation as induced by IL-1 by preventing DNA-binding. Similar results have been reported by other researchers. For example, curcumin has been shown to concentration-dependently inhibit the DNA-binding of NF-κB as induced by TNF-α, in human colon epithelial cells (Plummer *et al.* 1999). Similar results are reported by Jobin *et al.* (1999), where curcumin also inhibited the NF-κB DNA-binding as mediated by IL-1 in the human HT-29 colonic epithelial cell line. Furthermore, results presented by Singh and Aggarwal (1995) show that curcumin inhibits NF-κB DNA binding in the human myelomonoblastic leukemia cell line, ML-1a. Apigenin is also reported to prevent NF-κB DNA-binding (Liang *et al.* 1999; Romieu-Mourez *et al.* 2001; Cavin *et al.* 2003). However, to our knowledge this is the first study to show that these pharmacological agents may act as inhibitors of NF-κB in the Hep3B cell line.

Our EMSA data strongly indicates that NF-κB DNA-binding to a consensus κB oligonucleotide, is induced in response to IL-1 action in Hep3B cells. However, in order to extend these investigations to potentially provide further experimental evidence for a role of NF-κB in the regulation of C/EBPδ expression by IL-1, it is also necessary to determine whether NF-κB-DNA binding is induced by IL-1, to putative NF-κB binding sites as identified in the human C/EBPδ gene promoter, presented in Figure 7.13. Ideally this should be done in an *in vivo* setting with normal chromatin context, achieved by ChIP analysis, given that our results in chapter 3 suggest that the C/EBPδ gene promoter is not responsive to IL-1 action in an *in vitro* transfection assay. Identification of NF-κB subunits by ChIP analysis would entail Hep3B cells

being treated with IL-1 (or left untreated) for a requisite time period, and then incubated with formaldehyde to cross-link the NF-κB subunits to DNA *in vivo*. Nuclear fractions of the cells would then be prepared and following lysis, the chromatin would be fragmented by sonication and subjected to immunoprecipitation using antibodies specific to the NF-κB subunit(s) of interest. Immunoprecipitates would then be used to purify genomic DNA fragments which would then be subjected to PCR analysis. Specifically, PCR analysis would be carried out using primers that would amplify the region containing the putative p50 and p65 NF-κB binding sites of the human C/EBPδ gene proximal promoter (Figure 7.13). After resolving the PCR products by agarose gel electrophoresis, densitometric analysis of the PCR products from untreated verses IL-1-treated samples would then reveal if NF-κB binding was indeed induced at the C/EBPδ gene promoter in response to IL-1 action. If indeed, the results from these experiments were positive then this would provide very strong evidence for a role for NF-κB in the regulation of C/EBPδ expression by IL-1, together with our other results presented in this chapter. In addition, these investigations could also be extended to the analysis of pharmacological inhibitor action on NF-κB-DNA-binding in this context, to confirm the inhibitory action of apigenin and curcumin on NF-κB DNA-binding.

Furthermore, to establish whether the inhibitory action of apigenin and curcumin on NF-κB DNA-binding occurs by the direct action of these inhibitors on NF-κB, EMSA could be carried out using *in vitro* generated p50 and p65, for example. Similarly, cell-free *in vitro* transcription assays, using extracts depleted of CK2 or JNK MAPK, could be useful to determine whether CK2 or JNK MAPK were involved in the regulation of NF-κB-mediated gene transcription, thus helping to definitively establish whether there is a requirement for these signalling mediators in the regulation of NF-κB activity.

In summary, the results presented in this chapter suggest a role for NF-κB in the regulation of C/EBPδ expression by IL-1, a novel finding. We show that IL-1 activates NF-κB in stimulated Hep3B cells and that this is inhibited by the pharmacological agents apigenin and curcumin, through the interference of DNA-binding. In further support for a role of NF-κB in the regulation of C/EBPδ expression by IL-1, both these inhibitors were shown to inhibit the IL-1-mediated increase in C/EBPδ expression at both the mRNA and protein level in chapter 4. In

addition, preliminary data also presented in chapter 4 shows that an independent NF-κB inhibitor (NF-κB Activation Inhibitor) also attenuates the IL-1-mediated induction of C/EBPδ expression at the protein level.

# CHAPTER EIGHT: GENERAL DISCUSSION

## 8.1 OVERVIEW OF RESULTS PRESENTED IN THIS THESIS

C/EBPδ is an important transcription factor involved in the regulation of the inflammatory response, where its expression/activity is controlled by many inflammatory mediators including the cytokines IL-1 and IL-6. It is therefore of interest to examine the mechanisms governing its expression during this process. Unfortunately, only a limited number of studies have focused on discerning the signalling events leading to its expression and activation in response to inflammatory mediators.

Evidence also strongly suggests that C/EBPδ is a major regulator of cellular growth and proliferation (O'Rourke *et al.* 1999b; Sivko *et al.* 2004). Its expression in LNCaP prostrate cancer cells is modulated by IL-6 action and its induction by this cytokine, induces growth arrest in these cells (Sanford and DeWille 2005). Therefore, studying the regulation of C/EBPδ expression by cytokines such as IL-6 should not only have an impact on understanding mechanisms of inflammation but may also have a broader impact on our knowledge of other processes that it regulates, such as cell growth and proliferation.

The work detailed in this study has used several different approaches in order to elucidate mechanisms into the regulation of C/EBPδ expression by IL-1 and to a lesser extent, IL-6. The work was carried out in hepatocytes as these cells play a key role in the regulation of the inflammatory response and are known targets for the action of IL-1 and IL-6.

Our initial studies were designed to analyse the effects of various inflammatory mediators on the expression of C/EBPδ. Of the mediators examined, we found that IL-1, IL-6 and IFN-γ caused a significant increase in its expression, consistent with previous observations (Juan *et al.* 1993; Ramji *et al.* 1993a; Cantwell *et al.* 1998) and we extended our investigations to include a time-course analysis of C/EBPδ expression by IL-1 and IFN-γ. Additionally, we confirmed that the effect of IL-1 action on C/EBPδ expression occurred in a concentration-dependent manner. Because we, like others (Cantwell *et al.* 1998), noted that IFN-γ was less efficient at inducing C/EBPδ expression, than other cytokines like IL-1 and IL-6, we did not continue our investigations with this cytokine. However, this is an area that may be

the focus of future studies in our laboratory, given that IFN-γ is a major inflammatory cytokine.

In line with the original aims of this study, whereby we intended to fully delineate the regulatory elements present in the human C/EBPδ gene promoter that were responsible for mediating its induction in response to IL-1, we also initiated investigations into the regulation of C/EBPδ promoter activity. Using a transient transfection system that we had established, we examined the effects of both IL-1 and IL-6 on the promoter activity of both human and murine C/EBPδ genes. Whilst IL-6 action caused a significant increase in promoter activity from both species, we did not observe a similar response with IL-1. Consequently, we verified that our transfection system was able to produce a response to IL-1 action at the level of promoter activity with a positive control in our experiments. Therefore, although we clearly demonstrated that the C/EBPδ gene promoters were active in transfected cells, in contrast to the observed increase in mRNA and protein levels of C/EBPδ by IL-1, the promoters were not responsive to this cytokine. There are several possible reasons for this, but in our view, one of the most likely explanations for this is that for maximum activation of the C/EBPδ gene *in vivo*, there may be a requirement for a specific chromatin context, that is absent from transfected plasmid DNA. Indeed, recent studies have shown that C/EBPδ gene activation is highly dependent on the methylation status of its promoter *in vivo* (Tang *et al.* 2006; Agrawal *et al.* 2007).

Additionally, our other transfection data strongly indicates that the human C/EBPδ gene is auto-regulated, a novel finding. We further demonstrated that the proximal promoter region of the gene is sufficient to mediate this effect and have suggested candidate *cis*-acting elements that may be responsible for auto-regulation. However, in order to more firmly pin-point the *cis*-acting elements that are responsible for mediating auto-activation of the gene, further deletion/mutation constructs of the gene promoter would have to be generated and analysed (see section 8.3).

In light of the findings presented in chapter 3, it was therefore decided, in line with our original aims, to focus on investigating the signalling pathways by which IL-1 regulates the expression of C/EBPδ. Experiments based on the use of pharmacological inhibitors of potential mediators of IL-1 signalling, identified a selective and novel role for CK2, JNK MAPK and NF-κB in the regulation of this

response. A role for JNK MAPK was subsequently supported through the use of western blot analysis and associated kinase assays. We showed that IL-1 transiently activated the JNK MAPK in stimulated hepatocytes and confirmed that pharmacological inhibitors of JNK MAPK acted specifically to block the activation of this kinase by IL-1. Using the same approach, we also showed that CK2 was not involved in regulating the activation of JNK MAPK by IL-1, suggesting the existence of an independent IL-1-regulated signalling pathway. Therefore, our work was also extended to the examination of CK2 activation by IL-1. Consistent with the findings of Bird *et al.* (1997) and a very recent study by Parhar *et al.* (2007), the only studies to our knowledge, to have examined the effect of IL-1 action on CK2 activation, we did not note any consistently marked increase in overall CK2 activity in response to IL-1 action, from CK2α immunoprecipitates. However, we could not rule out a role for CK2 in the regulation of C/EBPδ expression by IL-1, based on these results alone given that our kinase assay was limited to the immunoprecipitation of CK2α and to the use of a single substrate, β-casein. Furthermore, the Bird *et al.* (1997) study had implicated CK2 as a regulator of p65 NF-κB phosphorylation, in IL-1 treated HepG2 hepatocytes, a closely related cell line to our model hepatocyte cell line, Hep3B. This observation was consistent with our pharmacological inhibitor data that suggested a role for both CK2 and NF-κB in the regulation of C/EBPδ expression by IL-1.

We also noted that expression of DN mutants of CK2, JNK and c-Jun did not attenuate the IL-1-mediated induction of C/EBPδ expression, potentially suggesting the existence of some functional redundancy between the two signalling pathways. Extensive cross-talk and overlap amongst signalling pathways has previously been documented and the existence of functionally redundant signalling pathways is therefore plausible and indeed has been reported (Fambrough *et al.* 1999; Mestre *et al.* 2001).

Therefore, in an attempt to more definitively establish the role of CK2 and JNK MAPK in the expression of C/EBPδ by IL-1, we adopted an alternative strategy. One of the most effective ways of elucidating the functional properties of a component of a signalling pathway is to suppress its expression with for example, the use of siRNA. As discussed in chapter 6, the use of RNAi as a method for pathway dissection has been tremendously successful. To illustrate this, a recent literature search has identified over 12,000 studies in which the authors have made use of RNAi as a

research tool (see chapter 6 for examples). RNAi is particularly useful because it is highly specific and also allows the researcher to overcome barriers associated with functional redundancy. More specifically, with the use of multiple siRNAs, one can examine the effects of simultaneous gene knock-down on a response of interest. We therefore examined the effect of siRNA-mediated knock-down of CK2 and components of the JNK MAPK pathway on the IL-1-mediated induction of C/EBPδ expression. We set up an effective transfection system to successfully deliver siRNA into Hep3B cells, where in most cases the knock-down of gene expression, as assessed at the mRNA and/or protein level, was more than 50%. However, siRNA-mediated knock-down of either CK2 (CK2α and CK2α') or JNK (JNK1 and JNK2) and also c-Jun, could not confirm a role for these signalling mediators in the regulation of C/EBPδ expression by IL-1. We therefore addressed the issue of functional redundancy by investigating the effect of simultaneous knock-down of CK2α and JNK (JNK1 and JNK2) with siRNA. Concomitant knock-down of CK2α and JNK (JNK1 and JNK2) also did not attenuate the IL-1-mediated increase in C/EBPδ expression. Based on these data alone however, we cannot completely rule-out the existence of functional redundancy between the JNK and CK2 pathways, as we were unable to examine the effect of depleting levels of both catalytic subunits of CK2 and JNK simultaneously, given the high concentration of siRNA required for this particular experiment. Therefore, the issue of functional redundancy may have to be readdressed in future investigations. Of further note, as discussed in chapter 6, siRNA-mediated suppression of gene expression only results in a knock-down of expression, not a complete knock-out, therefore there is always the possibility that any remaining protein, escaping the silencing process, is sufficient for function, thus also potentially explaining our results.

In addition to analysing putative signalling pathways by which IL-1 action resulted in an increase in C/EBPδ expression using siRNA, we also carried out investigations, using the same approach, to determine which signalling mediators led to the induction of C/EBPδ expression by IL-6. Previous studies have pointed to a role of STAT3 in the regulation of this response, primarily with data generated from promoter-reporter assays (Yamada *et al.* 1997; Cantwell *et al.* 1998) and therefore we took this into consideration with the design of our experiments. Simultaneous siRNA-mediated knock-down of both STAT1 and STAT3 significantly attenuated the endogenous

expression of C/EBPδ by IL-6, and to our knowledge we are the first to report this. Furthermore, we additionally showed that IL-6 stimulation of Hep3B cells led to the transient activation of both these transcription factors, albeit with different kinetics. However, further investigations are required to ascertain the exact signalling events resulting in their activation.

Using the same siRNA-based approach, we have also identified NF-κB as a regulator of C/EBPδ expression by IL-1, consistent with our inhibitor data that suggested a requirement for this transcription factor in the regulation of this response. These are novel data, as to our knowledge we are the first to report such a finding. We further report that this response is not completely dependent on a previously identified upstream regulator of IL-1-mediated NF-κB activation, the MAPKKK, TAK1. Consistent with our data, we show that NF-κB is activated by IL-1 using a transfection-based assay and by EMSA. Our data also suggests that the pharmacological inhibitors apigenin and curcumin inhibit NF-κB activation by IL-1, possibly by preventing its binding to DNA in Hep3B cells, also a novel finding. The role of JNK MAPK and CK2 in the regulation of NF-κB activation was also investigated given that both these signalling mediators have been reported to modulate NF-κB and because the pharmacological inhibitors of these kinases (curcumin and apigenin, respectively) attenuated NF-κB activation by IL-1 (see chapter 7 for details). The role of CK2 in the regulation of this response was of particular interest, given that the Bird *et al.* (1997) study had suggested a direct involvement of CK2 in the regulation of NF-κB phosphorylation by IL-1, in HepG2 hepatocytes. Our data suggest that CK2 is, at least in part, responsible for the regulation of NF-κB by IL-1 in Hep3B cells, as a DN mutant of this kinase significantly prevented *trans*-activation of NF-κB by IL-1, in a transient transfection assay.

Therefore, in summary, the work presented in this thesis has uncovered several key findings in relation to the expression of C/EBPδ during the inflammatory response. These being:

- Induction of C/EBPδ expression by the inflammatory cytokines IL-1, IL-6 and IFN-γ
- Auto-regulation of the human C/EBPδ gene
- Identification of STAT1 and STAT3 as mediators of C/EBPδ expression by IL-6

- Identification of NF-κB as a regulator of C/EBPδ expression by IL-1

Furthermore, these data can be approached with a good degree of confidence given that we have carried out all our experiments with appropriate controls, accurately reproduced previous findings, and verified novel findings with the use of a number of experimental approaches.

During the final stages of completion of this Ph.D. project, a study was published by Dong *et al.* (2007), in which the role of C/EBPδ in the regulation of PAI-1 expression by IL-1 and IL-6 was examined in HepG2 hepatocytes. Consistent with our data, the authors showed that both IL-1 and IL-6 induced the expression of C/EBPδ in human hepatocytes and additionally in primary murine hepatocytes. In conjunction, their data also indicated that C/EBPδ was the major transcription factor involved in inducing PAI-1 expression in response to both IL-1 and IL-6, consistent with its key role in the APR.

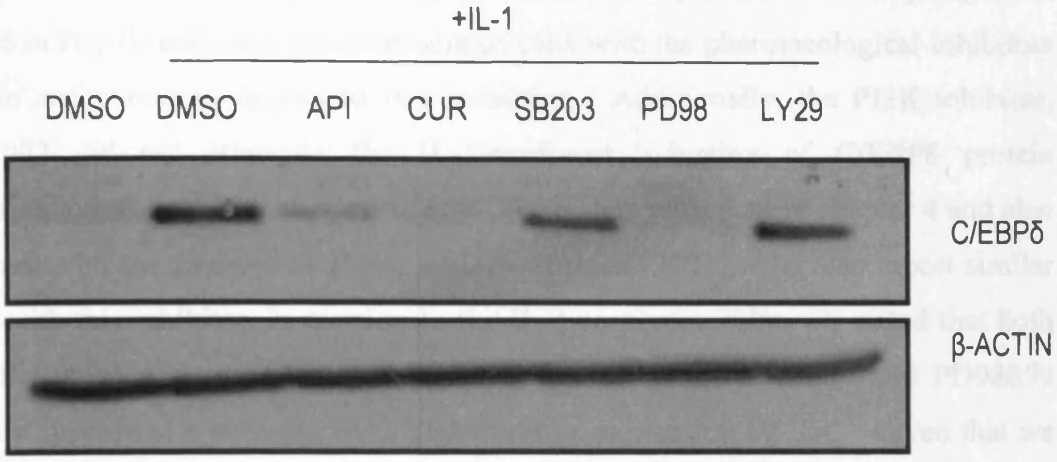
Similar to our initial investigations, in an effort to identify the key signal transduction pathways by which C/EBPδ, and in association PAI-1, were induced by both cytokines, a panel of pharmacological inhibitors were employed for analysis. Indeed, some of the inhibitors used in this study were also used in the Dong *et al.* (2007) study. These included the JNK MAPK inhibitor, SP600125; the p38 MAPK inhibitor, SB203580 and the PI3K inhibitor LY294002. In addition, the ERK MAPK inhibitor, U0126; the JAK inhibitor, JAK inhibitor 1; the NF-κB inhibitor, SN50 and the PKB/Akt inhibitor, API-2 were also used in their study. By contrast, we used in this study, a second JNK MAPK inhibitor, curcumin; a second p38 MAPK inhibitor SB202190; the ERK MAPK/MEK1 inhibitor, PD98059; the NF-κB inhibitor, NF-κB Activation Inhibitor and the CK2 inhibitor, apigenin.

Again, using a similar approach to ours, Dong and colleagues (2007) examined the effect of blocking the aforementioned signalling pathways on C/EBPδ expression (mRNA and protein) as induced by IL-1 and IL-6. The JNK MAPK inhibitor, SP600125 was shown to significantly impair the induction of C/EBPδ expression by IL-1, in HepG2 hepatocytes. Consistent with these findings, we report similar findings in Hep3B hepatocytes and we additionally confirmed these results with a second JNK MAPK inhibitor, curcumin. However, Dong and colleagues (2007) also

report that the IL-1-mediated induction of C/EBPδ expression was attenuated by the p38 MAPK inhibitor, SB203580 and the ERK MAPK inhibitor U0126, at both the mRNA and protein level. In contrast, we did not note similar results by examining the effects of the two p38 MAPK inhibitors (SB202190 and SB203580) and the ERK MAPK/MEK1 inhibitor, PD98059 on the IL-1-mediated induction of C/EBPδ mRNA expression as determined by semi-quantitative RT-PCR (see chapter 4). Therefore, in light of these recent results we decided to determine the effects of the p38 MAPK inhibitor, SB203580 and the ERK MAPK/MEK1 inhibitor, PD98059 on the induction of C/EBPδ protein as mediated by IL-1, by western blotting given that we have not previously carried out this experiment. In addition, we also decided to examine the effect of the PI3K inhibitor, LY294002 on this response given that we have also not previously determined its effect on C/EBPδ protein expression as mediated by IL-1. Furthermore, as a positive control, we also included the inhibitors apigenin and curcumin in our experiments as we have previously demonstrated that both inhibitors significantly attenuate the IL-1-mediated induction of C/EBPδ expression at both the mRNA and protein level (Chapter 4).

Therefore, Hep3B cells were pre-treated with the aforementioned inhibitors or DMSO (vehicle-control) for 1h. They were then either treated with IL-1 or left untreated for 3h before being harvested for protein extraction (Section 2.7.2) and subsequent western blotting (Sections 2.7.7 and 2.7.8). Immunodectection of C/EBPδ and β-actin was carried out as described in section 2.7.9 using C/EBPδ and β-actin specific antibodies. The results of these experiments are shown in Figure 8.1.

A.




B.

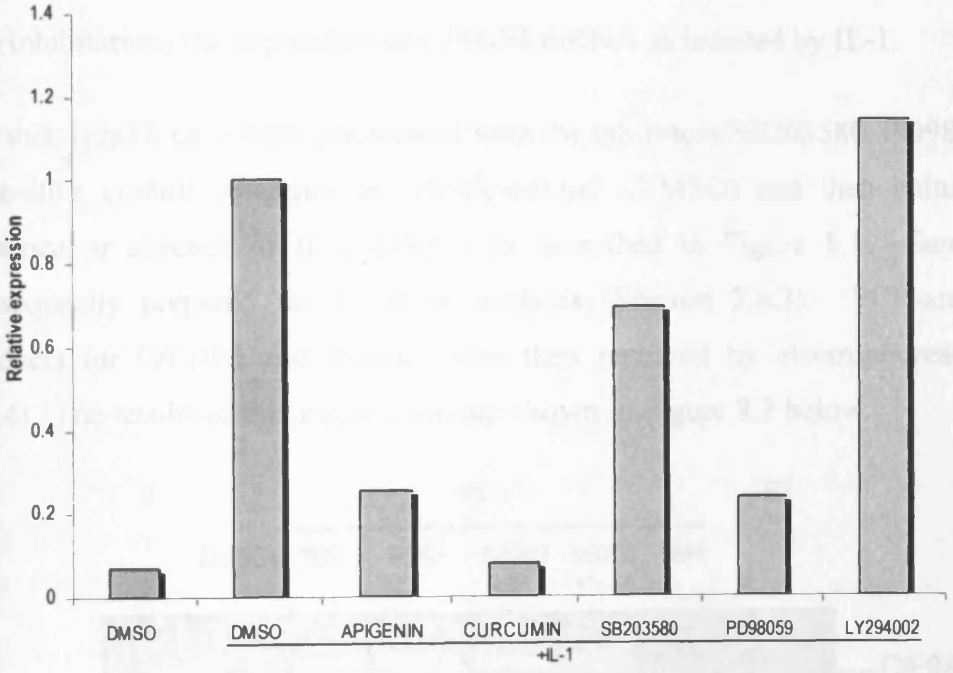



**FIGURE 8.1. Effect of pharmacological inhibitors on the IL-1-mediated induction of C/EBPδ protein expression**. Hep3B cells were pre-treated with pharmacological inhibitors apigenin; API (20μM), curcumin; CUR (30μM), SB203580; SB203 (5μM), PD98059; PD98 (50μM) and LY294002; LY29 (5μM) or DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated for 3h and harvested for protein extraction (Section 2.7.2). Total cell protein extracts were used for western blot analysis with 60μg of each sample subjected to 10% (w/v) SDS PAGE and subsequent western blotting. Immunodetection of C/EBPδ was carried out using the anti-C/EBPδ antibody as described in section 2.7.9. The same blot was then re-probed with the anti-β-actin antibody (A). Panel B displays the ratios of C/EBPδ:β-actin, normalised to the IL-1 treated DMSO control as determined by densitometric analysis. The data shown is the mean from two independent experiments.

Consistent with our previous findings, IL-1 dramatically induced the expression of C/EBPδ in Hep3B cells and pre-treatment of cells with the pharmacological inhibitors apigenin and curcumin decreased this induction. Additionally, the PI3K inhibitor, LY294002 did not attenuate the IL-1-mediated induction of C/EBPδ protein expression, consistent with our previous RT-PCR data presented in chapter 4 and also consistent with the findings of Dong and co-workers (2007), who also report similar results with this inhibitor, in relation to the IL-1 response. Also, we noted that both the p38 MAPK and the ERK MAPK/MEK1 inhibitors, SB203580 and PD98059 respectively, caused a decrease in C/EBPδ protein expression by IL-1. Given that we had not previously noted a similar effect at the level of mRNA, as assessed by semi-quantitative RT-PCR (Chapter 4), we also decided to re-examine the effects of these two inhibitors on the expression of C/EBPδ mRNA as induced by IL-1.

For this, Hep3B cells were pre-treated with the inhibitors SB203580, PD98059 and as a positive control, apigenin or vehicle-control (DMSO) and then cultured in the presence or absence of IL-1 exactly as described in Figure 8.1. Samples were subsequently prepared for RT-PCR analysis (Section 2.6.3). PCR-amplification products for C/EBPδ and β-actin were then resolved by electrophoresis (Section 2.6.4). The results of this experiment are shown in Figure 8.2 below.

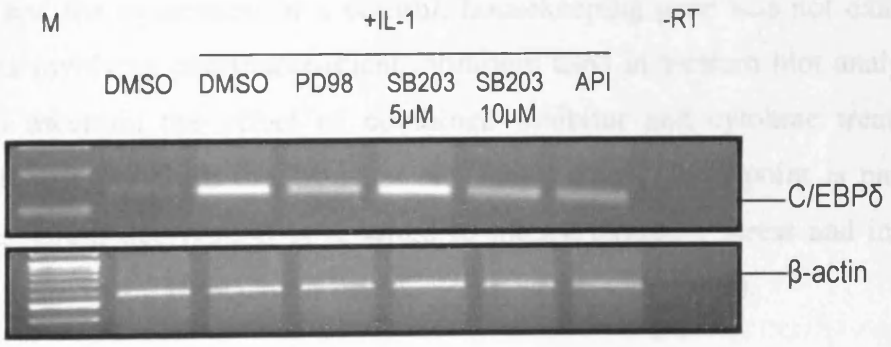



**FIGURE 8.2. Effect of PD98059, SB203580 and apigenin on the IL-1-mediated induction of C/EBPδ mRNA expression.** Hep3B cells were pre-treated with pharmacological inhibitors PD98059; PD98 (50μM), SB203580; SB203 (5μM and 10μM), apigenin; API (20μM) or DMSO as a vehicle control for 1h. They were then either treated with IL-1 (100U/ml) or left untreated for 3h. Total RNA was then extracted and used for RT-PCR analysis. The PCR amplification products for C/EBPδ and β-actin were resolved on 1.5% (w/v) agarose gels by electrophoresis. M denotes the 100bp DNA ladder (Appendix II). –RT denotes the RT-PCR in which no reverse transcriptase was included for the preparation of cDNA (using DMSO –IL-1 treated RNA).

Consistent with our previous findings, the IL-1-mediated induction of C/EBPδ was inhibited by apigenin and not affected by pre-treatment of cells with SB203580 at a concentration of 5μM (Chapter 4). However, we noted that at the higher concentration of 10μM, SB203580 did attenuate the response. Given that we have not previously carried out this experiment using this higher concentration of SB203580, this result requires confirmation. Therefore, although we, like Dong and co-workers (2007), have previously only used SB203580 at the concentration of 5μM, at this concentration of the inhibitor the IL-1-mediated induction of C/EBPδ expression is not attenuated, at least at the mRNA level in Hep3B cells. In contrast, the same concentration of the inhibitor caused a significant decrease in the IL-1-mediated induction of C/EBPδ expression at the mRNA level as assessed by quantitative RT-PCR in HepG2 cells, data presented by Dong and colleagues (2007). However, one should note that the experimental conditions vary between this study and that of Dong *et al.* (2007). Whilst in our study, all our experiments were carried out in Hep3B cells cultured in medium containing 10% (v/v) serum (FCS), in the Dong *et al.* (2007) study, HepG2 cells were cultured in medium without serum for 24h prior to experimentation. Furthermore, HepG2 cells were then cultured in the presence of pharmacological agents for 1h and subsequently exposed to IL-1 or IL-6 for a further 4h, again in the absence of serum. Given that no cell viability assay was carried out in this study and the expression of a control, housekeeping gene was not examined in experiments involving pharmacological inhibitors used in western blot analysis, it is difficult to ascertain the effect of combined inhibitor and cytokine treatment on HepG2 cell viability from the Dong *et al.* (2007) study. This point is particularly relevant, as serum deprivation is reported to induce oxidative stress and in extreme cases cell death, in the HepG2 cell line (Bai and Cederbaum 2006).

In addition, to the above mentioned observations, we also noted (Figure 8.2) that unexpectedly, the inhibitor ERK MAPK/MEK1 inhibitor, PD98059 also caused a decrease in the IL-1-mediated induction of C/EBPδ expression at the mRNA level. Given that we have not observed this decrease in our previous experiments (Chapter 4), this result requires further investigation. It is also worthy of note that owing to the constant expression of β-actin, that none of the inhibitors used in our experiments were having a global affect on cellular gene expression, and therefore the results presented in Figure 8.1 and 8.2 were specific to C/EBPδ expression. Therefore, if the

results presented in Figures 8.1 and 8.2 are confirmed, in relation to the inhibitory action of both SB203580 and PD98059, on the IL-1-mediated induction of C/EBPδ expression, further experiments will be required to ascertain the roles of the p38 and ERK1/2 MAPK pathways in the regulation of this response.

The other result presented by Dong *et al.* (2007) that merits discussion is that these authors did not observe a decrease in the expression of C/EBPδ as induced by IL-1, in response to the inhibition of NF-κB by a pharmacological agent, SN50. This is in contrast to our data, in which we show that, not only does a pharmacological inhibitor of NF-κB attenuate this response (Chapter 4) but also confirm this by using siRNA targeting NF-κB (Chapter 7). In addition, we have also carried out a series of investigations confirming that NF-κB is indeed activated by IL-1 in Hep3B cells, consistent with previous reports (see chapter 7 for details). Although the role of NF-κB in the regulation of C/EBPδ expression by IL-1 was investigated by Dong *et al.* (2007), their data is restricted to the use of the pharmacological inhibitor SN50, and additionally these authors present no evidence to suggest that this inhibitor does indeed inhibit NF-κB in HepG2 cells at the concentration they have used. In addition, no experiments were carried out to determine the effect of different concentrations of this inhibitor on the expression of C/EBPδ as mediated by IL-1. Another potential explanation for these contrasting results may be in the use of the different cell lines between this study (Hep3B) and that of Dong and co-workers (HepG2). Although both these cell lines are closely related, differences in signalling, in response to cytokine action have been reported. For example, in Hep3B cells, IL-6 leads to the activation of the PI3K/Akt pathway (Chen *et al.* 1999), whereas no significant Akt activation is observed in IL-6-treated HepG2 cells (Kortylewski *et al.* 2003). Thus, cell-type specific signalling events may also potentially explain the differences in the results.

In relation to the expression of C/EBPδ by IL-6, the Dong *et al.* (2007) study reports a major role for the JAKs in the regulation of this response, because a generic JAK inhibitor attenuates this response. However, this result is in contrast to Dr S.A. Rogers' (personal communication) data (Figure 6.15, Chapter 6), who showed that the JAK2 inhibitor, AG490 did not attenuate this response in Hep3B cells. Therefore, although a role for JAK2 in the regulation of this response is unlikely, given that Dr S.A. Rogers' pharmacological inhibitor data does not support this, the role of other

JAKs, like JAK1 and Tyk2 should be investigated further. This is also important given that our siRNA data fully supports a role for the STATs in the regulation of C/EBPδ expression by IL-6. Additionally, it is worthy of note that the Dong *et al.* (2007) study did not investigate a role for the JNK MAPK or CK2 in the regulation of the IL-6-mediated induction of C/EBPδ expression, or investigate a role for CK2 in the regulation of the IL-1 response.

One should also take into consideration that in the Dong *et al.* (2007) study, the identification of the signalling pathways by which IL-1 and IL-6 mediate the induction of C/EBPδ expression, is purely based on results obtained with the use of pharmacological agents and no other follow-up experiments (for example, with the use of siRNA) were carried out to investigate these initial findings. This point is particularly significant in the light of the findings of this study and stresses the importance of carrying out additional experiments to back-up preliminary findings. In further support of this, it should also be noted that preliminary RNAi data presented in chapter 6 (Figure 6.12), does not suggest an immediate role for the p38 MAPK in the regulation of C/EBPδ expression by IL-1 in Hep3B cells. siRNA mediated knock-down of p38 MAPK did not attenuate the induction of C/EBPδ expression by IL-1 at the mRNA level, compared to controls.

The following section will aim to discuss the findings of this thesis in relation to recently published reports relevant to the various aspects of our studies and summarises the significance of our findings in the context of regulation of the APR by C/EBPδ.

## 8.2 PERSPECTIVES

Studies presented in this thesis have provided evidence for a role of NF-κB, as a mediator of IL-1 signalling in Hep3B cells and have also implicated this factor as a regulator of C/EBPδ expression by IL-1. In addition our investigations also indicate that the protein kinase, CK2 may also be involved, at least in part, in regulating NF-κB activation by IL-1. A requirement for CK2 in the regulation of IL-1-mediated NF-κB activation has also been demonstrated in the recently reported study by Parhar and colleagues (2007). Whilst these authors showed that overall CK2 activity was unaffected by IL-1 treatment of HCT116 cells (human colon cancer cell line, intestinal epithelial cells), consistent with our data, they report kinase activation by

IL-1, in pools of CK2 bound to either p65 NF-κB or IKKγ/NEMO. Protein extracts from HCT116 cells treated with IL-1, immumnoprecipitated with antibodies for p65 or IKKγ, were subjected to a CK2 kinase assay using GTP as a phosphate donor and casein as a substrate. In both sets of immunoprecipitates, a modest increase in CK2 kinase activity, in response to IL-1 was observed. These findings are also consistent with those reported by the Bird *et al.* (1997) study that reported a marked increase in CK2 kinase activity as mediated by IL-1, in protein extracts immunoprecipitated with NF-κB-specific antibodies, but not in extracts immunoprecipitated using CK2-specific antibodies. Also consistent with our data, Parhar *et al.* (2007) report that the CK2 inhibitor apigenin, prevented the IL-1-induced activation of NF-κB in a transient transfection assay that mirrored the transfection-based assay we used in this study (Chapter 7, Figure 7.6). These researchers further report that DN mutants of CK2 also attenuated the activation of NF-κB as mediated by IL-1, consistent with our data. Moreover, another very significant result reported by these authors is that in response to IL-1, CK2 increases the *trans*-activation potential of the p65 NF-κB subunit by its phosphorylation of its C-terminal *trans*-activation domain at serine[529]. In contrast to our results however, which show that pre-treatment of Hep3B cells with apigenin prevents IL-1-induced NF-κB DNA binding, these researchers report that neither of the CK2 inhibitors, apigenin or DRB (5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole) affect the NF-κB DNA binding as mediated by IL-1 in Caco2 cells, although it should be noted that these experiments were only carried out with a single concentration of each inhibitor (Parhar *et al.* 2007). In light of these recent findings, a role for CK2 in the regulation of NF-κB activity by IL-1 in Hep3B cells may require further investigation as discussed in section 8.3.

As already discussed in chapter 1, NF-κB is a key mediator of IL-1 signalling. In addition, IL-1 is also known to activate all three branches of MAPK signalling, JNK, p38 and the ERK1/2 MAPKs. Indeed, we have shown that this cytokine activates both NF-κB and the JNK MAPK in Hep3B cells. A flow of recent studies have indicated that activation of the MAPKs, in some cases all three modules, and NF-κB is necessary for the induction of certain genes, in response to IL-1 action. This is of particular interest in light of the results presented in Figures 8.1. and 8.2. For example, induction of the matrix metalloproteinase-9 (MMP-9) gene by IL-1 is dependent on the activation of all three MAPKs and NF-κB for its expression in

human tracheal smooth muscle cells (Liang *et al.* 2007). Interestingly, activation of NF-κB is independent of MAPK activation, thus the MAPKs are likely to activate other transcription factors important for MMP-9 expression (Liang *et al.* 2007). In contrast, other studies suggest that a degree of cross-talk does exist between NF-κB and the MAPKs and that such cross-talk is important for IL-1-mediated gene expression. For example, IL-1 induces IL-8 production in lung epithelial cells, derived from cystic fibrosis patients, and its production is dependent on p38, ERK MAPK and NF-κB activation. However, the roles of these signalling mediators in the regulation of this response only became apparent with combined inhibition of either p38 or ERK MAPKs with NF-κB, determined with the use of pharmacological agents. Used individually, none of the pharmacological inhibitors of p38, ERK MAPK or NF-κB markedly impaired the production of IL-8 by IL-1 (Muselet-Charlier *et al.* 2007). As well as indicating that a degree of cross-talk exits between the p38, ERK MAPK and NF-κB dependent signalling pathways, this study also suggests the existence of some functional redundancy between the pathways.

Regulation of C/EBPδ expression by IL-1 may also be a complex process. Recent evidence suggests that its expression in response to certain inflammatory stimuli is also likely to be dependent on the activation of both NF-κB and the MAPKs. Although, there are no published studies examining in detail the signalling pathways by which IL-1 regulates C/EBPδ expression, NF-κB and MAPK activation by peptidoglycan and LPS is required for its expression in murine macrophages (Liu *et al.* 2006b; Huang *et al.* 2007). For maximal C/EBPδ expression as mediated by peptidoglycan, three transcription factors are required; Sp1, c-Rel and c-Jun. Combined inhibition of all three MAPK pathways, with the use of pharmacological inhibitors, impairs the increase of c-Jun noted in nuclear fractions of cells as mediated by peptidoglycan, but does not affect c-Rel. Conversely, nuclear levels of c-Rel are reduced by an NF-κB inhibitor, but c-Jun levels are not. Therefore, in response to peptidoglycan, C/EBPδ expression is likely to be regulated by two independent signalling pathways, one dependent on NF-κB, c-Rel activation and the other dependent on MAPK activation of c-Jun (Huang *et al.* 2007).

Additionally, as mentioned in section 7.3, receptors for IL-1 and LPS are members of the Toll/interleukin-1 receptor family and therefore are able to regulate gene expression by activating many of the same signalling pathways. It is therefore

intriguing that both the MAPKs and NF-κB are involved in regulating C/EBPδ expression by LPS, in murine macrophages. Combined inhibition of all three MAPKs or NF-κB with the use of pharmacological agents attenuates its expression and DNA binding activity as mediated by LPS (Liu *et al.* 2006b). Moreover, the existence of potential cross-talk between these pathways in relation to their regulation of C/EBPδ expression by LPS cannot be ruled out as in this study, no experiments were carried out to determine the effects of different inhibitor combinations on C/EBPδ expression by LPS. Therefore it appears, by evaluating the current literature that the mechanisms governing gene expression as modulated by IL-1 can be complex. It is emerging that genes like C/EBPδ can be regulated by a combination of signalling pathways involving the MAPKs and NF-κB in response to inflammatory mediators like IL-1 and LPS and that the activation of all these signalling pathways contributes to gene expression in response to the single stimulus. Such pathways can either function independently of one another or can act together via cross-talk. Where there is a necessity for the activation of multiple signalling pathways required for the expression of a certain gene, then the existence of some functional redundancy between these pathways is always plausible and indeed this notion is supported by previous work (Mestre *et al.* 2001).

Finally in relation to the APR, it should be noted that the collective results of this study provide experimental evidence to support a model for C/EBPδ action in APP gene regulation similar to that described by Valeria Poli (1998) (See section 1.3.1.4 and Figure 1.5). In our scheme of events, IL-1 and IL-6 transiently activate the transcription factors NF-κB and STAT3 respectively and these transcription factors in turn induce the expression of C/EBPδ and other APP genes in 'early' stage of the APR. However, unlike NF-κB and STAT3 whose activation is short-lived, C/EBPδ expression is maintained over many hours following its initial induction. This sustained expression is probably due to auto-activation of the gene and its expression is likely to support APP gene transcription in the 'late' phase of the APR. A schematic representation of the events likely to regulate C/EBPδ expression during the inflammatory response is shown in Figure 8.3.

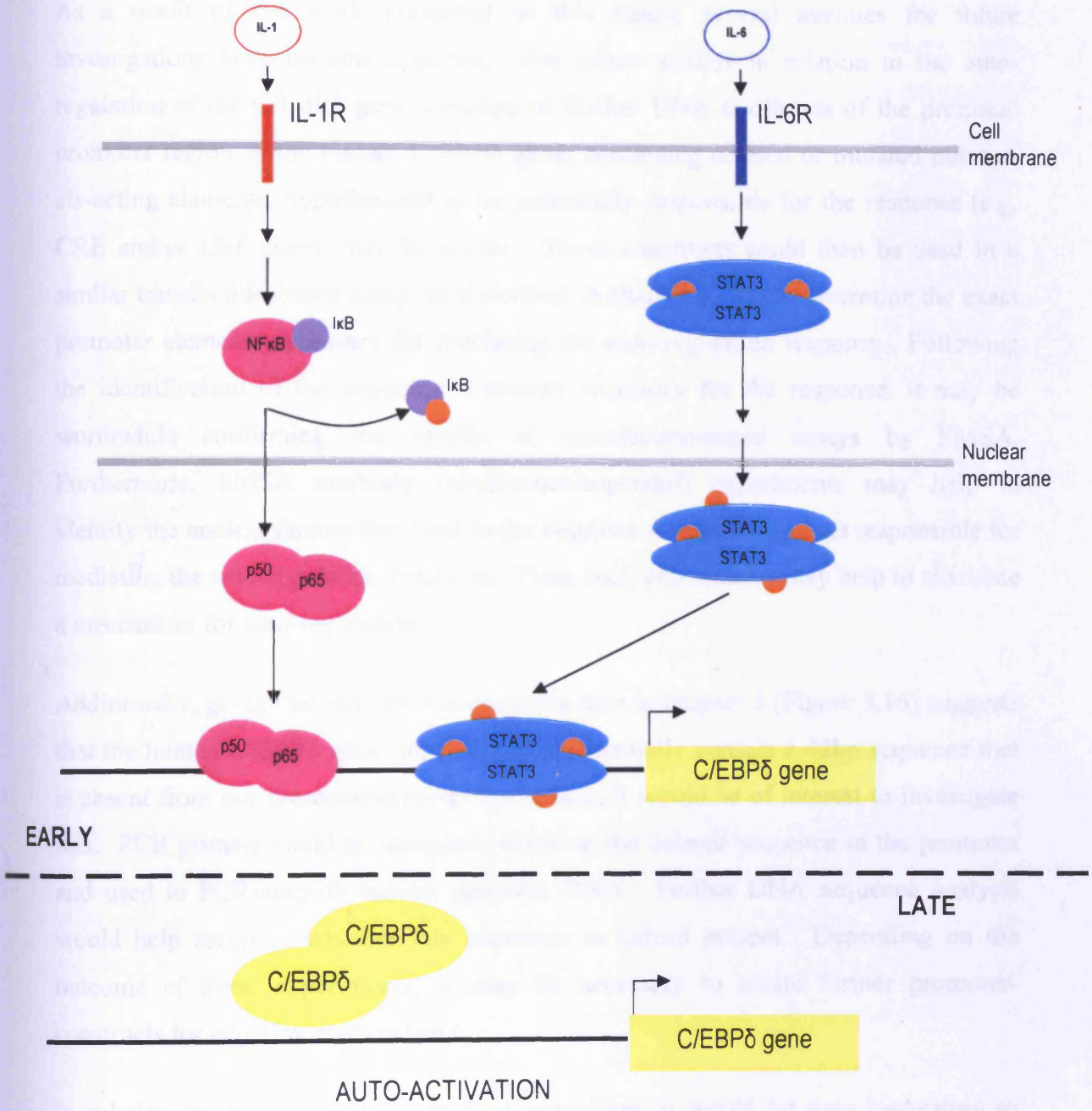



**FIGURE 8.3. Regulation of C/EBPδ gene expression during the inflammatory response in human hepatocytes.** Binding of IL-1 and IL-6 to their cognate receptors activates various signalling pathways leading to the activation of NF-κB and STAT3, respectively. Both NF-κB and STAT3 induce C/EBPδ gene transcription (EARLY). Because the activation of NF-κB and STAT3 is transient, it is unlikely that these nuclear factors drive the transcription of the C/EBPδ gene during the later stages of the response. C/EBPδ either as a homodimer or as a heterodimer (with other C/EBPs like C/EBPβ), is likely to drive its own expression by auto-activation (although the precise nature of this process, whether it occurs by a direct or indirect mechanism, is presently unclear) during the latent phase (LATE). Filled orange circles represent phosphate groups.

## 8.3 FUTURE STUDIES

As a result of the work presented in this thesis, several avenues for future investigations have become apparent. For future studies in relation to the auto-regulation of the C/EBPδ gene, creation of further DNA constructs of the proximal promoter region of the human C/EBPδ gene, containing deleted or mutated putative *cis*-acting elements, hypothesised to be potentially responsible for the response (e.g. CRE and/or USF sites), may be useful. These constructs could then be used in a similar transfection-based assay, as described in chapter 3 to help determine the exact promoter elements necessary for mediating the auto-regulation response. Following the identification of the sequence elements necessary for the response, it may be worthwhile confirming the results of transfection-based assays by EMSA. Furthermore, EMSA antibody interference/supershift experiments may help to identify the nuclear factors that bind to the required sequence elements responsible for mediating the auto-regulation response. Thus, such experiments may help to elucidate a mechanism for auto-regulation.

Additionally, given that our DNA sequencing data in chapter 3 (Figure 3.16) suggests that the human C/EBPδ gene promoter may potentially contain a 42bp sequence that is absent from our promoter-reporter constructs, it would be of interest to investigate this. PCR primers could be designed, flanking the deleted sequence in the promoter and used to PCR-amplify human genomic DNA. Further DNA sequence analysis would help ascertain whether this sequence is indeed present. Depending on the outcome of these experiments, it may be necessary to create further promoter-constructs for experimental analysis.

In relation to the role of CK2 in IL-1 signalling, it would be very interesting to determine whether specific pools of cellular CK2 were activated by IL-1 as suggested by Parhar *et al.* (2007). Evidence in this study suggests that the activity of CK2 associated with p65 NF-κB and IKKγ is increased in response to IL-1. Given that a role for NF-κB and a potential role for CK2 had been suggested in this study, in the regulation of C/EBPδ expression by IL-1, studies in this area may be particularly fruitful. Therefore, it may first be of interest to determine whether IL-1 action induces an association between CK2 and p65 NF-κB in human hepatocytes, as suggested by Bird *et al.* (1997). For this, co-immunoprecipitation assays could be

carried out. This would entail using an anti-p65 NF-κB antibody to immunopreciptiate p65 NF-κB from protein extracts derived from Hep3B cells harvested either in the presence or absence of IL-1. These immunoprecipitates would then be subjected to western blotting and subsequent immunodetection using anti-CK2 antibodies. Results from these experiments would reveal whether IL-1 action caused a physical interaction between CK2 and p65 NF-κB. For kinase assays, the CK2 activity assay used in this study could be modified slightly (Chapter 5). For this, rather than producing immunoprecipitates using anti-CK2 antibodies for the assay, an anti-p65 antibody could be used instead, for example. These immunoprecipitates could then be subjected to the kinase assay, in much the same way as described in section 2.7.6. It could then be determined if IL-1 does indeed induce CK2 activation in NF-κB-associated CK2 pools in treated Hep3B cells. Additionally, it may also be worthwhile determining whether the Hep3B cell line expresses a third catalytic isoform of CK2, CK2α'', given that its expression has been reported in hepatocytes (Shi *et al.* 2001). This could be done by RT-PCR and western blot analysis. Depending on the outcome of these experiments, further studies, for example with the use of siRNA, may be undertaken to further investigate a role for CK2 in the regulation of C/EBPδ expression by IL-1.

Although our data has shown that NF-κB is necessary for the induction of C/EBPδ expression by IL-1 in Hep3B cells, further investigations into the identification of the upstream mediators regulating this response are required. Our data suggests that the MAPKKK, TAK1 is not required for this response but the role of another MAPKKK, MEKK3, which has also been implicated as regulator of NF-κB activation by IL-1 (Yao *et al.* 2007), requires investigation. Studies utilising siRNA or DN mutants of this kinase may help determine its role (if any), in the regulation of C/EBPδ expression by IL-1. Future investigations should also aim to determine the precise nature of the inhibitory action of apigenin and curcumin on NF-κB activation by IL-1, with suggested experiments discussed in section 7.3. In conjunction, the effect of SP600125 on NF-κB activation by IL-1 should also be examined using a similar experimental approach as described in chapter 7, given that this pharmacological agent has also been shown to significantly inhibit the expression of C/EBPδ as induced by IL-1 (Chapter 4). Studies should also be carried out to determine if there is any functional redundancy between CK2 and JNK in the regulation of NF-κB

activation by IL-1, as suggested in section 7.3, given that we were unable to address this issue due to time constraints. Finally, as we have identified putative NF-κB binding sites in the promoter region of the human C/EBPδ gene promoter it would be of interest to determine whether these sites were indeed target binding sites for NF-κB *in vivo*, in IL-1 stimulated Hep3B cells. ChIP assays carried out as described in section 7.3 and EMSA analysis would help determine this.

In relation to the IL-6-mediated induction of C/EBPδ expression, future studies should aim to determine the upstream activators of STAT3 [given that Dr S.A. Rogers' (personal communication) data points to a predominant role of STAT3 over STAT1 in the regulation of this response] and should first focus on discerning a role of the JAK1 or Tyk2 in this process. Given that Dr S.A. Rogers' data suggests a potential requirement for CK2 and JNK in the regulation of C/EBPδ expression by IL-6, a role for these kinases in the regulation of this response should be investigated with the use of siRNA and DN mutants of these kinases.

Future studies should also concentrate on extending the results presented in this thesis to primary hepatocyte cell cultures in order for these findings to be fully relevant to the inflammatory response. Such experiments should be carried out to ensure that the responses we have characterised in Hep3B cells are not just specific to this cell line but are also significance in other hepatocyte cell cultures. This is of particular importance because there have been reported instances where differences in certain cellular responses between primary cell cultures and cell lines have been noted (Rao 2001).

If the results presented in Figures 8.1 and 8.2 are confirmed, in relation to the inhibitory action of the p38 MAPK inhibitor, SB203580 and the ERK MAPK inhibitor, PD98059, on the IL-1-mediated induction of C/EBPδ expression, then future studies should also investigate these signalling pathways. Studies should first aim to determine whether these kinases act as upstream regulators of NF-κB, as previous reports have suggested this (Chen *et al.* 2004; Larsen *et al.* 2005). This could be achieved with the use of pharmacological inhibitors of these kinases, used in a transfection-based assay; similar to the assay described in section 7.2.3, used to assess their effect on *trans*-activation of NF-κB by IL-1. Additionally, the effect of inhibiting these kinases on NF-κB DNA binding as induced by IL-1 could be assessed

by EMSA. Further experiments should aim to determine a role for these kinases in the regulation of C/EBPδ expression by IL-1 with the use of DN mutant constructs and siRNA. Additionally, use of phospho-specific antibodies of p38 and ERK MAPK in western blot analysis and associated kinase activity assays, would help determine whether these pathways are indeed activated in Hep3B cells, in response to IL-1.

Additional investigations could also be carried out to confirm the role of NF-κB and STAT3 in the IL-1- and IL-6-mediated increase of C/EBPδ expression, respectively. For example, the expression of C/EBPδ as mediated by IL-1 could be examined in NF-κB deficient cells. Indeed, Kravchenko *et al.* (2003) showed that the LPS-mediated induction of C/EBPδ was impaired in mouse embryonic fibroblasts deficient in p65 NF-κB compared to controls, and therefore studies of a similar nature could be extended to the analysis of the IL-1 response. Unfortunately, the analysis of C/EBPδ expression by IL-1 in p65 knock-out mice is not a feasible option, as these animals are embryonic lethal (Beg *et al.* 1995), although a p50 knock-out mouse model is viable (Sha *et al.* 1995). Therefore, the creation of a conditional knock-out mouse model for p65 NF-κB may be required for future studies. A similar approach may also be used to verify the role of STAT3 in the regulation of C/EBPδ expression by IL-6, although it should be noted that Alonzi *et al.* (2001) report that C/EBPδ expression as induced by IL-6 was only minimally impaired in STAT3 deficient mouse cells, indicating that STAT3 independent mechanisms of regulating C/EBPδ expression by IL-6 may also exist in murine cells. Potentially, the absence of STAT3 in these cells could be compensated for by STAT1, as functional redundancy between these two STATs has previously been reported. STAT3 activity can, at least in part, replace STAT1 activity in STAT1 deficient cells (Ramana *et al.* 2005).

To evaluate more 'globally' the role of NF-κB, STAT3 and C/EBPδ in the regulation of the inflammatory response gene expression, ChIP-on-chip assays could be carried out using hepatocytes stimulated with and without IL-1 or IL-6. Essentially, ChIP-on-chip assays combine chromatin immunoprecipitation assays with DNA microarray technology. Similar to convential ChIP analysis, ChIP-on-chip is used to investigate interactions between DNA and proteins *in vivo*. However, in contrast to conventional ChIP analysis, where one is required to have an idea about which DNA sequence that the protein of interest may bind prior to experimentation, ChIP-on-chip requires no prior knowledge about potential DNA binding sites. Therefore this technique allows,

by a non biased approach, the identification of DNA binding sites of the protein of interest, on a large, genome-wide scale. In brief, the protein of interest is immunoprecipitated from chromatin, in much the same way as conventional ChIP analysis. Following the reversal of cross-linking and DNA purification, the DNA is subjected to an amplification and denaturation step. At this point, the DNA is fluorescently labelled and hybridised to a DNA microarray which is spotted with short, single-stranded sequences that cover the genomic portion of interest. Subsequently, the DNA microarray data can be anlaysed to identify DNA sequences to which the protein of interest was bound to. Further computational analysis of the DNA sequences, would help determine the genes associated with the identified DNA sequences. In theory, ChIP-on-chip assays could be used to calculate the proportion of genes and potentially help identify novel genes, which may require NF-κB, STAT3 or C/EBPδ for their regulation in response to IL-1 or IL-6 in hepatocytes.

## 8.4 CONCLUDING REMARKS

The work presented here demonstrates that the expression of the transcription factor C/EBPδ is subject to transcriptional regulation by a number of mechanisms during the inflammatory response. As this transcription factor is key to the regulation of inflammatory gene expression, it is hoped that studies of this nature will in the long term lead to the eventual development of therapeutic strategies to combat inflammatory disease.

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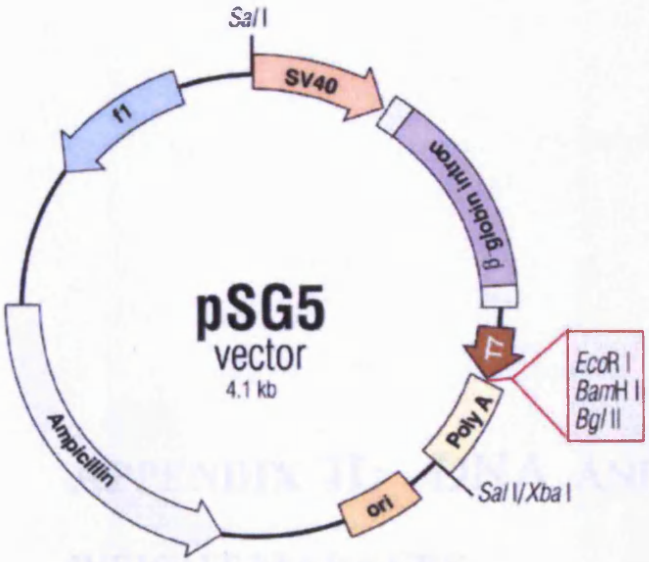
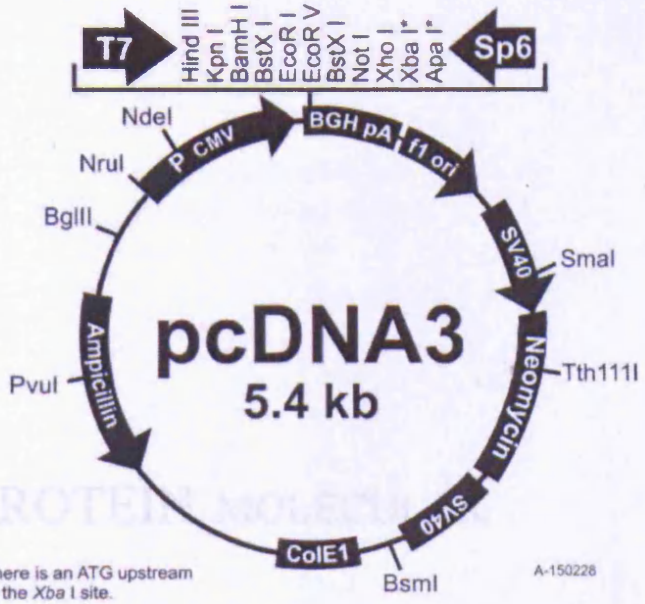
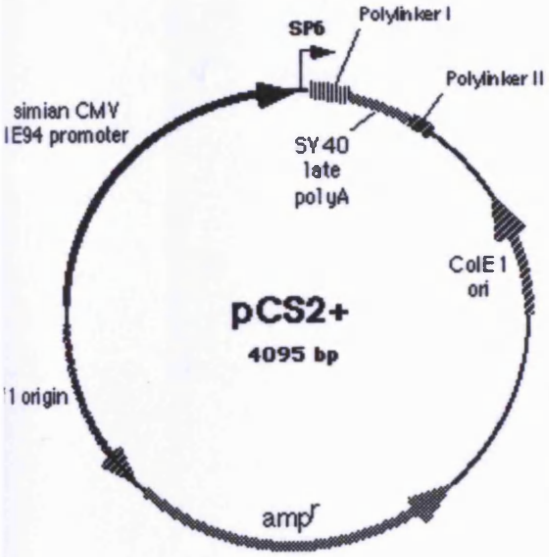
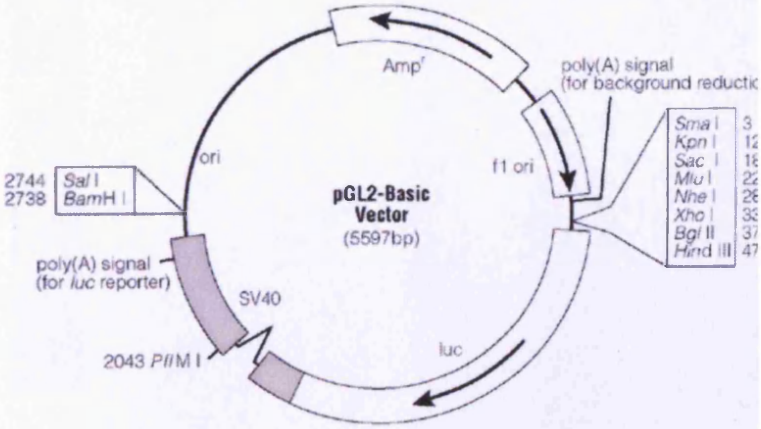
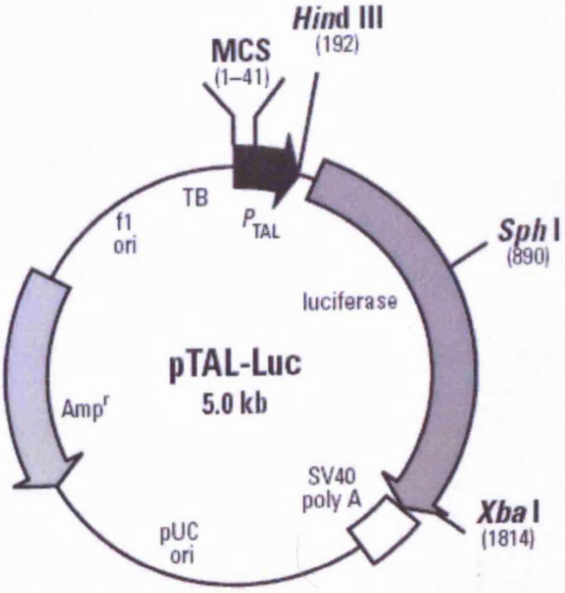
# APPENDIX I: MAPS FOR PLASMID VECTORS



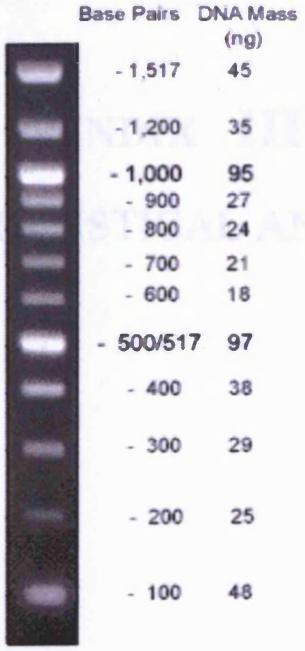



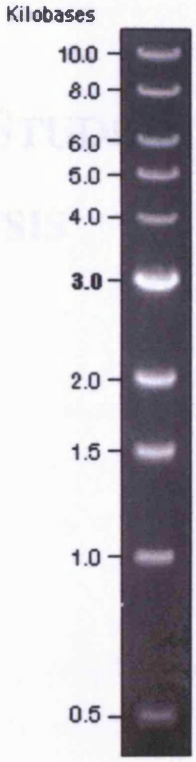

= Transcription blocker

# APPENDIX II: DNA AND PROTEIN MOLECULAR WEIGHT MARKERS

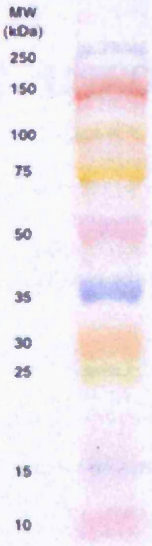
NEB 100bp ladder




NEB 1kb ladder




GE Healthcare Full Range Protein Marker



# APPENDIX III: STUDENT'S T-TEST USED FOR STATISTICAL ANALYSIS

## t-Test

Mean 1 = $\bar{x}_1$ Standard deviation 1 = $\sigma_1$

Mean 2 = $\bar{x}_2$ Standard deviation 2 = $\sigma_2$

Variance = $\sigma d^2$

$$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sigma_d}$$

## t-Table

A difference between two means is significant (at the given probability level) if the calculated t value is greater than the value given in this table. A probability of $p$ = 0.05 (95% probability of making a correct statement) is usually acceptable for biological work.

When comparing two means, the number of degrees of freedom is $(n_1 + n_2)$-2, where $n_1$ is the number of replicates of treatment 1, and $n_2$ is the number of replicates of treatment 2 (adapted from http://helios.bto.ed.ac.uk/bto/statistics/tress4a.html#Student's%20t-test).

| DEGREES OF FREEDOM | t-VALUE | | | |
|---|---|---|---|---|
| P-Value | 0.1 | 0.05 | 0.01 | 0.001 |
| 1 | 6.31 | 12.71 | 63.66 | 636.62 |
| 2 | 2.92 | 4.30 | 9.93 | 31.60 |
| 3 | 2.35 | 3.18 | 5.84 | 12.92 |
| 4 | 2.13 | 2.78 | 4.60 | 8.61 |
| 5 | 2.02 | 2.57 | 4.03 | 6.87 |
| 6 | 1.94 | 2.45 | 3.71 | 5.96 |
| 7 | 1.89 | 2.37 | 3.50 | 5.41 |
| 8 | 1.86 | 2.31 | 3.36 | 5.04 |
| 9 | 1.83 | 2.26 | 3.25 | 4.78 |
| 10 | 1.81 | 2.23 | 3.17 | 4.59 |

# APPENDIX IV: PRIMERS USED FOR SEQUENCING PLASMID INSERTS

F1 and F2 primers were used to sequence the proximal promoter region of the human C/EBPδ gene promoter insert in the plasmid vector pLUC. This plasmid is termed pHuC/EBPδ[1.6kb]-Luc throughout this thesis.

F1 5'-AGGAGCGAGGAGGTTCCAAG-3'

F2 5'-TCCGAGGAGAGCAGCGAGAA-3'

GL1 and GL2 universal primers were used to sequence DNA inserts in the reporter vector pGl2-Basic.

GL1 5'-TGTATCTTATGGTACTGTAACTG-3'

GL2 5'-CTTTATGTTTTTGGCGTCTTCCA-3'

# APPENDIX V: TRANSCRIPTION FACTOR BINDING SITE SEARCHES OF THE HUMAN C/EBPδ GENE PROMOTER

TESS Job W0391022515 : Tabulated Results

Home | Site Searches | Query Transfac | Query Matrices | Other Stuff
About | Strings | Filtered Strings | Combined | Recall Search

***Need help? Check our FAQ page then please send questions and comments to TessMaster@cbil.upenn.edu.***

**Result Navigation**

Annotated Sequence | GBrowse Viewer | Email Results | Small Java Applets | Poisson Significance
Tabular Results | Excel File | Search Again | Legend | Model/Class Associations

*Use these checkboxes and the 'Select' button to choose which columns to display.*

Select | # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_q$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$

*Click on a column heading to sort results on that column.*

[1 .. 50] of 105 1 | 11 | 21 | 31 | 41 | 51 | 61 | 71 | 81 | 91 | 101 | 111 | 121 | 131 | 141 | 151 | 161

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_q$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | _00000 LVc | I00085 (LVc) | 1 | N | 5 | CCTGC | 8.50 | 1.70 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 0.0e+00 | 2.9e-01 |
| 2 | T00478 LVc | R01644 () | 1 | N | 5 | CCTGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{al}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | _00000 USF | I00292 (USF) | 7 | R | 6 | ACGTGG | 9.67 | 1.61 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 0.0e+00 | 1.6e-01 |
| 4 | T00788 T-Ag | R01372 () | 10 | N | 5 | TGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 5 | T00752 Sp1<br>T00753 Sp1<br>T00754 Sp1<br>T00755 Sp1<br>T00757 Sp1<br>T00758 Sp1<br>T00759 Sp1<br>T01228 Sp1 | R02245 () | 10 | N | 9 | KRGGCKRRK | 12.00 | 1.33 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 6 | T00033 AP-2alphaA<br>T00034 AP-2<br>T00035 AP-2alphaA<br>T00952 AP-2<br>T01142 AP-2<br>T02466 AP-2alphaB | R02121 () | 11 | R | 8 | SSSNKGGG | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 7 | T00821 TFIID | R01943 () | 12 | N | 8 | GGCTGGGG | 16.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 8 | _00000 MIG1 | I00321 (MIG1) | 14 | N | 6 | CTGGGG | 9.79 | 1.63 | 1.000 | 0.00 | 1.6e-01 | 1.00 | 1.00 | 0.0e+00 | 9.4e-17 |
| 9 | T00302 GAL4 | R00496 () | 16 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 10 | _00000 H4TF2 | I00180 (H4TF2) | 19 | N | 5 | GGTCC | 7.92 | 1.58 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 0.0e+00 | 3.0e-01 |
| 11 | T00392 H4TF-2 | R00681 () | 19 | N | 5 | GGTCC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 | T00033 AP-2alphaA T00034 AP-2 T00035 AP-2alphaA T00952 AP-2 T01142 AP-2 T02466 AP-2alphaB | R05065 () | 21 | N | 10 | TCCCCMNSSS | 14.00 | 1.40 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 13 | T00302 GAL4 | R00496 () | 22 | R | 4 | CCCC | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 14 | _00000 MIG1 | I00321 (MIG1) | 22 | R | 6 | CCCCAG | 9.79 | 1.63 | 1.000 | 0.00 | 1.6e-01 | 1.00 | 1.00 | 0.0e+00 | 9.4e-17 |
| 15 | _00000 LBP-1 | I00191 (LBP-1) | 24 | R | 5 | CCAGG | 7.52 | 1.50 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 0.0e+00 | 2.3e-02 |
| 16 | T00320 GCF | R02159 () | 27 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 17 | T00302 GAL4 | R00496 () | 32 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 18 | T00759 Sp1 | R01758 () | 32 | R | 11 | GGGGAGGGGGC | 22.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 19 | T00490 MAZ | R02306 () | 33 | N | 7 | GGGAGGG | 14.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 20 | T00033 AP-2alphaA T00034 AP-2 T00035 AP-2alphaA T00952 AP-2 T01142 AP-2 T02466 AP-2alphaB | R02121 () | 33 | R | 8 | SSSNKGGG | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 21 | T00172 CTCF | R02137 () | 35 | R | 5 | GAGGG | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{al}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 22 | T00302 GAL4 | R00496 () | 37 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 23 | T00788 T-Ag | R01372 () | 38 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 24 | T00320 GCF | R02159 () | 41 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 25 | T00302 GAL4 | R00496 () | 42 | R | 4 | CCCC | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 26 | T00033 AP-2alphaA T00034 AP-2 T00035 AP-2alphaA T00952 AP-2 T01142 AP-2 T02466 AP-2alphaB | R02121 () | 42 | R | 8 | SSSNKGGG | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 27 | T00302 GAL4 | R00496 () | 46 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 28 | _00000 PU.1 | I00047 (PU.1) | 49 | R | 6 | GAGGAA | 10.93 | 1.82 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 8.2e-02 | 7.7e-02 |
| 29 | T00702 PU.1 | R04413 () | 49 | R | 6 | GAGGAA | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 30 | T00172 CTCF | R02137 () | 59 | R | 5 | GAGGG | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 31 | T00302 GAL4 | R00496 () | 61 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 32 | T00788 T-Ag | R01372 () | 62 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 33 | T00320 GCF | R02159 () | 62 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 34 | T02349 ZF5 | R01772 () | 64 | N | 6 | GGCGCG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 35 | T00788 T-Ag | R01372 () | 69 | R | 5 | GCCCC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 36 | T00320 GCF | R02159 () | 69 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 37 | T00302 GAL4 | R00496 () | 70 | R | 4 | CCCC | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 38 | T00113 c-Ets- | R04343 () | 74 | N | 6 | GGGAAG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | | | | | | | | | | | | | | | |
| 39 | T00302 GAL4 | R00495 () | 78 | R | 5 | AGGCT | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 40 | T00714 RAF | R00256 () | 82 | R | 4 | TCGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 41 | T00302 GAL4 | R00496 () | 84 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 42 | T00788 T-Ag | R01372 () | 84 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 43 | T00320 GCF | R02159 () | 84 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 44 | _00000 Sp1 | I00295 (Sp1) | 85 | N | 6 | GGGCGG | 8.19 | 1.36 | 1.000 | 0.00 | 1.6e-01 | 1.00 | 1.00 | 0.0e+00 | 1.7e-05 |
| 45 | T00752 Sp1<br>T00754 Sp1<br>T00755 Sp1 | R00017 ()<br>R00447 ()<br>R02891 () | 85 | R | 6 | GGGCGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 46 | T00270 ETF<br>T00753 Sp1<br>T00759 Sp1<br>T01171 CP1 | R01375 ()<br>R01545 ()<br>R03039 ()<br>R04790 () | 85 | N | 6 | GGGCGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 47 | T00788 T-Ag | Q00168 (-) | 87 | N | 5 | GCGGC | 6.50 | 1.30 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 0.0e+00 | 2.2e-02 |
| 48 | T00320 GCF | R02159 () | 93 | N | 7 | SCGSSSC | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 49 | T00302 GAL4 | R00496 () | 95 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 50 | T00788 T-Ag | R01372 () | 95 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

[1 .. 50] of 105 1 | 11 | 21 | 31 | 41 | 51 | 61 | 71 | 81 | 91 | 101 | 111 | 121 | 131 | 141 | 151 | 161

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | _00000 WT1+KTS | I00016 (WT1+KTS) | 99 | R | 9 | CGCCCCCGC | 15.30 | 1.70 | 1.000 | 0.00 | 1.3e-03 | 1.00 | 1.00 | 0.0e+00 | 3.0e-06 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 52 | _00000 EGR-1 | I00117 (EGR-1) | 99 | N | 9 | CGCCCCCGC | 16.37 | 1.82 | 1.000 | 0.00 | 1.3e-03 | 1.00 | 1.00 | 0.0e+00 | 2.4e-03 |
| 53 | T00243 EGR3<br>T00244 Egr-1<br>T00454 Krox-20 | R02147 () | 99 | N | 9 | CGCCCSCGC | 17.00 | 1.89 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 54 | T01841 WT1-del2<br>T01842 WT1 I-del2 | R02262 () | 99 | N | 9 | CGCCCCCGC | 18.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 55 | T00620 NGFI-C<br>T00759 Sp1<br>T00899 WT1<br>T00900 WT1 I -KTS<br>T01839 WT1 -KTS<br>T01840 WT1 I | R03332 ()<br>R03385 ()<br>R04861 () | 99 | R | 9 | CGCCCCCGC | 18.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 56 | T00788 T-Ag | R01372 () | 100 | R | 5 | GCCCC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 57 | T00302 GAL4 | R00496 () | 101 | R | 4 | CCCC | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 58 | T00033 AP-2alphaA<br>T00034 AP-2<br>T00035 AP-2alphaA<br>T00952 AP-2<br>T01142 AP-2<br>T02466 AP-2alphaB | R02121 () | 101 | N | 8 | CCCMNSSS | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 59 | T00714 RAF | R00256 () | 118 | R | 4 | TCGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 60 | T00302 GAL4 | R00496 () | 120 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 61 | T00788 T-Ag | R01372 () | 120 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 62 | T00320 GCF | R02159 () | 120 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 63 | T00753 Sp1<br>T00755 Sp1<br>T00757 Sp1<br>T00758 Sp1<br>T01228 Sp1 | R02245 () | 120 | N | 9 | KRGGCKRRK | 12.00 | 1.33 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 64 | _00000 Sp1 | I00032 (Sp1) | 120 | N | 10 | GGGGCGGGGC | 16.91 | 1.69 | 1.000 | 0.00 | 3.3e-04 | 1.00 | 1.00 | 0.0e+00 | 4.2e-11 |
| 65 | T00752 Sp1<br>T00759 Sp1<br>T00798 TBP<br>T00820 TFIID | R00281 ()<br>R01764 ()<br>R02102 () | 120 | N | 10 | GGGGCGGGGC | 20.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 66 | T00754 Sp1 | R02572 () | 120 | R | 10 | GGGGCGGGGC | 20.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 67 | _00000 Sp1 | I00295 (Sp1) | 121 | N | 6 | GGGCGG | 8.19 | 1.36 | 1.000 | 0.00 | 1.6e-01 | 1.00 | 1.00 | 0.0e+00 | 1.7e-05 |
| 68 | T00270 ETF<br>T01171 CP1 | R01375 ()<br>R03039 () | 121 | N | 6 | GGGCGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 69 | T00033 AP-2alphaA<br>T00034 AP-2<br>T00035 AP-2alphaA<br>T00952 AP-2<br>T01142 AP-2<br>T02466 AP-2alphaB | R02121 () | 121 | R | 8 | SSSNKGGG | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 70 | T00261 ER | R04883 () | 122 | N | 6 | GGCGGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 71 | _00000 GCF | I00152 (GCF) | 123 | N | 8 | GCGGGGCG | 11.13 | 1.39 | 1.000 | 0.00 | 2.1e-02 | 1.00 | 1.00 | 0.0e+00 | 1.1e-21 |
| 72 | T00302 GAL4 | R00496 () | 125 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 73 | T00788 T-Ag | R01372 () | 125 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 74 | _00000 Sp1 | I00295 (Sp1) | 126 | N | 6 | GGGCGT | 8.19 | 1.36 | 1.000 | 0.00 | 1.6e-01 | 1.00 | 1.00 | 0.0e+00 | 1.7e-05 |
| 75 | T00495 MBF-I | R02198 () | 128 | R | 7 | GYGYGCA | 12.00 | 1.71 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 76 | T00515 MTF-1 | R02204 () | 128 | R | 7 | GNGYGCA | 11.00 | 1.57 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 77 | T00752 Sp1 | R01021 () | 130 | R | 5 | GTGCA | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 78 | T02789 bZIP910 | R08444 () | 133 | R | 7 | CACGTCA | 14.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 79 | _00000 ATF-1 | I00004 (ATF-1) | 134 | R | 6 | ACGTCA | 10.21 | 1.70 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 8.2e-02 | 8.9e-02 |
| 80 | _00000 LRF-1 | I00087 (LRF-1) | 134 | N | 6 | ACGTCA | 9.51 | 1.58 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 0.0e+00 | 8.9e-02 |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 81 | _00000 ASF-1<br>_00000 MSN4<br>_00000 deltaCREB | I00322 (MSN4)<br>I00344 (ASF-1)<br>I00416 (deltaCREB) | 134 | R | 6 | ACGTCA | 9.51 | 1.58 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 0.0e+00 | 8.9e-02 |
| 82 | _00000 HBP-1 | I00349 (HBP-1) | 134 | R | 6 | ACGTCA | 9.67 | 1.61 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 0.0e+00 | 1.7e-01 |
| 83 | _00000 TREB-1 | I00410 (TREB-1) | 134 | N | 6 | ACGTCA | 10.21 | 1.70 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 8.2e-02 | 8.9e-02 |
| 84 | T00968 ATF-1 | Q00199 (-) | 134 | N | 6 | ACGTCA | 10.90 | 1.82 | 1.000 | 0.00 | 8.2e-02 | 1.00 | 1.00 | 8.2e-02 | 8.9e-02 |
| 85 | T00049 ATF<br>T00050 ATF1<br>T00132 c-Jun | R00362 ()<br>R01492 () | 134 | N | 6 | ACGTCA | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| # | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{al}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T00163 CREB<br>T00164 CREB<br>T00166 deltaCREB<br>T00167 CRE-BP1<br>T00846 TREB-1<br>T00942 EivF<br>T01095 ATF-3 | | | | | | | | | | | | | | |
| 86 | T00051 ATF<br>T00354 HBP-1<br>T00937 HBP-1a<br>T00938 HBP-1b<br>T01393 HBP-1b(c1)<br>T01394 HBP-1a(1)<br>T01395 HBP-1a(c14) | R01562 ()<br>R01915 () | 134 | R | 6 | ACGTCA | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 87 | T00605 NF-S | R02219 () | 135 | N | 7 | YGTCAGC | 13.00 | 1.86 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 88 | T00029 AP-1 | R00368 () | 136 | R | 4 | GTCA | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 89 | T00320 GCF | R02159 () | 141 | N | 7 | SCGSSSC | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 90 | T00302 GAL4 | R00496 () | 143 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 91 | T00788 T-Ag | R01372 () | 143 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 92 | T00386 HSTF | M00028 (mid_c) | 149 | N | 5 | AGAAA | 7.82 | 1.56 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 2.9e-01 | 3.0e-01 |
| 93 | T00802 TCF-1alpha<br>T00803 TCF-2alpha<br>T00930 LEF-1 | R02248 () | 151 | N | 5 | MAMAG | 8.00 | 1.60 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T00999 TCF-1A<br>T01000 TCF-1B<br>T01001 TCF-1C<br>T01002 TCF-1<br>T01109 TCF-1<br>T01979 TCF-1E<br>T01981 TCF-1F<br>T01982 TCF-1G | | | | | | | | | | | | | | | |
| 94 | T01059 MNB1a<br>T02690 Dof2<br>T02691 Dof3<br>T02692 PBF | R08440 ()<br>R08441 ()<br>R08442 ()<br>R08443 () | 152 | N | 4 | AAAG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 95 | T00759 Sp1 | R02860 () | 154 | N | 6 | AGGCGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 96 | T00913 Yi | R03154 () | 154 | R | 10 | AGNNNGNGGG | 12.00 | 1.20 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 97 | T00788 T-Ag | Q00168 (-) | 156 | N | 5 | GCGGC | 6.50 | 1.30 | 1.000 | 0.00 | 2.9e-01 | 1.00 | 1.00 | 0.0e+00 | 2.2e-02 |
| 98 | T00320 GCF | R02159 () | 156 | R | 7 | GSSSCGS | 10.00 | 1.43 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 99 | T00033 AP-2alphaA<br>T00034 AP-2<br>T00035 AP-2alphaA<br>T00952 AP-2<br>T01142 AP-2<br>T02466 AP-2alphaB | R02121 () | 157 | R | 8 | SSSNKGGG | 10.00 | 1.25 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 100 | T00261 ER | R04883 () | 158 | N | 6 | GGCGGG | 12.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

| | Factor | Model | Beg | Sns | Len | Sequence | $L_a$ | $L_{a/}$ | $L_g$ | $L_d$ | $L_{pv}$ | $S_c$ | $S_m$ | $S_{pv}$ | $P_{pv}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 101 | _00000 GCF | I00152 (GCF) | 159 | N | 8 | GCGGGGCT | 11.13 | 1.39 | 1.000 | 0.00 | 2.1e- | 1.00 | 1.00 | 0.0e+00 | 1.1e-21 |

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| | | | | | | | | | | | 02 | | | | |
| 102 | T00302 GAL4 | R00496 () | 161 | N | 4 | GGGG | 8.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 103 | T00788 T-Ag | R01372 () | 161 | N | 5 | GGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| 104 | T00752 Sp1 | R02245 () | 161 | N | 9 | KRGGCKRRK | 12.00 | 1.33 | 1.000 | 0.00 | nc | ? | ? | nc | nc |
| | T00753 Sp1 | | | | | | | | | | | | | | |
| | T00754 Sp1 | | | | | | | | | | | | | | |
| | T00755 Sp1 | | | | | | | | | | | | | | |
| | T00757 Sp1 | | | | | | | | | | | | | | |
| | T00758 Sp1 | | | | | | | | | | | | | | |
| | T00759 Sp1 | | | | | | | | | | | | | | |
| | T01228 Sp1 | | | | | | | | | | | | | | |
| 105 | T00788 T-Ag | R01372 () | 166 | N | 5 | TGGGC | 10.00 | 2.00 | 1.000 | 0.00 | nc | ? | ? | nc | nc |

FAQ | Projects | Protocol | Help

**GEMS Launcher Task: *MatInspector*: Search for transcription factor binding sites working on Ali2humandeltapromoter.seq (1830 bp)**

## Please be aware:

Individual binding sites in a promoter are NEVER sufficient to indicate transcriptional function. Functional assessment of binding sites can be carried out by our other tools, e.g. ModelInspector, Comparative Genomics, FrameWorker, BiblioSphere

If MatInspector does **not** identify a known site, please send an email to support@genomatix.de citing the corresponding paper!

## Search Results ( 307 matches)

**MatInspector Release professional 7.4.8.2, July 2007** **Tue Aug 28 16:56:50 2007**

## Solution parameters:

**Sequence file:** Ali2humandeltapromoter.seq (1830 bp)
**Family matches:** yes
**MatInspector library:** Matrix Family Library Version 6.3 (March 2007)
**Selected groups** (core/matrix sim)
- ALL vertebrates.lib (0.75/Optimized)

## Inspecting sequence Ali2humandeltapromoter (1 - 1830):

| Family/matrix | Further Information | Opt. | Position from - to | Str. | Core sim. | Matrix sim. | Sequence (red: ci-value > 60 capitals: core sequence) |
|---|---|---|---|---|---|---|---|

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V$GREF/PRE.01 | Progesterone receptor binding site, IR3 sites | 0.84 | 8 - 26 | (+) | 1.000 | 0.850 | agaaatatgttTGTTcttt |
| V$HOXF/BARX2.01 | Barx2, homeobox transcription factor that preferentially binds to paired TAAT motifs | 0.95 | 27 - 43 | (-) | 1.000 | 0.956 | tattTAATaggtttcct |
| V$BRNF/BRN3.01 | Brn-3, POU-IV protein class | 0.78 | 31 - 49 | (+) | 0.750 | 0.809 | aacctattaAATAtcccat |
| V$HOXF/HOXC13.01 | Homeodomain transcription factor HOXC13 | 0.91 | 31 - 47 | (+) | 1.000 | 0.914 | aacctatTAAAtatccc |
| V$HOMF/EN1.01 | Homeobox protein engrailed (en-1) | 0.77 | 32 - 44 | (-) | 1.000 | 0.775 | ataTTTAatagt |
| V$OCTP/OCT1P.01 | Octamer-binding factor 1, POU-specific domain | 0.86 | 38 - 50 | (+) | 1.000 | 0.864 | taaATATcccata |
| V$STAF/STAF.02 | Se-Cys tRNA gene transcription activating factor | 0.82 | 41 - 63 | (+) | 1.000 | 0.859 | atatCCCAtagtgcaacgttaga |
| V$MYBL/VMYB.04 | v-Myb, AMV v-myb | 0.85 | 51 - 63 | (-) | 1.000 | 0.879 | tctAACGttgcac |
| V$RXRF/RAR_RXR.01 | Retinoic acid receptor / retinoid X receptor heterodimer, DR1 sites | 0.78 | 58 - 82 | (-) | 1.000 | 0.793 | gtcagttccagAGGTcatttctaac |
| V$NR2F/ARP1.01 | Apolipoprotein AI regulatory protein 1, NR2F2, DR1 sites | 0.82 | 60 - 84 | (-) | 1.000 | 0.898 | cagtcagttccagaGGTCatttcta |
| V$TCFF/TCF11.01 | TCF11/LCR-F1/Nrf1 homodimers | 1.00 | 63 - 69 | (-) | 1.000 | 1.000 | GTCAttt |
| V$MYBL/CMYB.02 | c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb | 0.96 | 94 - 106 | (-) | 1.000 | 0.971 | acTAACtgctgtg |
| V$GREF/ARE.02 | Androgene receptor binding site, IR3 sites | 0.89 | 105 - 123 | (+) | 1.000 | 0.926 | gtagagcttggtGTTCcga |
| V$SRFF/SRF.02 | Serum response factor | 0.84 | 121 - 139 | (+) | 0.822 | 0.840 | cgacgCAGAtctggtgaga |
| V$ETSF/ETS1.01 | c-Ets-1 binding site | 0.92 | 142 - 162 | (+) | 1.000 | 0.952 | cctgcgcaGGAAgagactcga |
| V$ZF35/ZNF35.01 | Human zinc finger protein ZNF35 | 0.96 | 146 - 158 | (+) | 1.000 | 0.963 | cgcaggAAGAgac |
| V$BRAC/TBX5.01 | T-Box factor 5 site (TBX5), mutations related to Holt-Oram syndrome | 0.99 | 162 - 182 | (+) | 1.000 | 0.995 | acatggagcaGGTGtgactag |
| V$PAX3/PAX3.02 | Pax-3 paired domain protein | 0.85 | 164 - | (-) | 1.000 | 0.867 | ctagTCACacctgctccat |

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|---|---|---|---|---|---|---|---|---|
| | | | | 182 | | | | |
| | V$MYOD/E47.01 | MyoD/E47 and MyoD/E12 dimers | 0.92 | 165 - 181 | (+) | 1.000 | 0.968 | tggaGCAGgtgtgacta |
| | V$MEF2/SL1.01 | Member of the RSRF (related to serum response factor) protein family from Xenopus laevis | 0.84 | 173 - 195 | (-) | 1.000 | 0.848 | taaatcaCTATcgctagtcacac |
| | V$GATA/GATA1.06 | Complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2 | 0.96 | 180 - 192 | (+) | 1.000 | 0.960 | tagcGATAgtgat |
| | V$GFI1/GFI1.02 | Growth factor independence 1 | 0.90 | 182 - 196 | (-) | 1.000 | 0.916 | ttaAATCactatcgc |
| | V$PARF/TEF.01 | Thyrotrophic embryonic factor | 0.85 | 184 - 200 | (+) | 0.772 | 0.864 | gatagtgatTTAAtgga |
| | V$HOXC/PBX_HOXA9.01 | PBX - HOXA9 binding site | 0.79 | 185 - 201 | (+) | 1.000 | 0.838 | atagTGATttaatggat |
| | V$PARF/TEF_HLF.01 | Thyrotrophic embryonic factor / hepatic leukemia factor | 0.78 | 185 - 201 | (-) | 0.784 | 0.784 | atccaTTAAatcactat |
| | V$HOXF/HOXC13.01 | Homeodomain transcription factor HOXC13 | 0.91 | 186 - 202 | (-) | 1.000 | 0.945 | aatccatTAAAtcacta |
| | V$HOMF/EN1.01 | Homeobox protein engrailed (en-1) | 0.77 | 189 - 201 | (+) | 1.000 | 0.773 | tgaTTTAatggat |
| | V$HOXF/BARX2.01 | Barx2, homeobox transcription factor that preferentially binds to paired TAAT motifs | 0.95 | 190 - 206 | (+) | 1.000 | 0.960 | gattTAATggatttggg |
| | V$CLOX/CLOX.01 | Cut-like homeo box | 0.81 | 191 - 209 | (-) | 0.804 | 0.812 | gggcccaaATCCattaaat |
| | V$HNF6/HNF6.01 | Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT) | 0.82 | 192 - 208 | (-) | 0.833 | 0.887 | ggcccaaaTCCAttaaa |
| | V$NKXH/HMX2.01 | Hmx2/Nkx5-2 homeodomain transcription factor | 0.83 | 262 - 276 | (-) | 1.000 | 0.936 | gggCTTAagagattc |
| | V$NKXH/HMX2.01 | Hmx2/Nkx5-2 homeodomain transcription factor | 0.83 | 265 - 279 | (+) | 1.000 | 0.913 | tctCTTAagcccagg |
| | V$CHRF/CHR.01 | Cell cycle gene homology region (CDE/CHR tandem elements regulate cell | 0.92 | 279 - 291 | (+) | 1.000 | 0.956 | gagtTTGAaacca |

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|---|---|---|---|---|---|---|---|---|
| | | cycle dependent repression) | | | | | | |
| | V$SORY/SOX5.01 | Sox-5 | 0.87 | 293 - 309 | (+) | 1.000 | 0.871 | cctggaCAATattgtga |
| | V$FKHD/FHXB.01 | Fork head homologous X binds DNA with a dual sequence specificity (FHXA and FHXB) | 0.83 | 297 - 313 | (-) | 0.909 | 0.840 | gatctcACAAtattgtc |
| | V$OCT1/OCT1.05 | Octamer-binding factor 1 | 0.89 | 313 - 327 | (+) | 0.950 | 0.910 | ccCATCtcaattttt |
| | V$SP1F/BTEB3.01 | Basic transcription element (BTE) binding protein, BTEB3, FKLF-2 | 0.93 | 333 - 347 | (+) | 1.000 | 0.934 | gagatGGAGtctcgc |
| | V$PAX5/PAX5.02 | B-cell-specific activator protein | 0.73 | 334 - 362 | (-) | 1.000 | 0.739 | agcctgggcgacagAGCGagactccatct |
| | V$AHRR/AHR.01 | Aryl hydrocarbon / dioxin receptor | 0.78 | 354 - 378 | (+) | 0.750 | 0.803 | gcccaggctgGAGTgcaatggaacg |
| | V$HEAT/HSF2.01 | Heat shock factor 2 | 0.88 | 361 - 385 | (+) | 1.000 | 0.880 | ctggagtgcaatgGAACgatctcgg |
| | V$PURA/PURALPHA.01 | Purine-rich element binding protein A | 0.97 | 394 - 406 | (-) | 1.000 | 0.985 | ggAGGCggaggtt |
| | V$GZF1/GZF1.01 | GDNF-inducible zinc finger protein 1 (ZNF336) | 0.73 | 450 - 462 | (-) | 1.000 | 0.760 | TGCGcctgtaatc |
| | V$AHRR/AHRARNT.01 | Aryl hydrocarbon receptor / Arnt heterodimers | 0.92 | 451 - 475 | (-) | 1.000 | 0.946 | aagcgtagtggCGTGcgcctgtaat |
| | V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 455 - 465 | (-) | 1.000 | 0.960 | gcgtGCGCctg |
| | V$HESF/HES1.01 | Drosophila hairy and enhancer of split homologue 1 (HES-1) | 0.92 | 456 - 470 | (-) | 1.000 | 0.932 | tagtggcGTGCgcct |
| | V$SORY/HMGIY.01 | HMGI(Y) high-mobility-group protein I (Y), architectural transcription factor organizing the framework of a nuclear protein-DNA transcriptional complex | 0.92 | 471 - 487 | (-) | 1.000 | 0.921 | caaaAATTagccaagcg |
| | V$CLOX/CUT2.01 | Cut repeat II | 0.67 | 472 - 490 | (+) | 0.750 | 0.687 | gcttggctaATTTttgtat |
| | V$MEF2/MEF2.04 | Myocyte-specific enhancer factor 2 | 0.80 | 481 - | (-) | 1.000 | 0.829 | atttctactaaAAATacaaaaat |

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| | | | | 503 | | | | |
| | V$BCL6/BCL6.02 | POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma | 0.77 | 490 - 506 | (+) | 1.000 | 0.835 | ttttagTAGAaatggg |
| | V$RUSH/SMARCA3.01 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3 | 0.96 | 497 - 507 | (-) | 1.000 | 0.961 | ccCCATttcta |
| | V$RXRF/VDR_RXR.04 | Bipartite binding site of VDR/RXR heterodimers, DR3 sites | 0.79 | 505 - 529 | (-) | 1.000 | 0.831 | cagcctGGTCaacatggtgaaaccc |
| | V$AARF/AARE.01 | Amino acid response element, ATF4 binding site | 0.95 | 507 - 515 | (+) | 1.000 | 0.952 | gTTTCacca |
| | V$AP1R/VMAF.01 | v-Maf | 0.82 | 510 - 534 | (+) | 1.000 | 0.829 | tcaccatgtTGACcaggctggtttc |
| | V$CHRF/CHR.01 | Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression) | 0.92 | 528 - 540 | (-) | 1.000 | 0.956 | gagtTTGAaacca |
| | V$RORA/VERBA.01 | vErbA, viral homolog of thyroid hormone receptor alpha1 | 0.92 | 533 - 555 | (-) | 1.000 | 0.921 | atcacttgaGGTCaggagtttga |
| | V$CREB/CJUN_ATF2.01 | c-Jun/ATF2 heterodimers | 0.99 | 536 - 556 | (+) | 1.000 | 0.992 | aactccTGACctcaagtgatc |
| | V$RXRF/LXRE.01 | Nuclear receptor involved in the regulation of lipid homeostasis, DR4 element | 0.90 | 537 - 561 | (-) | 0.763 | 0.935 | gggcgGATCacttgaggtcaggagt |
| | V$NKXH/NKX25.01 | Homeo domain factor Nkx-2.5/Csx, tinman homolog, high affinity sites | 1.00 | 543 - 557 | (+) | 1.000 | 1.000 | gacctcAAGTgatcc |
| | V$SP1F/SP1.02 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.85 | 551 - 565 | (-) | 1.000 | 0.865 | aggtGGGCggatcac |
| | V$STAF/STAF.01 | Se-Cys tRNA gene transcription activating factor | 0.77 | 568 - 590 | (+) | 1.000 | 0.803 | gcctCCCAaaatgctgggattac |
| | V$HOXF/GSC.01 | Vertebrate bicoid-type homeodomain protein Goosecoid | 0.98 | 577 - 593 | (-) | 1.000 | 0.985 | ccggTAATcccagcatt |
| | V$IKRS/IK3.01 | Ikaros 3, potential regulator of lymphocyte differentiation | 0.84 | 579 - 591 | (+) | 0.750 | 0.849 | tgctgGGATtacc |

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|---|---|---|---|---|---|---|---|---|
| V$PAX2/PAX2.01 | Zebrafish PAX2 paired domain protein | 0.78 | 599 - 621 | (+) | 0.789 | 0.806 | gccactgtgctcggctgAAAAaa |
| V$YY1F/YY1.02 | Yin and Yang 1 repressor sites | 0.94 | 618 - 636 | (+) | 1.000 | 0.944 | aaaaaCCATatttttaatt |
| V$BRNF/BRN2.01 | Brn-2, POU-III protein class | 0.86 | 620 - 638 | (-) | 0.933 | 0.875 | taAATTaaaaatatggttt |
| V$ATBF/ATBF1.01 | AT-binding transcription factor 1 | 0.79 | 623 - 639 | (+) | 1.000 | 0.804 | ccatattttAATTtaa |
| V$LHXF/LHX3.01 | Homeodomain binding site in LIM/Homeodomain factor LHX3 | 0.81 | 626 - 640 | (+) | 1.000 | 0.830 | tatttTTAAtttaaa |
| V$HOMF/MSX.01 | Homeodomain proteins MSX-1 and MSX-2 | 0.97 | 627 - 639 | (+) | 1.000 | 0.989 | attttTAATttaa |
| V$RBIT/BRIGHT.01 | Bright, B cell regulator of IgH transcription | 0.92 | 628 - 640 | (-) | 1.000 | 0.950 | tttaaATTAaaaa |
| V$ATBF/ATBF1.01 | AT-binding transcription factor 1 | 0.79 | 630 - 646 | (-) | 1.000 | 0.840 | tattatttaAATTaaa |
| V$HOMF/EN1.01 | Homeobox protein engrailed (en-1) | 0.77 | 631 - 643 | (-) | 1.000 | 0.773 | tatTTTAaattaa |
| V$FKHD/FHXB.01 | Fork head homologous X binds DNA with a dual sequence specificity (FHXA and FHXB) | 0.83 | 635 - 651 | (+) | 1.000 | 0.863 | tttaaaATAAtaaatgg |
| V$BRNF/BRN3.01 | Brn-3, POU-IV protein class | 0.78 | 636 - 654 | (-) | 1.000 | 0.887 | gacccatttATTAttttaa |
| V$LHXF/LMX1B.01 | LIM-homeodomain transcription factor | 0.91 | 636 - 650 | (+) | 1.000 | 0.955 | ttaaaaTAATaaatg |
| V$GKLF/GKLF.01 | Gut-enriched Krueppel-like factor | 0.86 | 640 - 652 | (+) | 0.798 | 0.868 | aataataaaTGGG |
| V$RORA/RORA2.01 | RAR-related orphan receptor alpha2 | 0.82 | 642 - 664 | (+) | 1.000 | 0.824 | taataaatgGGTCtgaccagaaa |
| V$RUSH/SMARCA3.01 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3 | 0.96 | 643 - 653 | (-) | 1.000 | 0.960 | acCCATttatt |
| V$HEAT/HSF1.03 | Heat shock factor 1 | 0.76 | 648 - | (+) | 1.000 | 0.787 | atgggtctgaccAGAAatgcctgcc |

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|---|---|---|---|---|---|---|---|---|
| | | | | 672 | | | | |
| | V$ETSF/FLI.01 | ETS family member FLI | 0.81 | 652 - 672 | (+) | 0.750 | 0.825 | gtctgaCCAGaaatgcctgcc |
| | V$HMTB/MTBF.01 | Muscle-specific Mt binding site | 0.90 | 674 - 682 | (-) | 1.000 | 0.906 | tgcaATTTg |
| | V$NR2F/TR4.02 | TR4 homodimer, DR1 site | 0.75 | 676 - 700 | (+) | 0.814 | 0.761 | aattgcAGGAcacatgacaaagccc |
| | V$BTBF/KAISO.01 | Transcription factor Kaiso, ZBTB33 | 0.92 | 677 - 687 | (-) | 1.000 | 0.976 | tgtcCTGCaat |
| | V$AP1R/TCF11MAFG.01 | TCF11/MafG heterodimers, binding to subclass of AP1 sites | 0.81 | 681 - 705 | (+) | 1.000 | 0.829 | caggacacaTGACaaagcccattct |
| | V$EBOX/USF.04 | Upstream stimulating factor 1/2 | 0.90 | 682 - 694 | (+) | 0.914 | 0.920 | aggaCACAtgaca |
| | V$NR2F/HNF4.03 | Hepatic nuclear factor 4, DR1 sites | 0.83 | 683 - 707 | (+) | 1.000 | 0.862 | ggacacatgaCAAAgcccattctta |
| | V$CREB/E4BP4.01 | E4BP4, bZIP domain, transcriptional repressor | 0.80 | 698 - 718 | (-) | 1.000 | 0.907 | aatgtcttaaGTAAgaatggg |
| | V$CREB/E4BP4.01 | E4BP4, bZIP domain, transcriptional repressor | 0.80 | 699 - 719 | (+) | 0.758 | 0.820 | ccattcttacTTAAgacattt |
| | V$PARF/TEF_HLF.01 | Thyrotrophic embryonic factor / hepatic leukemia factor | 0.78 | 700 - 716 | (+) | 1.000 | 0.788 | cattcTTACttaagaca |
| | V$PARF/TEF.01 | Thyrotrophic embryonic factor | 0.85 | 701 - 717 | (-) | 1.000 | 0.882 | atgtcttaaGTAAgaat |
| | V$CEBP/CEBP.02 | CCAAT/enhancer binding protein | 0.92 | 702 - 716 | (-) | 0.971 | 0.954 | tgtcttaaGTAAgaa |
| | V$NKXH/NKX31.01 | Prostate-specific homeodomain protein NKX3.1 | 0.84 | 702 - 716 | (-) | 1.000 | 0.893 | tgtcttAAGTaagaa |
| | V$TBPF/ATATA.01 | Avian C-type LTR TATA box | 0.78 | 703 - 719 | (+) | 1.000 | 0.833 | tcttactTAAGacattt |
| | V$NKXH/HMX2.01 | Hmx2/Nkx5-2 homeodomain transcription factor | 0.83 | 705 - 719 | (+) | 1.000 | 0.905 | ttaCTTAagacattt |
| | V$DMTF/DMP1.01 | Cyclin D-interacting myb-like protein, | 0.82 | 737 - | (+) | 1.000 | 0.903 | taccgGGATgtaa |

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|---|---|---|---|---|---|---|---|---|
| | | DMTF1 - cyclin D binding myb-like transcription factor 1 | | 749 | | | | |
| | V$PARF/VBP.01 | PAR-type chicken vitellogenin promoter-binding protein | 0.86 | 737 - 753 | (+) | 1.000 | 0.902 | taccgggatGTAAcccc |
| | V$E4FF/E4F.01 | GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter | 0.82 | 741 - 753 | (+) | 0.842 | 0.830 | gggATGTaacccc |
| | V$HAND/HAND2_E12.01 | Heterodimers of the bHLH transcription factors HAND2 (Thing2) and E12 | 0.75 | 762 - 776 | (+) | 0.758 | 0.795 | tcgaggCCCAtctgc |
| | V$HEN1/HEN1.01 | HEN1 | 0.82 | 762 - 782 | (-) | 0.826 | 0.840 | cagggtgCAGAtgggcctcga |
| | V$AP4R/TAL1BETAHEB.01 | Tal-1beta/HEB heterodimer | 0.86 | 764 - 780 | (-) | 1.000 | 0.862 | gggtgCAGAtgggcctc |
| | V$NEUR/NEUROG.01 | Neurogenin 1 and 3 (ngn1/3) binding sites | 0.92 | 766 - 778 | (+) | 1.000 | 0.944 | ggcCCATctgcac |
| | V$HESF/HES1.01 | Drosophila hairy and enhancer of split homologue 1 (HES-1) | 0.92 | 771 - 785 | (-) | 1.000 | 0.932 | tagcaggGTGCagat |
| | V$MTF1/MTF-1.01 | Metal transcription factor 1, MRE | 0.88 | 771 - 785 | (+) | 1.000 | 0.887 | atctGCACcctgcta |
| | V$BTBF/KAISO.01 | Transcription factor Kaiso, ZBTB33 | 0.92 | 776 - 786 | (+) | 1.000 | 0.921 | caccCTGCtat |
| | V$MEF2/SL1.01 | Member of the RSRF (related to serum response factor) protein family from Xenopus laevis | 0.84 | 776 - 798 | (+) | 1.000 | 0.846 | caccctgCTATagctactggtag |
| | V$CLOX/CDPCR3.01 | Cut-like homeodomain protein | 0.73 | 782 - 800 | (-) | 0.880 | 0.809 | atctaccagtagctATAGc |
| | V$GATA/GATA3.02 | GATA-binding factor 3 | 0.91 | 794 - 806 | (+) | 1.000 | 0.953 | ggtAGATcttacc |
| | V$RXRF/RAR_RXR.01 | Retinoic acid receptor / retinoid X receptor heterodimer, DR1 sites | 0.78 | 794 - 818 | (-) | 1.000 | 0.794 | ttttgggaaacAGGTaagatctacc |
| | V$PERO/PPAR_RXR.01 | PPAR/RXR heterodimers, DR1 sites | 0.76 | 799 - 821 | (-) | 1.000 | 0.822 | ccttttttgggaaacAGGTaagat |
| | V$ZFHX/AREB6.01 | AREB6 (Atp1a1 regulatory element binding | 0.93 | 799 - | (+) | 1.000 | 0.963 | atcttACCTgttt |

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|---|---|---|---|---|---|---|---|---|
| | | factor 6) | | 811 | | | | |
| | V$ZFHX/AREB6.04 | AREB6 (Atp1a1 regulatory element binding factor 6) | 0.98 | 803 - 815 | (+) | 1.000 | 0.988 | tacctGTTTccca |
| | V$STAT/STAT.01 | Signal transducers and activators of transcription | 0.87 | 804 - 822 | (-) | 1.000 | 0.924 | tcctttttgGGAAacaggt |
| | V$RBPF/RBPJK.02 | Mammalian transcriptional repressor RBP-Jkappa/CBF1 | 0.94 | 805 - 819 | (-) | 1.000 | 0.940 | ttttTGGGaaacagg |
| | V$IKRS/IK2.01 | Ikaros 2, potential regulator of lymphocyte differentiation | 0.98 | 806 - 818 | (-) | 1.000 | 0.992 | ttttGGGAaacag |
| | V$MOKF/MOK2.02 | Ribonucleoprotein associated zinc finger protein MOK-2 (human) | 0.98 | 814 - 834 | (-) | 1.000 | 0.983 | aaagtagttgggtCCTTtttg |
| | V$BARB/BARBIE.01 | Barbiturate-inducible element | 0.88 | 824 - 838 | (-) | 1.000 | 0.907 | acctAAAGtagttgg |
| | V$PLZF/PLZF.01 | Promyelocytic leukemia zink finger (TF with nine Krueppel-like zink fingers) | 0.86 | 824 - 838 | (-) | 0.958 | 0.870 | accTAAAgtagttgg |
| | V$MOKF/MOK2.02 | Ribonucleoprotein associated zinc finger protein MOK-2 (human) | 0.98 | 837 - 857 | (-) | 1.000 | 0.988 | ttccaaaaaagtgCCTTgaac |
| | V$RUSH/SMARCA3.02 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3 | 0.98 | 842 - 852 | (+) | 1.000 | 0.992 | aggcACTTttt |
| | V$BCL6/BCL6.01 | POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma | 0.76 | 844 - 860 | (-) | 1.000 | 0.783 | ataTTCCaaaaaagtgc |
| | V$STAT/STAT.01 | Signal transducers and activators of transcription | 0.87 | 845 - 863 | (+) | 1.000 | 0.876 | cacttttttGGAAtattta |
| | V$HNF1/HNF1.01 | Hepatic nuclear factor 1 | 0.80 | 850 - 866 | (-) | 1.000 | 0.819 | tGTTAaatattccaaaa |
| | V$OCTP/OCT1P.01 | Octamer-binding factor 1, POU-specific domain | 0.86 | 851 - 863 | (-) | 1.000 | 0.910 | taaATATtccaaa |
| | V$ZNFP/SZF1.01 | SZF1, hematopoietic progenitor-restricted KRAB-zinc finger protein | 0.82 | 864 - 888 | (+) | 0.875 | 0.846 | acaGGGAaagagcctggaactaaaa |
| | V$GRHL/GRHL3.01 | Grainyhead-like 3 (sister-of-mammalian | 0.82 | 885 - | (-) | 0.761 | 0.835 | tagaccTGTTttt |

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|---|---|---|---|---|---|---|---|---|
| | | grainyhead - SOM) | | 897 | | | | |
| | V$AHRR/AHR.01 | Aryl hydrocarbon / dioxin receptor | 0.78 | 887 - 911 | (-) | 1.000 | 0.782 | gcggcatcttGCGTtagacctgttt |
| | V$CEBP/CEBP.02 | CCAAT/enhancer binding protein | 0.92 | 892 - 906 | (+) | 1.000 | 0.922 | ggtctaacGCAAgat |
| | V$MYBL/VMYB.04 | v-Myb, AMV v-myb | 0.85 | 894 - 906 | (+) | 1.000 | 0.887 | tctAACGcaagat |
| | V$E2FF/E2F4_DP1.01 | E2F-4/DP-1 heterodimeric complex | 0.84 | 902 - 918 | (-) | 0.769 | 0.842 | tcgtgtGGCGgcatctt |
| | V$E2FF/E2F4_DP1.01 | E2F-4/DP-1 heterodimeric complex | 0.84 | 903 - 919 | (+) | 0.769 | 0.840 | agatgcCGCCacacgat |
| | V$YY1F/YY1.01 | Yin and Yang 1 activator sites | 0.82 | 908 - 926 | (-) | 1.000 | 0.828 | cagtgCCATcgtgtggcgg |
| | V$ZF35/ZNF35.01 | Human zinc finger protein ZNF35 | 0.96 | 936 - 948 | (+) | 1.000 | 0.962 | acctggAAGAaac |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 943 - 959 | (-) | 1.000 | 0.829 | cccGCGCccgcgtttct |
| | V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 943 - 965 | (-) | 1.000 | 0.899 | ccagctcCCGCgcccgcgtttct |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 944 - 960 | (+) | 0.750 | 0.786 | gaaACGCgggcgcggga |
| | V$E2FF/E2F.03 | E2F, involved in cell cycle regulation, interacts with Rb p107 protein | 0.85 | 948 - 964 | (+) | 1.000 | 0.916 | cgcggGCGCgggagctg |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 949 - 965 | (-) | 0.750 | 0.784 | ccaGCTCccgcgcccgc |
| | V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 949 - 959 | (+) | 1.000 | 0.957 | gcggGCGCggg |
| | V$STAF/ZNF76_143.01 | ZNF143 is the human ortholog of Xenopus Staf, ZNF76 is a DNA binding protein related to ZNF143 and Staf | 0.76 | 971 - 993 | (-) | 0.809 | 0.764 | ccgaCCCCgcagacccgggcgtc |

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|---|---|---|---|---|---|---|---|---|
| V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 986 - 1002 | (+) | 1.000 | 0.950 | ggggtcGGGGagtagcg |
| V$DEAF/NUDR.01 | NUDR (nuclear DEAF-1 related transcriptional regulator protein) | 0.73 | 987 - 1005 | (+) | 1.000 | 0.731 | gggTCGGggagtagcgtcc |
| V$SP1F/BTEB3.01 | Basic transcription element (BTE) binding protein, BTEB3, FKLF-2 | 0.93 | 989 - 1003 | (+) | 1.000 | 0.938 | gtcggGGAGtagcgt |
| V$CREB/TAXCREB.02 | Tax/CREB complex | 0.71 | 991 - 1011 | (-) | 0.750 | 0.711 | ccggccGGACgctactccccg |
| V$ETSF/CETS1P54.01 | c-Ets-1(p54) | 0.92 | 993 - 1013 | (-) | 1.000 | 0.922 | ctccggCCGGacgctactccc |
| V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 996 - 1006 | (-) | 1.000 | 0.975 | cggACGCtact |
| V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 1010 - 1032 | (+) | 1.000 | 0.915 | ggagagcCCGCacctcgaggagc |
| V$CDEF/CDE.01 | Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression) | 0.87 | 1039 - 1051 | (+) | 1.000 | 0.870 | aggaCGCGgtgtc |
| V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 1039 - 1049 | (+) | 1.000 | 0.965 | aggACGCggtg |
| V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 1054 - 1064 | (-) | 1.000 | 0.955 | gagACGCggtc |
| V$ZBPF/ZBP89.01 | Zinc finger transcription factor ZBP-89 | 0.93 | 1054 - 1076 | (+) | 1.000 | 0.938 | gaccgcgtctCCCCcatctgctc |
| V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1057 - 1079 | (+) | 1.000 | 0.912 | cgcgtctCCCCcatctgctctgc |
| V$AP4R/TAL1BETAHEB.01 | Tal-1beta/HEB heterodimer | 0.86 | 1062 - 1078 | (-) | 1.000 | 0.899 | cagagCAGAtgggggag |
| V$YY1F/YY1.01 | Yin and Yang 1 activator sites | 0.82 | 1062 - 1080 | (+) | 1.000 | 0.838 | ctcccCCATctgctctgct |
| V$NEUR/NEUROG.01 | Neurogenin 1 and 3 (ngn1/3) binding sites | 0.92 | 1064 - | (+) | 1.000 | 0.962 | cccCCATctgctc |

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|---|---|---|---|---|---|---|---|---|
| | | | | 1076 | | | | |
| | V$E2FF/E2F.02 | E2F, involved in cell cycle regulation, interacts with Rb p107 protein | 0.84 | 1077 - 1093 | (-) | 1.000 | 0.886 | cgctgtcgcCAAAagca |
| | V$E2FF/E2F4_DP1.01 | E2F-4/DP-1 heterodimeric complex | 0.84 | 1078 - 1094 | (+) | 0.769 | 0.868 | gctttGGCGacagcgt |
| | V$E2FF/RB_E2F1_DP1.01 | RB/E2F-1/DP-1 heterotrimeric complex | 0.71 | 1080 - 1096 | (+) | 0.765 | 0.757 | ttttgGCGAcagcgtcc |
| | V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 1087 - 1097 | (-) | 1.000 | 0.983 | gggACGCtgtc |
| | V$NR2F/HNF4.02 | Hepatic nuclear factor 4, DR2 sites | 0.76 | 1094 - 1118 | (-) | 1.000 | 0.800 | cgtcctggccgAAAGgccgacggga |
| | V$GLIF/GLI3.01 | GLI-Kruppel family member GLI3 | 0.86 | 1165 - 1179 | (+) | 0.822 | 0.866 | gggaCCTCcgaggag |
| | V$MAZF/MAZ.01 | Myc associated zinc finger protein (MAZ) | 0.90 | 1170 - 1182 | (+) | 1.000 | 0.930 | ctccGAGGagagc |
| | V$DMTF/DMP1.01 | Cyclin D-interacting myb-like protein, DMTF1 - cyclin D binding myb-like transcription factor 1 | 0.82 | 1193 - 1205 | (+) | 1.000 | 0.832 | ggcccGGATtccc |
| | V$NOLF/OLF1.01 | Olfactory neuron-specific factor | 0.82 | 1196 - 1218 | (+) | 1.000 | 0.846 | ccggatTCCCcgaggcgcccgag |
| | V$AP2F/AP2.02 | Activator protein 2 alpha | 0.92 | 1200 - 1214 | (-) | 1.000 | 0.931 | ggcGCCTcgggaat |
| | V$DEAF/NUDR.01 | NUDR (nuclear DEAF-1 related transcriptional regulator protein) | 0.73 | 1202 - 1220 | (-) | 1.000 | 0.743 | cacTCGGgcgcctcgggga |
| | V$HICF/HIC1.01 | Hypermethylated in cancer 1, transcriptional repressor containing five Krüppel-like C2H2 zinc fingers, for optimal binding multiple binding sites are required. | 0.93 | 1224 - 1236 | (-) | 1.000 | 0.985 | agggcTGCCcgca |
| | V$EGRF/CKROX.01 | Collagen krox protein (zinc finger protein 67 - zfp67) | 0.88 | 1227 - 1243 | (-) | 1.000 | 0.887 | ggcgGGGAgggctgccc |
| | V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1229 - 1243 | (-) | 0.807 | 0.891 | ggcgGGGAgggctgc |

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|---|---|---|---|---|---|---|---|---|
| V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1230 - 1246 | (-) | 1.000 | 0.933 | gcaggcGGGGagggctg |
| V$MYOD/MYOD.01 | Myogenic regulatory factor MyoD (myf3) | 0.88 | 1234 - 1250 | (-) | 1.000 | 0.922 | cctGGCAggcggggagg |
| V$MZF1/MZF1.01 | Myeloid zinc finger protein MZF1 | 0.99 | 1234 - 1242 | (-) | 1.000 | 0.991 | gcGGGGagg |
| V$AP4R/AP4.01 | Activator protein 4 | 0.85 | 1248 - 1264 | (-) | 1.000 | 0.874 | ggggaCAGCggcggcct |
| V$CREB/ATF6.02 | Activating transcription factor 6, member of b-zip family, induced by ER stress | 0.85 | 1256 - 1276 | (-) | 1.000 | 0.887 | gggaccaGACGtggggacagc |
| V$MZF1/MZF1.01 | Myeloid zinc finger protein MZF1 | 0.99 | 1258 - 1266 | (-) | 1.000 | 1.000 | gtGGGGaca |
| V$HIFF/HIF1.02 | Hypoxia inducible factor, bHLH / PAS protein family | 0.93 | 1262 - 1274 | (-) | 1.000 | 0.962 | gaccagaCGTGgg |
| V$SMAD/SMAD3.01 | Smad3 transcription factor involved in TGF-beta signaling | 0.99 | 1267 - 1275 | (+) | 1.000 | 0.993 | GTCTggtcc |
| V$AHRR/NXF_ARNT.01 | bHLH-PAS type transcription factors NXF/ARNT heterodimer | 0.90 | 1268 - 1292 | (-) | 1.000 | 0.944 | gcgggaagaggCGTGggggaccaga |
| V$EGRF/WT1.01 | Wilms Tumor Suppressor | 0.92 | 1268 - 1284 | (-) | 1.000 | 0.987 | aggcgTGGGggaccaga |
| V$EGRF/NGFIC.01 | Nerve growth factor-induced protein C | 0.80 | 1270 - 1286 | (-) | 1.000 | 0.887 | agagGCGTgggggacca |
| V$ETSF/GABP.01 | GABP: GA binding protein | 0.86 | 1277 - 1297 | (-) | 1.000 | 0.912 | cagatgcgGGAAgaggcgtgg |
| V$STAT/STAT1.01 | Signal transducer and activator of transcription 1 | 0.77 | 1280 - 1298 | (-) | 1.000 | 0.783 | ccagatgcgGGAAgaggcg |
| V$E2FF/E2F1_DP1.01 | E2F-1/DP-1 heterodimeric complex | 0.81 | 1281 - 1297 | (-) | 0.782 | 0.846 | cagaTGCGggaagaggc |
| V$ZF35/ZNF35.01 | Human zinc finger protein ZNF35 | 0.96 | 1281 - 1293 | (-) | 1.000 | 0.961 | tgcgggAAGAggc |
| V$PAX6/PAX6.01 | Pax-6 paired domain binding site | 0.75 | 1284 - 1302 | (+) | 0.779 | 0.753 | tcttcCCGCatctggtccc |

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|---|---|---|---|---|---|---|---|---|
| V$SRFF/SRF.03 | Serum response factor | 0.79 | 1285 - 1303 | (+) | 0.803 | 0.801 | cttcccgcatCTGGtccca |
| V$AP4R/PARAXIS.01 | Paraxis (TCF15), member of the Twist subfamily of Class B bHLH factors, forms heterodimers with E12 | 0.86 | 1286 - 1302 | (-) | 0.882 | 0.891 | gggACCAgatgcgggaa |
| V$DMTF/DMP1.01 | Cyclin D-interacting myb-like protein, DMTF1 - cyclin D binding myb-like transcription factor 1 | 0.82 | 1286 - 1298 | (+) | 0.801 | 0.832 | ttcccGCATctgg |
| V$NEUR/NEUROG.01 | Neurogenin 1 and 3 (ngn1/3) binding sites | 0.92 | 1288 - 1300 | (+) | 0.833 | 0.931 | cccGCATctggtc |
| V$PAX5/PAX5.01 | B-cell-specific activator protein | 0.79 | 1294 - 1322 | (+) | 0.857 | 0.818 | tctggtCCCAcggtggaggcgcggggcga |
| V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1302 - 1318 | (+) | 0.842 | 0.894 | cacggtggAGGCgcggg |
| V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 1302 - 1324 | (-) | 1.000 | 0.884 | cctcgccCCGCgcctccaccgtg |
| V$SP1F/SP1.02 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.85 | 1308 - 1322 | (+) | 0.750 | 0.863 | ggagGCGCggggcga |
| V$PLAG/PLAG1.01 | Pleomorphic adenoma gene (PLAG) 1, a developmentally regulated C2H2 zinc finger protein | 0.88 | 1309 - 1329 | (+) | 1.000 | 0.881 | GAGGcgcggggcgaggcctc |
| V$EBOX/MYCMAX.03 | MYC-MAX binding sites | 0.91 | 1310 - 1322 | (-) | 1.000 | 0.919 | tcgcccCGCGcct |
| V$MAZF/MAZ.01 | Myc associated zinc finger protein (MAZ) | 0.90 | 1310 - 1322 | (+) | 0.866 | 0.910 | aggcGCGGggcga |
| V$AHRR/AHRARNT.01 | Aryl hydrocarbon receptor / Arnt heterodimers | 0.92 | 1324 - 1348 | (+) | 1.000 | 0.953 | ggcctcacgcgCGTGcccacggtcg |
| V$HESF/HELT.01 | Hey-like bHLH-transcriptional repressor | 0.91 | 1325 - 1339 | (+) | 1.000 | 0.922 | gcctCACGcgcgtgc |
| V$HESF/HELT.01 | Hey-like bHLH-transcriptional repressor | 0.91 | 1328 - 1342 | (-) | 1.000 | 0.961 | tgggCACGcgcgtga |
| V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear | 0.78 | 1328 - 1344 | (-) | 0.750 | 0.834 | cgtGGGCacgcgcgtga |

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|---|---|---|---|---|---|---|---|---|
| | | genes encoding mitochondrial proteins | | | | | | |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1329 - 1345 | (+) | 1.000 | 0.842 | cacGCGCgtgcccacgg |
| | V$EBOX/MYCMAX.03 | MYC-MAX binding sites | 0.91 | 1330 - 1342 | (-) | 1.000 | 0.921 | tgggcaCGCGcgt |
| | V$PAX5/PAX5.01 | B-cell-specific activator protein | 0.79 | 1333 - 1361 | (+) | 0.857 | 0.804 | cgcgtgCCCAcggtcgggggcgtccttg |
| | V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1338 - 1360 | (-) | 1.000 | 0.946 | aaggacgCCCCccgaccgtgggc |
| | V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1343 - 1359 | (+) | 1.000 | 0.868 | cggtcgggGGGCgtcct |
| | V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 1349 - 1359 | (-) | 1.000 | 0.953 | aggACGCcccc |
| | V$SF1F/FTF.01 | Alpha (1)-fetoprotein transcription factor (FTF), liver receptor homologue-1 (LRH-1) | 0.94 | 1353 - 1365 | (-) | 1.000 | 0.951 | cgtcCAAGgacgc |
| | V$AHRR/NXF_ARNT.01 | bHLH-PAS type transcription factors NXF/ARNT heterodimer | 0.90 | 1370 - 1394 | (-) | 1.000 | 0.916 | gggtggagaccCGTGggagaggacc |
| | V$ZBPF/ZBP89.01 | Zinc finger transcription factor ZBP-89 | 0.93 | 1382 - 1404 | (+) | 1.000 | 0.968 | cgggtctccaCCCCccgccctcc |
| | V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1385 - 1407 | (+) | 1.000 | 0.986 | gtctccaCCCCccgccctccggc |
| | V$SP1F/TIEG.01 | TGFbeta-inducible early gene (TIEG) / Early growth response gene alpha (EGRalpha) | 0.83 | 1386 - 1400 | (-) | 1.000 | 0.858 | ggcGGGGggtggaga |
| | V$EGRF/WT1.01 | Wilms Tumor Suppressor | 0.92 | 1387 - 1403 | (-) | 0.953 | 0.963 | gagggCGGGgggtggag |
| | V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1387 - 1403 | (-) | 1.000 | 0.945 | gagggcGGGGggtggag |
| | V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1391 - 1405 | (-) | 1.000 | 0.934 | cggaGGGCgggggt |

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|---|---|---|---|---|---|---|---|---|
| V$PURA/PURALPHA.01 | Purine-rich element binding protein A | 0.97 | 1392 - 1404 | (-) | 1.000 | 0.974 | ggAGGGcgggggg |
| V$AP2F/AP2.01 | Activator protein 2 | 0.90 | 1396 - 1410 | (-) | 0.881 | 0.933 | gtcGCCGgagggcgg |
| V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1422 - 1438 | (-) | 1.000 | 0.797 | gcaGCGCgcgcgtccca |
| V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1423 - 1439 | (+) | 0.750 | 0.788 | gggACGCgcgcgctgcg |
| V$WHNF/WHN.01 | Winged helix protein, involved in hair keratinization and thymus epithelium differentiation | 0.95 | 1423 - 1433 | (+) | 1.000 | 0.952 | gggACGCgcgc |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1425 - 1435 | (-) | 1.000 | 0.966 | gcgcGCGCgtc |
| V$HESF/HES1.01 | Drosophila hairy and enhancer of split homologue 1 (HES-1) | 0.92 | 1426 - 1440 | (-) | 0.944 | 0.924 | ccgcagcGCGCgcgt |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1428 - 1438 | (+) | 1.000 | 0.960 | gcgcGCGCtgc |
| V$P53F/P53.05 | Tumor suppressor p53 | 0.78 | 1438 - 1460 | (-) | 0.800 | 0.799 | aaatCAAAaccaggacttggccg |
| V$IRFF/IRF7.01 | Interferon regulatory factor 7 (IRF-7) | 0.86 | 1445 - 1465 | (-) | 1.000 | 0.877 | gagtGAAAtcaaaaccaggac |
| V$GFI1/GFI1.02 | Growth factor independence 1 | 0.90 | 1448 - 1462 | (-) | 1.000 | 0.906 | tgaAATCaaaaccag |
| V$PRDF/PRDM1.01 | PRDI binding factor 1 | 0.81 | 1449 - 1467 | (-) | 1.000 | 0.835 | gggagtGAAAtcaaaacca |
| V$IRFF/IRF4.01 | Interferon regulatory factor (IRF)-related protein (NF-EM5, PIP, LSIRF, ICSAT) | 0.94 | 1451 - 1471 | (-) | 1.000 | 0.959 | ggttgggagtGAAAtcaaaac |
| V$NFKB/NFKAPPAB65.01 | NF-kappaB (p65) | 0.87 | 1480 - 1492 | (+) | 1.000 | 0.902 | cgaggaggTTCCa |
| V$ETSF/SPIB.01 | Spi-B transcription factor (Spi-1/PU.1 related) | 0.88 | 1497 - 1517 | (+) | 1.000 | 0.944 | cacaaacaGGAAgaagagaag |

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|---|---|---|---|---|---|---|---|---|
| V$EVI1/EVI1.07 | Evi-1 zinc finger protein, carboxy-terminal zinc finger domain | 0.90 | 1502 - 1518 | (+) | 1.000 | 0.913 | acaggAAGAagagaagg |
| V$MOKF/MOK2.02 | Ribonucleoprotein associated zinc finger protein MOK-2 (human) | 0.98 | 1511 - 1531 | (-) | 1.000 | 0.984 | cagcactccagggCCTTctct |
| V$THAP/THAP1.01 | THAP domain containing, apoptosis associated protein | 0.90 | 1525 - 1535 | (+) | 1.000 | 0.940 | agtgctGGCAg |
| V$MYOD/MYOD.01 | Myogenic regulatory factor MyoD (myf3) | 0.88 | 1528 - 1544 | (+) | 1.000 | 0.881 | gctGGCAgagggagtgt |
| V$SP1F/BTEB3.01 | Basic transcription element (BTE) binding protein, BTEB3, FKLF-2 | 0.93 | 1533 - 1547 | (+) | 1.000 | 0.958 | cagagGGAGtgtcat |
| V$TEAF/TEAD.01 | TEA domain-containing factors, transcriptional enhancer factors 1,3,4,5 | 0.90 | 1542 - 1554 | (+) | 1.000 | 0.921 | tgtCATTcccagc |
| V$IKRS/IK1.01 | Ikaros 1, potential regulator of lymphocyte differentiation | 0.92 | 1543 - 1555 | (-) | 1.000 | 0.937 | tgctGGGAatgac |
| V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1574 - 1590 | (+) | 1.000 | 0.939 | cgggccGGGGagggagc |
| V$EGRF/CKROX.01 | Collagen krox protein (zinc finger protein 67 - zfp67) | 0.88 | 1577 - 1593 | (+) | 1.000 | 0.905 | gccgGGGAgggagcagc |
| V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1577 - 1591 | (+) | 0.807 | 0.887 | gccgGGGAgggagca |
| V$NRSF/NRSF.01 | Neuron-restrictive silencer factor | 0.69 | 1588 - 1608 | (+) | 1.000 | 0.744 | agcAGCAgcgcgggcaccctc |
| V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1591 - 1607 | (-) | 0.750 | 0.788 | aggGTGCccgcgctgct |
| V$CDEF/CDE.01 | Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression) | 0.87 | 1592 - 1604 | (+) | 1.000 | 0.876 | gcagCGCGggcac |
| V$HICF/HIC1.01 | Hypermethylated in cancer 1, transcriptional repressor containing five Krüppel-like C2H2 zinc fingers, for optimal binding multiple binding sites are required. | 0.93 | 1596 - 1608 | (-) | 1.000 | 0.992 | gagggTGCCcgcg |
| V$HESF/HES1.01 | Drosophila hairy and enhancer of split | 0.92 | 1597 - | (-) | 1.000 | 0.962 | caggaggGTGCccgc |

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|---|---|---|---|---|---|---|---|---|
| | | homologue 1 (HES-1) | | 1611 | | | | |
| | V$CHRE/CHREBP_MLX.01 | Carbohydrate response element binding protein (CHREBP) and Max-like protein X (Mlx) bind as heterodimers to glucose-responsive promoters | 0.83 | 1602 - 1618 | (-) | 1.000 | 0.855 | CACGttgcaggagggtg |
| | V$BTBF/KAISO.01 | Transcription factor Kaiso, ZBTB33 | 0.92 | 1605 - 1615 | (+) | 1.000 | 0.981 | cctcCTGCaac |
| | V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1612 - 1634 | (-) | 1.000 | 0.920 | ctgggaCCCCcagcccacgttg |
| | V$NFKB/NFKAPPAB50.01 | NF-kappaB (p50) | 0.83 | 1621 - 1633 | (+) | 0.750 | 0.876 | ctgGGGGtcccca |
| | V$NFKB/NFKAPPAB50.01 | NF-kappaB (p50) | 0.83 | 1622 - 1634 | (-) | 1.000 | 0.934 | ctgGGGAccccca |
| | V$NOLF/OLF1.01 | Olfactory neuron-specific factor | 0.82 | 1622 - 1644 | (+) | 1.000 | 0.846 | tggggTCCCcagggccggggag |
| | V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1633 - 1649 | (+) | 1.000 | 0.947 | agggccGGGGaggggc |
| | V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1633 - 1655 | (-) | 1.000 | 0.925 | cccggggCCCCctccccggccct |
| | V$EGRF/CKROX.01 | Collagen krox protein (zinc finger protein 67 - zfp67) | 0.88 | 1636 - 1652 | (+) | 1.000 | 0.920 | gccgGGGAgggggcccc |
| | V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1636 - 1650 | (+) | 0.807 | 0.911 | gccgGGGAgggggcc |
| | V$EGRF/WT1.01 | Wilms Tumor Suppressor | 0.92 | 1638 - 1654 | (+) | 0.837 | 0.930 | cggggAGGGggccccgg |
| | V$MAZF/MAZR.01 | MYC-associated zinc finger protein related transcription factor | 0.88 | 1638 - 1650 | (+) | 1.000 | 0.914 | cgggaGGGGgcc |
| | V$ETSF/SPI1_PU1.02 | Spleen focus forming virus (SFFV) proviral integration oncogene Spi1/transcription factor PU.1 | 0.96 | 1650 - 1670 | (+) | 1.000 | 0.969 | cccggggaGGAActgagaggg |
| | V$MOKF/MOK2.01 | Ribonucleoprotein associated zinc finger protein MOK-2 (mouse) | 0.74 | 1652 - 1672 | (-) | 0.750 | 0.752 | cccctctcagttCCTCcccg |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V$PLAG/PLAG1.01 | Pleomorphic adenoma gene (PLAG) 1, a developmentally regulated C2H2 zinc finger protein | 0.88 | 1656 - 1676 | (+) | 1.000 | 0.908 | GAGGaactgagaggggcgcg |
| V$GABF/GAGA.01 | GAGA-Box | 0.78 | 1660 - 1684 | (+) | 1.000 | 0.780 | aactgAGAGggggcgcgccccggga |
| V$EGRF/WT1.01 | Wilms Tumor Suppressor | 0.92 | 1662 - 1678 | (+) | 0.837 | 0.925 | ctgagAGGGggcgcgcc |
| V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1663 - 1679 | (+) | 1.000 | 0.912 | tgagagGGGGcgcgccc |
| V$INSM/INSM1.01 | Zinc finger protein insulinoma-associated 1 (IA-1) functions as a transcriptional repressor | 0.90 | 1663 - 1675 | (+) | 1.000 | 0.936 | tgagaGGGGgcgc |
| V$E2FF/E2F.03 | E2F, involved in cell cycle regulation, interacts with Rb p107 protein | 0.85 | 1666 - 1682 | (-) | 1.000 | 0.863 | ccgggGCGCgccccctc |
| V$E2FF/E2F.03 | E2F, involved in cell cycle regulation, interacts with Rb p107 protein | 0.85 | 1667 - 1683 | (+) | 1.000 | 0.872 | aggggGCGCgccccggg |
| V$HESF/HES1.01 | Drosophila hairy and enhancer of split homologue 1 (HES-1) | 0.92 | 1668 - 1682 | (-) | 0.944 | 0.943 | ccgggcGCGCcccc |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1668 - 1678 | (+) | 1.000 | 0.959 | ggggGCGCgcc |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1669 - 1679 | (-) | 1.000 | 0.965 | gggcGCGCccc |
| V$HEAT/HSF2.01 | Heat shock factor 2 | 0.88 | 1670 - 1694 | (+) | 1.000 | 0.887 | gggcgcgccccggGAAGgctcgggg |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1670 - 1680 | (+) | 1.000 | 0.965 | gggcGCGCccc |
| V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 1682 - 1704 | (-) | 1.000 | 0.934 | cccggagCCGCcccgagccttcc |
| V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1688 - 1702 | (+) | 1.000 | 0.887 | ctcgGGGCggctccg |
| V$PLAG/PLAG1.01 | Pleomorphic adenoma gene (PLAG) 1, a developmentally regulated C2H2 zinc finger protein | 0.88 | 1691 - 1711 | (+) | 0.958 | 0.896 | GGGGcggctccgggggctcc |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V$NRSF/NRSE.01 | Neural-restrictive-silencer-element | 0.67 | 1692 - 1712 | (-) | 1.000 | 0.728 | gggagccccCGGAgccgccc |
| V$GLIF/ZIC2.01 | Zinc finger transcription factor, Zic family member 2 (odd-paired homolog, Drosophila) | 0.89 | 1698 - 1712 | (-) | 1.000 | 0.899 | gggagccCCCCggag |
| V$NFKB/NFKAPPAB.01 | NF-kappaB | 0.89 | 1702 - 1714 | (-) | 1.000 | 0.908 | ctGGGAgccccccc |
| V$ZBPF/ZNF219.01 | Kruppel-like zinc finger protein 219 | 0.91 | 1712 - 1734 | (+) | 1.000 | 0.996 | cagggcgCCCCcgccccttcccc |
| V$EGRF/WT1.01 | Wilms Tumor Suppressor | 0.92 | 1713 - 1729 | (-) | 0.953 | 0.980 | aggggCGGGggcgccct |
| V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1713 - 1729 | (+) | 1.000 | 0.797 | aggGCGCccccgccct |
| V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1713 - 1723 | (-) | 1.000 | 0.956 | gggGCGCcct |
| V$PLAG/PLAG1.01 | Pleomorphic adenoma gene (PLAG) 1, a developmentally regulated C2H2 zinc finger protein | 0.88 | 1715 - 1735 | (-) | 0.958 | 0.894 | GGGGgaaggggcgggggcgcc |
| V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 1715 - 1737 | (+) | 1.000 | 0.940 | ggcgcccCCGCcccttcccccgc |
| V$MAZF/MAZR.01 | MYC-associated zinc finger protein related transcription factor | 0.88 | 1717 - 1729 | (-) | 1.000 | 0.902 | aggggcGGGGgcg |
| V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1717 - 1731 | (-) | 1.000 | 0.986 | gaagGGGCgggggcg |
| V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1718 - 1734 | (-) | 1.000 | 0.945 | ggggaaGGGGcgggggc |
| V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1719 - 1735 | (-) | 1.000 | 0.911 | gggggaagGGGCggggg |
| V$ETSF/SPI1_PU1.02 | Spleen focus forming virus (SFFV) proviral integration oncogene Spi1/transcription factor PU.1 | 0.96 | 1720 - 1740 | (-) | 1.000 | 0.960 | gccgcgggGGAAggggcgggg |
| V$STAF/ZNF76_143.01 | ZNF143 is the human ortholog of Xenopus | 0.76 | 1727 - | (+) | 0.809 | 0.817 | ccttCCCCcgcggccccgggcg |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Staf, ZNF76 is a DNA binding protein related to ZNF143 and Staf | | 1749 | | | | |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1743 - 1759 | (-) | 0.750 | 0.847 | accGCGGgggcgccccg |
| | V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1744 - 1760 | (-) | 1.000 | 0.910 | caccgcggGGGCgcccc |
| | V$NRF1/NRF1.01 | Nuclear respiratory factor 1 (NRF1), bZIP transcription factor that acts on nuclear genes encoding mitochondrial proteins | 0.78 | 1744 - 1760 | (+) | 1.000 | 0.783 | gggGCGCccccgcggtg |
| | V$ZF5F/ZF5.01 | Zinc finger / POZ domain transcription factor | 0.95 | 1744 - 1754 | (-) | 1.000 | 0.956 | ggggGCGCccc |
| | V$NRSF/NRSE.01 | Neural-restrictive-silencer-element | 0.67 | 1745 - 1765 | (-) | 0.782 | 0.687 | tccggcaccgCGGGggcgccc |
| | V$ZBPF/ZF9.01 | Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers | 0.87 | 1760 - 1782 | (-) | 1.000 | 0.894 | gcacgccCCGCcccgactccggc |
| | V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1762 - 1778 | (+) | 1.000 | 0.881 | cggagtcgGGGCggggc |
| | V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1763 - 1779 | (+) | 1.000 | 0.940 | ggagtcGGGGcggggcg |
| | V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1766 - 1780 | (+) | 1.000 | 1.000 | gtcgGGGCggggcgt |
| | V$AHRR/AHRARNT.01 | Aryl hydrocarbon receptor / Arnt heterodimers | 0.92 | 1767 - 1791 | (+) | 1.000 | 0.934 | tcggggcggggCGTGcacgtcagcc |
| | V$EGRF/EGR1.02 | EGR1, early growth response 1 | 0.86 | 1767 - 1783 | (+) | 1.000 | 0.870 | tcgggcgGGGCgtgca |
| | V$EKLF/KKLF.01 | Kidney-enriched kruppel-like factor, KLF15 | 0.91 | 1768 - 1784 | (+) | 1.000 | 0.972 | cggggcGGGGcgtgcac |
| | V$MAZF/MAZ.01 | Myc associated zinc finger protein (MAZ) | 0.90 | 1768 - 1780 | (+) | 0.866 | 0.910 | cgggGCGGggcgt |
| | V$SP1F/TIEG.01 | TGFbeta-inducible early gene (TIEG) / Early growth response gene alpha (EGRalpha) | 0.83 | 1771 - 1785 | (+) | 1.000 | 0.859 | ggcGGGGcgtgcacg |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V$HESF/HES1.01 | Drosophila hairy and enhancer of split homologue 1 (HES-1) | 0.92 | 1772 - 1786 | (+) | 1.000 | 0.920 | gcggggcGTGCacgt |
| V$CREB/ATF6.02 | Activating transcription factor 6, member of b-zip family, induced by ER stress | 0.85 | 1774 - 1794 | (-) | 1.000 | 0.990 | cccggctGACGtgcacgcccc |
| V$CREB/CREB.02 | cAMP-responsive element binding protein | 0.89 | 1777 - 1797 | (-) | 1.000 | 0.975 | agccccggcTGACgtgcacgc |
| V$HIFF/HIF1.02 | Hypoxia inducible factor, bHLH / PAS protein family | 0.93 | 1780 - 1792 | (-) | 1.000 | 0.976 | cggctgaCGTGca |
| V$BARB/BARBIE.01 | Barbiturate-inducible element | 0.88 | 1797 - 1811 | (+) | 1.000 | 0.887 | tagaAAAGgcggcgg |
| V$SP1F/GC.01 | GC box elements | 0.88 | 1802 - 1816 | (+) | 1.000 | 0.907 | aaggcGGCGgggctg |
| V$SP1F/SP1.01 | Stimulating protein 1, ubiquitous zinc finger transcription factor | 0.88 | 1807 - 1821 | (+) | 1.000 | 0.898 | ggcgGGGCtgggccc |

307 matches found in this sequence.

*Problems displaying the Java graphics? See our Technical FAQ*

**A total of 307 matches were found in 1 sequences!**

**Sequences searched: 1 (1830 bp).**

For comments, questions, or bug reports, please contact support@genomatix.de.

## TFSEARCH Search Result

```
** TFSEARCH  ver.1.3 **     (c)1995 Yutaka Akiyama (Kyoto Univ.)

 This simple routine searches highly correlated sequence fragments
 versus TFMATRIX transcription factor binding site profile database
 by E.Wingender, R.Knueppel, P.Dietze, H.Karas (GBF-Braunschweig).

  <Warning> Scoring scheme is so straightforward in this version.
            score = 100.0 * ('weighted sum' - min) / (max - min)
            The score does not properly reflect statistical significance!

 Database:  TRANSFAC MATRIX TABLE, Rel.3.3 06-01-1998
 Query:     delta promoter (1830 bases)
 Taxonomy:  ALL
 Threshold: 85.0 point


 TFMATRIX entries with High-scoring:

   1 ACTCTAAAGA AATATGTTTG TTCTTTAGGA AACCTATTAA ATATCCCATA entry        score
          ----->                                          M00028 HSF    100.0
                     <-----                               M00029 HSF    100.0
          ----->                                          M00029 HSF    96.0
                                        <-------          M00101 CdxA   92.9
                <------                                   M00148 SRY    90.9
         ------->                                         M00148 SRY    90.0
                ------------>                             M00131 HNF-3b 89.6
                ------------>                             M00130 HFH-2  88.9
                     <-----                               M00028 HSF    88.5
                                           <----------    M00076 GATA-2 88.1
                                               ----->     M00142 NIT2   87.5
                           ----->                         M00029 HSF    86.9
                     <-------                             M00148 SRY    86.4
                           ----->                         M00028 HSF    85.9
                        ------>                           M00100 CdxA   85.9
        <------------                                     M00131 HNF-3b 85.5
                        ------------>                     M00087 Ik-2   85.5
            ------->                                      M00101 CdxA   85.0
```

```
 51 GTGCAACGTT AGAAATGACC TCTGGAACTG ACTGCAGAAA CTGCACAGCA entry        score
              ---->                                        M00028 HSF    100.0
                                    ---->                  M00028 HSF    100.0
              ---->                                        M00029 HSF    96.0
                                    ---->                  M00029 HSF    96.0
            <-------                                       M00101 CdxA   94.3
                         <--------                         M00253 cap    93.9
              <-------                                     M00253 cap    91.0
                         ---->                             M00029 HSF    90.9
                                     <--------             M00253 cap    89.0
                                              -------      M00175 AP-4   87.5
                                                 <---      M00183 c-Myb  86.6
            <-------                                       M00100 CdxA   85.9
          <----------------                                M00197 ABF1   85.4
                    <---------                             M00111 CF1 /  85.3

101 GTTAGTAGAG CTTGGTGTTC CGACGCAGAT CTGGTGAGAG GCCTGCGCAG entry        score
                     <-----                                M00029 HSF    90.9
    -->                                                    M00175 AP-4   87.5
    ------                                                 M00183 c-Myb  86.6

151 GAAGAGACTC GACATGGAGC AGGTGTGACT AGCGATAGTG ATTTAATGGA entry        score
                                     -------->             M00077 GATA-3 92.8
                                              ------>      M00101 CdxA   92.1
                                       <--------           M00253 cap    91.6
                                     <-----                M00142 NIT2   91.2
                                     --------->            M00075 GATA-1 90.6
                                          ---------------- M00018 Ubx    90.6
                        ---------------->                  M00002 E47    90.4
                              <-------                     M00253 cap    90.4
                                     --------->            M00076 GATA-2 90.1
                        ----->                             M00048 ADR1   89.2
                           <----------                     M00184 MyoD   88.3
                            <--------                      M00217 USF    85.5

201 TTTGGGCCCG ACATGGTGGC TCACGCCTGT AATCCCAGCA CTTTGGGAGG entry        score
                                              --------> M00141 Lyf-1  100.0
                                                   <--- M00253 cap    96.2
                                  <--------             M00140 Bcd    93.7
    ---->                                              M00018 Ubx    90.6
                              <----------------        M00019 Dfd    89.4
                                   <------------       M00087 Ik-2   87.3
```

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                                                  -----> M00048 ADR1   86.2

251 CTGAGGCCGG CGAATCTCTT AAGCCCAGGA GTTTGAAACC AGCCTGGACA entry        score
    ----                                                  M00253 cap    96.2
                                  ------>                 M00048 ADR1   89.2
                                    <--------             M00253 cap    86.2
               ---->                                      M00028 HSF    85.4

301 ATATTGTGAG ATCCCATCTC AATTTTTTTT TTGAGATGGA GTCTCGCTCT entry        score
                                           ------>         M00047 ADR1   93.8
                                <----------                M00022 Hb     92.0
                                 <----------               M00021 Hb     91.1
                               <----------                 M00022 Hb     88.4
                             ------------>                 M00136 HFH-2  87.8
                          -------->                        M00253 cap    87.4
                                        --------->         M00077 GATA-3 87.2
                  <--------                                M00077 GATA-3 86.2
                  <----------                              M00075 GATA-1 86.1
                                      ---------->          M00075 GATA-1 86.1
                         ------------>                     M00130 HFH-2  85.1

351 GTCGCCCAGG CTGGAGTGCA ATGGAACGAT CTCGGCTCAC TGCAACCTCC entry        score
                 ----->                                   M00048 ADR1   93.8
                             ---->                        M00029 HSF    90.9
                                                  <----   M00048 ADR1   90.8
                             ---------->                  M00147 HSF2   90.4
                               <----------                M00075 GATA-1 89.4

401 GCCTCCCGGG TTCAAGCGAT TCTCCTGCCT CAGCCTCCCG AGTAGCTGGG entry        score
                                 -------->                M00253 cap    96.2
                        <-----                            M00029 HSF    95.4
                        <-----                            M00028 HSF    94.3
                                                    ---   M00140 Bcd    93.7
    -                                                     M00048 ADR1   90.8
                                                  ------  M00019 Dfd    89.4
                                                  -------M00087 Ik-2    89.0
            <-----                                        M00029 HSF    86.3
     <-----                                               M00048 ADR1   86.2
                                       <-----             M00046 ADR1   86.2

451 ATTACAGGCG CACGCCACTA CGCTTGGCTA ATTTTTGTAT TTTTAGTAGA entry        score
                                                    ---   M00028 HSF   100.0
```

```
                                                    --- M00029 HSF    96.0
                                                   <--- M00101 CdxA   94.3
    ---->                                               M00140 Bcd    93.7
    ---------->                                         M00019 Dfd    89.4
    ---->                                               M00087 Ik-2   89.0
                                      ------>           M00100 CdxA   87.2
                                                   <--- M00100 CdxA   85.9
                                            <-------   M00101 CdxA   85.7

501 AATGGGGTTT CACCATGTTG ACCAGGCTGG TTTCAAACTC CTGACCTCAA entry        score
    ->                                                  M00029 HSF    100.0
      ----->                                            M00048 ADR1   100.0
                                                   ---- M00240 Nkx-2. 100.0
    ->                                                  M00029 HSF    96.0
    ---                                                 M00101 CdxA   94.3
                                            <------     M00048 ADR1   89.2
                                                 <-----  M00271 AML-1a 88.7
                                                   <---- M00220 SREBP- 88.7
                                               <---------  M00111 CF1 /  88.2
                                                  ------->  M00041 CRE-BP 87.5
      ----->                                            M00271 AML-1a 87.4
                                                  ------->  M00039 CREB   86.6
                                                  <-------  M00041 CRE-BP 86.2
                                         ------->           M00253 cap    86.2
    ---                                                 M00100 CdxA   85.9
                                                  <-------  M00039 CREB   85.6
                                                       <--- M00253 cap    85.3

551 GTGATCCGCC CACCTCAGCC TCCCAAAATG CTGGGATTAC CGGCATGAGC entry        score
    -->                                                 M00240 Nkx-2. 100.0
                        <---------                      M00141 Lyf-1  100.0
                     -------->                          M00253 cap    96.2
                                      ----------------->  M00019 Dfd    93.9
                                          ------->       M00140 Bcd    93.7
                 <-----                                 M00271 AML-1a 88.7
     ------                                             M00220 SREBP- 88.7
                                     ---------->        M00088 Ik-3   88.1
                                     ---------->        M00087 Ik-2   87.3
            ----------->                                M00073 deltaE 87.0
                        <------                         M00048 ADR1   86.2
                                        ---------->     M00053 c-Rel  86.0
     ----                                               M00253 cap    85.3
```

```
601 CACTGTGCTC GGCTGAAAAA AACCATATTT TTAATTTAAA ATAATAAATG entry        score
                                          ------->      M00101 CdxA    98.6
                                                 <      M00253 cap     93.1
                                             <------    M00101 CdxA    90.0
                                             <------    M00100 CdxA    89.7
           <--------                                    M00253 cap     89.4
                                      ------>           M00100 CdxA    88.5
                                        ------>         M00101 CdxA    86.4
                 ----------->                           M00126 dl      86.0
                ---------->                             M00022 Hb      85.7
                     ------------------>                M00059 YY1     85.7
                           <-------                     M00161 CdxA    85.7
                                    <---------------    M00019 Dfd     85.4
                              <------                   M00101 CdxA    85.0

651 GGTCTGACCA GAAATGCCTG CCTCAAATTG CAGGACACAT GACAAAGCCC entry        score
             ----->                                     M00028 HSF     100.0
             ----->                                     M00029 HSF     96.0
             <-------                                   M00101 CdxA    93.6
    ------- <                                          M00253 cap     93.1
             <-------                                   M00100 CdxA    89.7
                                                  --    M00253 cap     88.4
                                  ---------->           M00122 USF     88.0
                                  <----------           M00122 USF     88.0
                                <--------               M00009 Ttk 69  86.7
                                    ---------->         M00187 USF     85.5
    -                                                   M00019 Dfd     85.4

701 ATTCTTACTT AAGACATTTT CAGTGTGCGA TGGGTCTACC GGGATGTAAC entry        score
                                                 <-     M00048 ADR1    100.0
                                                  <     M00075 GATA-1  95.5
    <----                                               M00029 HSF     95.4
    <----                                               M00028 HSF     94.3
                             ---------->                M00075 GATA-1  91.0
                        --------->                      M00253 cap     89.2
                                                  <     M00076 GATA-2  88.5
    ----->                                              M00253 cap     88.4
        -------->                                       M00040 CRE-BP  87.9
                                                 <-     M00271 AML-1a  87.4
        <--------                                       M00040 CRE-BP  86.7
                             ---------->                M00076 GATA-2  86.6
```

```
        <-------                                          M00240 Nkx-2. 86.0
                                                       < M00077 GATA-3 85.6

751 CCCATCGTGA GTCGAGGCCC ATCTGCACCC TGCTATAGCT ACTGGTAGAT entry        score
    ----                                                 M00048 ADR1   100.0
    ---------                                            M00075 GATA-1 95.5
                  <----------                            M00075 GATA-1 92.2
    ---------                                            M00076 GATA-2 88.5
                  <----------                            M00076 GATA-2 88.5
                                              <--------  M00226 P      87.8
    ----                                                 M00271 AML-1a 87.4
    --------                                             M00077 GATA-3 85.6

801 CTTACCTGTT TCCCAAAAAG GACCCAACTA CTTTAGGTTC AAGGCACTTT entry        score
          <---------                                     M00141 Lyf-1  93.5
         <------------                                   M00087 Ik-2   89.9
            --------->                                   M00223 STATx  88.5
          <-----                                         M00029 HSF    86.9
                                          <-----         M00029 HSF    86.3
                                                -------  M00253 cap    86.2
          <-----                                         M00028 HSF    85.9
                                    ------>              M00100 CdxA   85.9
                                                   --    M00159 C/EBP  85.4

851 TTTGGAATAT TTAACAGGGA AAGAGCCTGG AACTAAAAAC AGGTCTAACG entry        score
                               ----->                    M00029 HSF    90.9
                      ----->                             M00029 HSF    86.9
       ---->                                             M00029 HSF    86.3
    >                                                    M00253 cap    86.2
                      ----->                             M00028 HSF    85.9
    ----------->                                         M00159 C/EBP  85.4

901 CAAGATGCCG CCACACGATG GCACTGCTTC CGTAGACCTG GAAGAAACGC entry        score
                                              ---->      M00028 HSF    100.0
                                              ---->      M00029 HSF    96.0
                                                ------   M00263 StuAp  90.8
                 ---------->                             M00075 GATA-1 89.0
    --------->                                           M00075 GATA-1 88.2
    --------->                                           M00076 GATA-2 87.0
                 ---------->                             M00076 GATA-2 85.8
                       <--------                         M00253 cap    85.2
```

```
 951 GGGCGCGGGA GCTGGCGGCG GACGCCCGGG TCTGCGGGGT CGGGGAGTAG entry        score
                                        ----->             M00048 ADR1   98.5
                                              ----->       M00048 ADR1   92.3
     --->                                                  M00263 StuAp  90.8
                                                ------>    M00048 ADR1   87.7
                                   <---------              M00253 cap    86.8
                                            -------->      M00083 MZF1   86.1
                                                     <-    M00032 c-Ets- 85.3

1001 CGTCCGGCCG GAGAGCCCGC ACCTCGAGGA GCCGGCGGAG GACGCGGTGT entry        score
                                                <----------  M00263 StuAp  93.8
                                             ------>         M00048 ADR1   90.8
          ------>                                           M00048 ADR1   86.2
     --------                                               M00032 c-Ets- 85.3

1051 CGTGACCGCG TCTCCCCCAT CTGCTCTGCT TTTGGCGACA GCGTCCCGTC entry        score
                  <-----                                   M00048 ADR1   98.5
                   <----------                             M00075 GATA-1 95.5
                   <----------                             M00076 GATA-2 90.9
     <--------                                             M00111 CF1 /  90.8
        ---------->                                        M00263 StuAp  90.8
                  <-------                                 M00083 MZF1   89.6
                    <---------                             M00077 GATA-3 89.1
     <--------                                             M00112 CF1 /  87.9
                  <-----                                   M00048 ADR1   87.7
                                    ------->               M00050 E2F    87.4
                      ---------->                          M00184 MyoD   86.2

 1101 GGCCTTTCGG CCAGGACGCA GCAGCCGCGT CTCTGGGCCT CGACCCCCAG entry        score
                                                  <-----   M00048 ADR1   98.5
                                                 <-----    M00048 ADR1   93.8
          <----                                            M00028 HSF    91.1
                            ------->                       M00253 cap    87.4

 1151 GGCCGGCCGG ACCCGGGACC TCCGAGGAGA GCAGCGAGAA CCGGCCCGGA entry        score
                                          ----->           M00029 HSF    100.0
                         <------                           M00048 ADR1   90.8
                                          ----->           M00028 HSF    88.5
           <--------                                       M00009 Ttk 69 86.7
                                                        <  M00029 HSF    86.3

 1201 TTCCCCGAGG CGCCCGAGTG CGGTGCGGGC AGCCCTCCCC GCCTGCCAGG entry        score
```

```
                                        <--------        M00083 MZF1   93.0
      <-----                                             M00048 ADR1   92.3
                                        <------          M00048 ADR1   92.3
                        ------>                          M00271 AML-1a 92.0
                                  -------->              M00253 cap    89.0
     ----                                                M00029 HSF    86.3
                                     <-----              M00048 ADR1   86.2
     ------------>                                       M00189 AP-2   85.0

1251 CCGCCGCTGT CCCCACGTCT GGTCCCCCAC GCCTCTTCCC GCATCTGGTC entry        score
                           <-----                        M00048 ADR1   98.5
              <--------                                  M00083 MZF1   95.7
              <------                                    M00048 ADR1   93.8
                                           <----------   M00075 GATA-1 92.2
                         <-------                        M00083 MZF1   87.8
                         <-----                          M00048 ADR1   87.7
                                     ------------->      M00270 GCM    86.5
                                           <----------   M00076 GATA-2 86.2

1301 CCACGGTGGA GGCGCGGGGC GAGGGCCTCA CGCGCGTGCC CACGGTCGGG entry        score
                                                   ----  M00048 ADR1   96.9
                    ----->                               M00048 ADR1   93.8
            ------>                                      M00048 ADR1   92.3
                                                  ---    M00048 ADR1   92.3
                                                   --    M00048 ADR1   89.2
                                  <----------------      M00067 Hairy  88.2
                                               <------  M00189 AP-2    87.0

1351 GGGCGTCCTT GGACGCGCGG GTCCTCTCCC ACGGGTCTCC ACCCCCCGCC entry        score
     ->                                                  M00048 ADR1   96.9
                                             <-----      M00048 ADR1   96.9
                                                  ----   M00189 AP-2   94.0
                                            <-----       M00048 ADR1   93.8
     -->                                                 M00048 ADR1   92.3
                                             <-----      M00048 ADR1   92.3
                                                    <    M00048 ADR1   90.8
                                             <--------   M00196 Sp1    89.8
     --->                                                M00048 ADR1   89.2
                                        <------          M00048 ADR1   87.7
     -----                                               M00189 AP-2   87.0
                                             <-------    M00008 Sp1    86.3
                                  ---------->            M00187 USF    85.5
```

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                                  ------------>              M00055 N-Myc  85.5

1401 CTCCGGCGAC AGGAGCCGGC CTGGGACGCG CGCGCTGCGG CCAAGTCCTG entry        score
     ------->                                                M00189 AP-2   94.0
     -----                                                   M00048 ADR1   90.8
     ----                                                    M00196 Sp1    89.8
                                  <----------                M00263 StuAp  87.7
     --                                                      M00008 Sp1    86.3
                               ---------->                   M00263 StuAp  86.2

1451 GTTTTGATTT CACTCCCAAC CCCGAGGAGC GAGGAGGTTC CAAGCCCACA entry        score
                         <------                             M00048 ADR1   98.5
                 -------->                                   M00253 cap    94.9
          <------                                            M00148 SRY    90.9
                                            <-----           M00029 HSF    90.9
                    <-----                                  M00048 ADR1   87.7
                                       ----->               M00048 ADR1   87.7
                                                          - M00074 c-Ets- 87.0
                      --------->                            M00226 P      86.9

1501 AACAGGAAGA AGAGAAGGCC CTGGAGTGCT GGCAGAGGGA GTGTCATTCC entry        score
             ----->                                         M00028 HSF    95.3
                   ---->                                    M00028 HSF    95.3
                                                  -------   M00253 cap    94.8
                            ----->                          M00048 ADR1   93.8
             ----->                                         M00029 HSF    93.7
                   ---->                                    M00029 HSF    93.7
          ---------->                                       M00032 c-Ets- 89.2
                                            ------>         M00048 ADR1   87.7
                                         --------------->   M00033 p300   87.1
     ------------>                                          M00074 c-Ets- 87.0
                                                <-------    M00088 Ik-3   86.8
                                            <--------       M00253 cap    86.7
                                                     <----  M00029 HSF    86.3
                                              ------------> M00229 Skn-1  85.2

1551 CAGCAGCGCA GCGCAGGCCG GCCCGGGCCG GGGAGGGAGC AGCAGCGCGG entry        score
     >                                                      M00253 cap    94.8
                                  ------>                   M00048 ADR1   92.3
     -----                                                  M00088 Ik-3   86.8
                                     ----->                 M00048 ADR1   86.2
                                  --------->                M00083 MZF1   85.2
```

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1601 GCACCCTCCT GCAACGTGGG CTGGGGGTCC CCAGGGCCGG GGAGGGGGCC entry        score
                            ----->                          M00048 ADR1   98.5
                             ----->                         M00048 ADR1   93.8
                                  <------                   M00048 ADR1   93.8
                                                 ----->     M00048 ADR1   93.8
                                                     <--    M00048 ADR1   93.8
                                           ------>          M00048 ADR1   92.3
                                                  ----->    M00048 ADR1   89.2
                             <----------                    M00051 NF-kap 88.9
                 <--------------                            M00182 GBP    88.4
                             ---------->                    M00051 NF-kap 88.3
                                  <--------                 M00083 MZF1   87.8
          <------                                           M00048 ADR1   87.7
       -------->                                            M00253 cap    86.7
                                               ------>      M00048 ADR1   86.2
                                                 ------->   M00154 STRE   85.8
                 -------------->                            M00064 PHO4   85.5
                               ---------->                  M00111 CF1 /  85.4
                                        -------->           M00083 MZF1   85.2
                                                    <---    M00189 AP-2   85.0

1651 CCGGGGAGGA ACTGAGAGGG GGCGCGCCCC GGGAAGGCTC GGGGCGGCTC entry        score
             <--------                                      M00253 cap    93.9
       ---                                                  M00048 ADR1   93.8
                        ------>                             M00048 ADR1   93.8
                                <------                     M00048 ADR1   93.8
                                               ------>      M00048 ADR1   93.8
        ----->                                              M00048 ADR1   92.3
                                                    <--     M00189 AP-2   91.0
             ----->                                         M00029 HSF    90.9
                          ------>                           M00048 ADR1   89.2
                                       ---------->          M00147 HSF2   89.1
                        -------->                           M00154 STRE   87.7
                                                    <---    M00048 ADR1   87.7
          ----->                                            M00048 ADR1   86.2
       --------                                             M00189 AP-2   85.0

1701 CGGGGGGCTC CCAGGGCGCC CCCGCCCCTT CCCCCGCGGC CCCGGGGCGC entry        score
       ----->                                               M00048 ADR1   96.9
                          <------                           M00048 ADR1   96.9
                                     <-----                 M00048 ADR1   96.9
```

```
                                                    < M00048 ADR1   96.9
                                              <------  M00048 ADR1   93.8
                                                   -----> M00048 ADR1   93.8
                        <---------------                  M00255 GC box 92.5
                         <-------------                   M00196 Sp1    92.4
      ----->                                              M00048 ADR1   92.3
                          <----------                     M00008 Sp1    91.8
     ---------                                            M00189 AP-2   91.0
                                <-----                    M00048 ADR1   90.8
       ----->                                             M00048 ADR1   89.2
                         <------                          M00048 ADR1   89.2
                                                    <-    M00048 ADR1   89.2
                                           ------------>   M00189 AP-2   89.0
                                <--------                  M00154 STRE   88.7
     --                                                   M00048 ADR1   87.7
                                     <------               M00048 ADR1   87.7
                                             <------------ M00189 AP-2   87.0
                                     <--------             M00083 MZF1   87.0

1751 CCCCGCGGTG CCGGAGTCGG GGCGGGGCGT GCACGTCAGC CGGGGCTAGA entry        score
                                                     ---  M00028 HSF    100.0
      -----                                               M00048 ADR1   96.9
                                                     ---  M00029 HSF    96.0
                          ---------->                     M00008 Sp1    94.5
                         ------>                          M00048 ADR1   93.8
                               ----->                     M00048 ADR1   93.8
                                                ------>   M00048 ADR1   93.8
                        ------------>                     M00196 Sp1    93.5
                        -------------->                   M00255 GC box 92.6
                  ----->                                  M00048 ADR1   92.3
                                  <------------           M00113 CREB   91.8
                                       -------->          M00253 cap    90.0
      ----                                                M00048 ADR1   89.2
                                                   <---   M00101 CdxA   87.1
                                                  -----   M00252 TATA   86.1
                                             ---------->          M00175 AP-4   85.4
                                                   ------ M00216 TATA   85.4

1801 AAAGGCGGCG GGGCTGGGCC CAGCGAGGTG                     entry        score
      ->                                                  M00028 HSF    100.0
      ->                                                  M00029 HSF    96.0
             ------>                                      M00048 ADR1   93.8
```

```
      -------------->                    M00255 GC box 91.3
            ---------->                  M00008 Sp1    89.0
      -------------->                    M00196 Sp1    88.6
        ---------->                      M00008 Sp1    87.7
    ---                                  M00101 CdxA   87.1
           <---------                    M00253 cap    86.6
    --------->                           M00252 TATA   86.1
          -------------->                M00196 Sp1    85.4
    --->                                 M00216 TATA   85.4


Total 326 high-scoring sites found.
Max score: 100.0 point, Min score: 85.0 point
```

- TRANSFAC database document (Notice to users)
- TFMATRIX: binding site distribution matrix
- Parallel Application Tsukuba Laboratory Home Page
- PAPIA system Top Page

## PUBLICATIONS ARISING AS A RESULT OF THIS STUDY

**Ali, S.**, Kockar, F. T., Ramji, D. P. " The proximal promoter region of the human C/EBPδ gene is sufficient to mediate auto-regulation of the gene." (pending submission)

**Ali, S.**, Foka P. N., Rogers S.A., Ramji, D. P. "STATs regulate IL-6-mediated regulation of C/EBPα and C/EBPδ genes in hepatocytes." (in preparation)

**Ali, S.**, Ramji, D. P. "NF-κB regulates transcriptional induction of C/EBPδ in response to IL-1 in hepatocytes." (in preparation)

### Reviews

**Ali, S.**, Ramji, D. P. "CCAAT enhancer binding proteins in atherosclerosis." (in preparation)

### Abstracts

**Ali, S.**, Ramji, D. P. (2005). "Regulation of CCAAT Enhancer Binding Protein δ Gene Transcription". 6th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology (Washington D.C., U.S.A.). 25: E-59

Foka, P., Singh, N. N., Irvine, S. A., Greenow, K., Evans, S., Rogers, S., Harvey, E., **Ali, S.**, Arnaoutakis, K. and Ramji, D. P. (2005). "Molecular Mechanisms Involved in the Cytokine-Regulated Expression of Genes in Macrophages Implicated in Foam Cell Formation and Atherosclerosis". 6th Annual Conference on Arteriosclerosis, Thrombosis and Vascular Biology (Washington D.C., U.S.A.).25: E-71

Ramji, D. P. , Foka, P., Irvine, S, A., Hughes, T. R., Rogers, S. A., Singh, N. N., Greenow, K., Evans, S., Harvey, E. and **Ali, S.** (2004). "Signal transduction pathways and transcriptional control mechanisms involved in the cytokine-mediated regulation of key genes in macrophages implicated in foam cell formation and atherosclerosis". Atherosclerosis 5:29-30

Irvine, S. A., Singh, N. N., Foka, P., Greenow, K., Hughes, T. R., Evans, S., Rogers, S., Harvey, E., **Ali, S.**, Huwait, E. and Ramji, D. P. (2004). "Molecular mechanisms involved in the cytokine-mediated regulation of genes implicated in lipid homeostasis and atherosclerosis". The 2004 HDL Workshop, Heraklion, Crete, Greece