CHARACTERISATION OF A SMALL ACIDIC PROTEIN- A NOVEL POTENTIAL REGULATOR OF CHONDROCYTE HYPERTROPHY DURING ENDOCHONDRAL OSSIFICATION

A thesis submitted to the University of Wales, Cardiff for the degree of *Philosophiae Doctorae.*

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August 2006

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In the name of Allah,
Most Gracious, Most Merciful

This thesis is dedicated to

My parents, my wife,
My daughter

Nermean W. Al-Amoudi

&

My son

Mohammed W. Al-Amoudi

Thank you for your love, encouragement and
Support throughout my studies,
I could not have done it without you.

Acknowledgements

I would like to thank my supervisor Dr Alvin Kwan for his guidance and support.

I would like to thank my co-supervisor Professor Vic Duance for his guidance, support and encouragement, he always had a few minutes to spare me.

Members of Laboratory past and present*, have made the past few years a good memorable experience. I was fortunate to be surrounded by a group of bright knowledgeable people and I am grateful to them all.

* Dr Ilyas Khan
* Dr Sophie Gilbert
* Dr Emma Blain
* Dr Debbie Mason
* Amanda Hall

Dr Steve luckman

Dr Sam Webster

Your good advice, practical assistance, time and friendship have been very much appreciated.

Thank you to Dr Ant Hann for offering advice, support and encouragement. *Cheers Hann*

Abstract

Endochondral ossification is the process of cell proliferation and differentiation leading to bone formation. It requires temporal and spatial coordination of the actions of various cell types and is mediated by many signaling molecules amongst which Ihh and PTHrP are included. A novel gene product identified recently as SAP (Small Acidic Protein) has also been proposed to play a major role in endochondral ossification. The main aim of this study is to characterize the functional roles of SAP that are potentially associated with new bone formation. The embryonic chick sternum has been rarely used in the previous studies of the development of cartilage. Thus, both tissues (tibia and sternum) from 17 days old embryonic chicks are included in this project. The areas of investigation are as follows: (1) Localising the expression of SAP, Ihh and type X collagen using an *in situ* hybridisation technique. (2) To investigate whether the expression of SAP is associated with the Ihh/PTHrP loop. (3) Studying the over expression of SAP in chick sternal chondrocytes using Replication-Competent Retrovirus.

Tibia and sternum show a close similarity in terms of their cell growth structure as confirmed by histological investigation. The tissues are composed of three characteristic zones (caudal, middle and cephalic) distinguishable by their respective chondrocyte phenotypes: proliferative, prehypertrophic, and hypertrophic. As revealed by *in situ* hybridisation, SAP is predominantly localised in the proliferative chondrocytes of both, tibia and sternum, although low intensity of expression is also observed in the prehypertrophic and hypertrophic cells. Ihh is highly expressed in the prehypertrophic chondrocytes in both tissues and its expression continues throughout this transitional zone where the majority of cells are stained strongly, but the reactivity becomes less pronounced throughout the hypertrophic part of the tissues. Unlike SAP and Ihh, type X collagen is found to accumulate exclusively in the hypertrophic zone.

Under normal culture conditions where the samples are not treated with cyclopamine, the proliferative chondrocytes show high levels of SAP, PTHrP and Patched expression, but do not express Ihh and type X collagen. When treated with cyclopamine, the proliferative chondrocytes no longer express SAP and PTHrP but unexpectedly show the expression of Ihh and type X collagen. The initiation of the expression of Ihh and type X collagen by the proliferative chondrocytes following cyclopamine treatment is also accompanied by a decline in the production of GAG and hydroxyproline, suggesting the onset of cell differentiation. Cyclopamine is a naturally-occurring chemical that belong to the group of steroidal jerveratrum alkaloids. It has been shown that cyclopamine blocks the hedgehog signalling pathway by influencing the balance between active and inactive forms of the Smoothened protein (Taipale *et al.,* 2002) Cyclopamine inhibits SAP and PTHrP expression in the prehypertrophic and hypertrophic chondrocytes where Ihh and type X collagen remains unaffected. As SAP expression is predominantly found in the proliferative zone and downregulated in prehypertrophic and hypertrophic chondrocytes, this may indicate its involvement in endochondral ossification as a promoter of cell proliferation and as a transcription regulator preventing the cells from reaching hypertrophy or maturation. An attempt to investigate the biological role of SAP by over expressing the SAP gene in cell cultures has not been successful and it remains to be confirmed whether SAP is an integral part of the Ihh/PTHrP feedback control loop.

Characterisation of a Small Acidic Protein - a Novel Potential Regulator of Chondrocyte Hypertrophy during Endochondral Ossification

Abbreviation

ALP	Alkaline phosphatase
A^{260}	Absorbance at 260nm
A^{280}	Absorbance at 280nm
AER	Apical ectodermal ridge
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CMP	Cartilage matrix protein
COMP	Cartilage oligomeric matrix protein
CTGF	Connective tissue growth factor
C_t	Threshold cycle
Ce	Cephalic
CCN	Ctgf/cyr61/nov family
Co	Carboxyl group
CS	Chondroitin sulphate
CD	Campomelic dysplasia
Da	Dalton
DMEM	Dulbecco's modified eagles's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiotheitol
dNTP	Deoxynucleotide 5'-triphosphate
Dhh	Desert hedgehog
DIG	Digoxigenin
ddH_2O	Double distilled water
MMSO	Dimethyl sulfoxide
MCS	Mesenchymal stem cell
DMEM-SF	Dulbecco's modified eagles's medium,serum free
DMEM-10%FCS	Dulbecco's modified eagles's medium with 10% foetal calf serum
ECM	Extracellular matrix
E.coli	*Escherichia coli*
EDTA	Ethylene diaminetetra-acetic acid
EGF	Epidermal growth factor
EO	Endochondral ossification
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
G	Globular
GH	Growth hormone
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hyaluronan
HAP	Hydroxyapatite
HEPES	N-2'hydroxyethyl-piperazine-N-2'ethane sulphonic acid
His	Histidine
HC	Hypertrophic chondrocyte
IGF	Insulin-like growth factor

GF	Growth factor
Ihh	Indian hedgehog
IPTG	Isopropyl β-D-thiogalactoside
kDa	Kilo dalton
KS	Keratan sulphate
KO	Knock-out
LB	Luria broth
MVs	Matrix Vesicles
MMP	Multiple cloning site
M_t	Melting temperature
mRNA	Messenger ribonucleic acid
MED	Multiple epiphyseal dysplasia
Mi	Middle
MOPS	3-[N-morpholino]-2-hydroxypropanesulfonic acid
MMLV	Moloney murine leukaemia virus
NBT/BCIP	Nitro blue tetrazolium/bromo-4-chloroindol-3-yl phosphate
NC	Non-collagenous
NH	Amino group
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTHrP	Parathyroid hormone related protein
PTH	Parathyroid hormone
PTHR	Parathyroid hormone receptor
PG	Proteoglycan
PDGF	Platelet-derived growth factor
PC	Proliferative chondrocyte
Ptc	Patched
PKB	Protein kinase B
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RER	Rough endoplasmic reticulum
PSACH	Pseudochondroplasia
RCDP	Rhizomelic chondrodyplasia punctata
rpm	Revolutions per minute
SC	Short chain
SMCD	Schmid metaphyseal chondrodysplasia
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
spnt	Supernatant
Smo	Smoothened
Shh	Sonic hedgehog
SAP	Small acidic protein
SDS	Sodium dodecyl-sulphate
T_m	Denaturation temperature
TD	Tibial dyschondroplasia
TBE	Tris borate EDTA
TFG	Transforming growth factor
TEMED	Tetramethylethylenediamine
x-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
VEGF	Vascular endothelial growth facto

CHAPTER 1

Introduction

1 Introduction

Cartilage is one of the major components of the vertebrate skeleton. This is a type of connective tissue closely associated with bone; it is tough, semi-transparent, elastic and flexible (Price *et al.,* 1994). This tissue contains chondrocytes that are embedded in a matrix composed of collagens, proteoglycans and glycoproteins. Covered by a dense fibrous membrane and perichondrium, the cartilage matrix has no nerve and blood vessels. According to their histological appearances and the biochemistry of the extracellular matrix, there are three types of cartilage: hyaline, elastic and fibro cartilage. The present work focuses on the hyaline cartilage since this tissue is known to be associated with endochondral ossification.

Two different mechanisms are known to be involved in the formation of the vertebrate skeleton. One of these is known as intramembranous ossification during which mesenchymal cells directly differentiate into osteoblasts. According to Ortega *et al* (2004), some of the skeletal elements, mainly craniofacial bones, are formed by this process. The second mechanism is known as endochondral ossification (EO), which involves the initial formation of a cartilage template from aggregated mesenchymal cells, and then the subsequent replacement of this cartilaginous template by bone.

1.1 Endochondral Ossification

This process of bone formation is highlighted in the formation of the long bone in Figure (1.1).

1.1.1 Formation of the Cartilage Template

During the initial stage of long bone formation, mesenchymal cells condense in the region where the bone is to be formed and they differentiate into chondrocytes. The second stage involves proliferation of the chondrocytes and secretion of a cartilage extracellular matrix which subsequently formed the shape of the future long bone. The third stage is the phase during which the chondrocytes in the main shaft of the anlagen stop dividing but increase their volume dramatically and are differentiated into mature hypertrophic chondrocytes. These large hypertrophic chondrocytes alter the

composition and organisation of the matrix and enable it to become mineralized by calcium hydroxyapatite deposition. The last stage involves the invasion of the calcified cartilage model by blood vessels and osteoprogenitor cells (Ortega *et al.,* 2004). At this last stage, the hypertrophic chondrocytes become apoptotic, leaving spaces which eventually turn into bone marrow. Osteoprogenitor cells then migrate to the mineralised cartilage and differentiate into mature osteoblasts which lay down an unmineralised bone matrix (osteoid). The osteoid is eventually mineralised to form woven bone but this structure is not mechanically strong enough to support the weight of the growing vertebrate (Cheung *et al.,* 2003). The woven bone is eventually remodelled by the concerted efforts of osteoblasts and osteoclasts to more rigid and compact bone. This strictly regulated process leads to the replacement of cartilage with bone (Hunziker *et al.,* 1987; Gibson, 1998; Steven and Lowe, Human Histology, 1996).

1.1.2 The Secondary Ossification Centre

The secondary ossification centre arises within the epiphysis (Figure 1.1F). The mechanism of this process is similar to that of the ossification of the metaphysis but the ossification propagates radially instead of longitudinally. As the embryo grows, the diaphysis (Fig 1.1) is eventually separated from the secondary ossification centre by a thin plate of cartilage known as the epiphyseal growth plate (Roach *et al.,* 1995; 2004; Steven Lowe, Human histology, 1996; Gartner and Hiatt, Atlas of histology, 2000) (Figure 1.2). This region of cartilage is the focal point of the present study and a good understanding of its histology is helpful in the investigation.

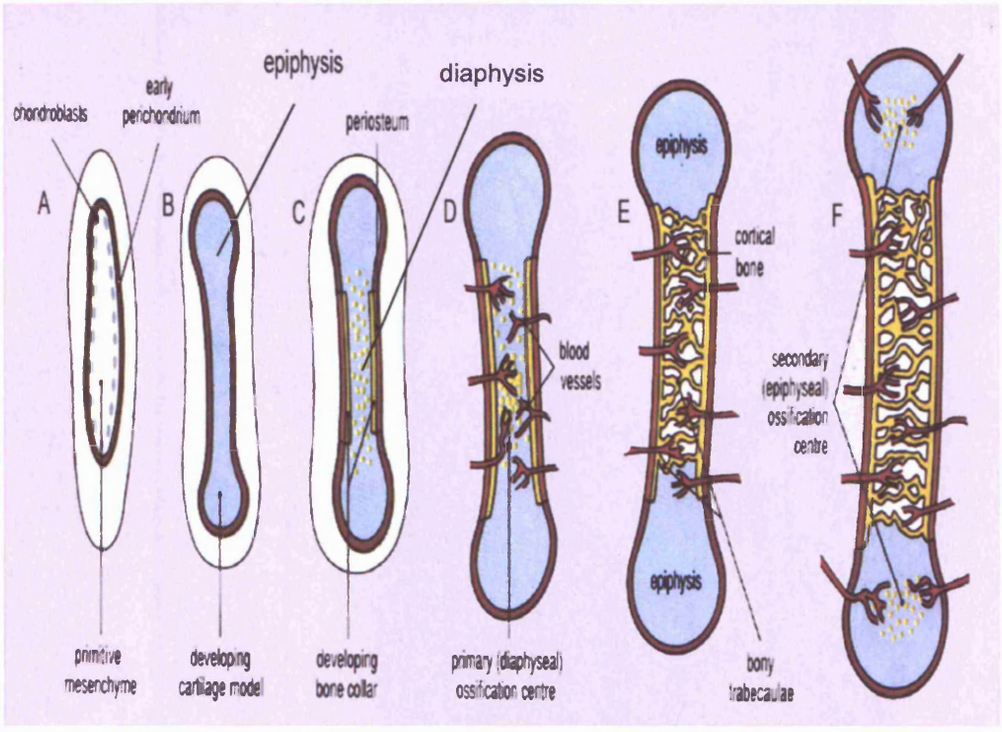


Figure 1.1: Diagram showing the formation of a long bone. The process starts as a condensation of mesenchymal cells in the place where the long bone will be formed **(A)**. These mesenchymal cells differentiate into chondrocytes, forming a cartilaginous template. The cells surrounding this cartilage template differentiate into perichondrial cells producing the perichondrium **(B).** The cells of the cartilage template proliferate and secret a proteoglycan-rich matrix which eventually form the shape of the long bone and the surrounding perichondrium becomes the periosteum forming a bony collar around the shaft of the developing long bone **(C).** At the middle region of the diaphysis, vascularisation occurs in the periosteum and grows through the bony collar. This process also brings the osteoprogenitor cells into the diaphysis **(D).** The bony trabeculae produced by mature osteoblasts spread throughout the primary ossification centre to occupy the entire diaphysis **(E).** The formation of the secondary ossification centre **(F).** (Adapted from Human histology, Steven and Lowe, 1996).

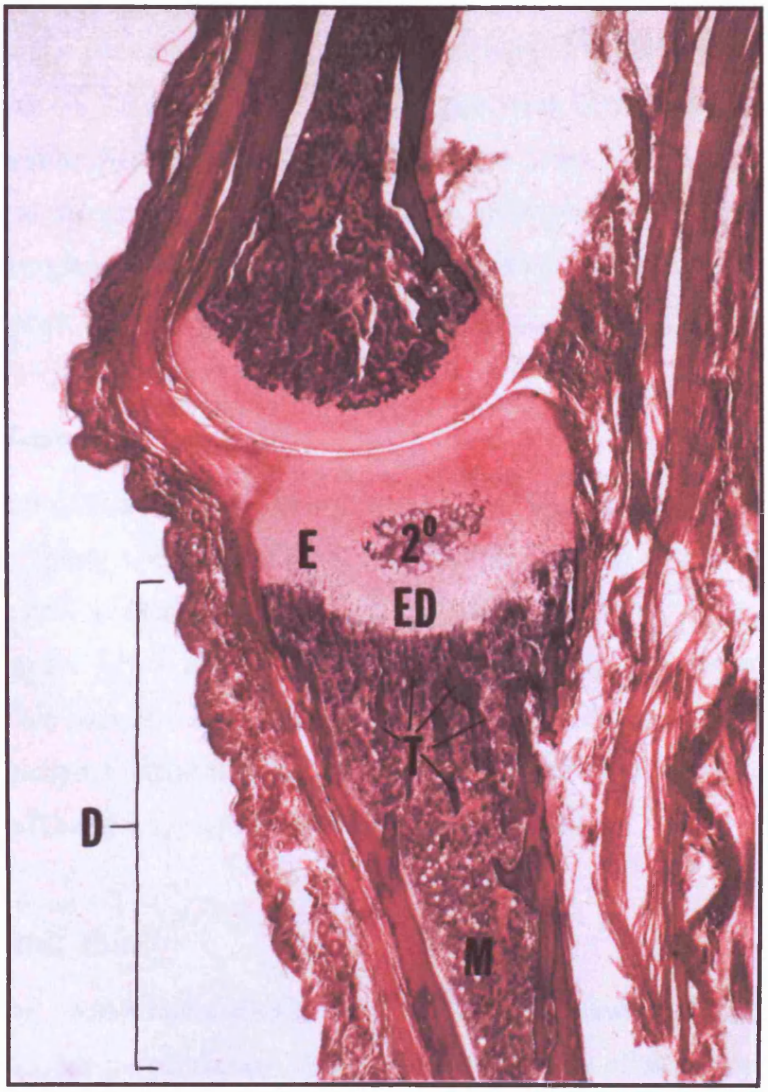


Figure 1.2: Photomicrograph of a longitudinal section of a human digit showing the distal interphalangeal joint. In this photomicrograph, the diaphysis **(D)** of the lower phalanx is replaced by bone and the marrow cavity is filled with marrow **(M).** The secondary ossification centre (2°) is shown developing within the epiphysis **(E).** The epiphyseal growth plate **(ED)** is the thin cartilaginous band between the diaphysis **(D)** and the epiphysis **(E).** Woven bone is formed as the result of ossification process (Taken from Color atlas of histology, Gartner and Hiatt, 2000).

1.2 The Epiphyseal Growth Plate

The epiphyseal growth plate is responsible for the longitudinal growth of long bones by providing a population of chondrocytes that undergo proliferation to give new cartilage at the ends of the diaphysis. Due to the biological sequence of their development, the chondrocytes within the epiphyseal plate can be histologically divided into five distinct zones. As shown in Figure 1.3, the morphological and histological appearances of chondrocytes within these zones are different. These zones are: (1) the resting zone which is close to the articular cartilage; (2) the proliferative zone; (3) the transitional zone (prehypertrophic zone); (4) the zone of terminal chondrocyte differentiation (hypertrophic zone); and (5) the ossifying zone (Kember, 1978; Gibson, 1998).

1.2.1 Resting Zone

The chondrocytes of this zone are round and small as compared to the other zones of the growth plate. These chondrocytes are surrounded by an abundant amount of extracellular matrix (Figure 1.3b). Morphologically, the resting zone appears as a narrow region at the top of the growth plate and the chondrocytes are not mature and non-dividing. This zone is a very important zone as recent evidence has shown that it contains mesenchymal stem cell (MCS) which can differentiate into other connective tissue cell types (Dowthwaite *et al.*, 2004; Veronica *et al.*, 2005).

1.2.2 Proliferating Zone

In this zone, the chondrocytes divide rapidly and form columns of disc-shaped cells whose long axes are perpendicular to the longitudinal axis of the growth plate. The chondrocytes proliferate in response to a number of regulatory factors that mediate their division. These factors include retinoic acid, thyroid hormone, vitamin D and growth factors such as FGFs, TGF, and BMPs (Tsumaki *et al.*, 2002). The chondrocytes in this zone appear slightly larger than those in the resting zone. On the other hand, the proliferating zone is responsible for providing a stream of chondrocytes for cell differentiation, therefore maintaining the constant width of the epiphyseal growth plate throughout the growth of the bone (Hunziker *et al.*, 1987; 1989; Goldring *et al.*, 2006).

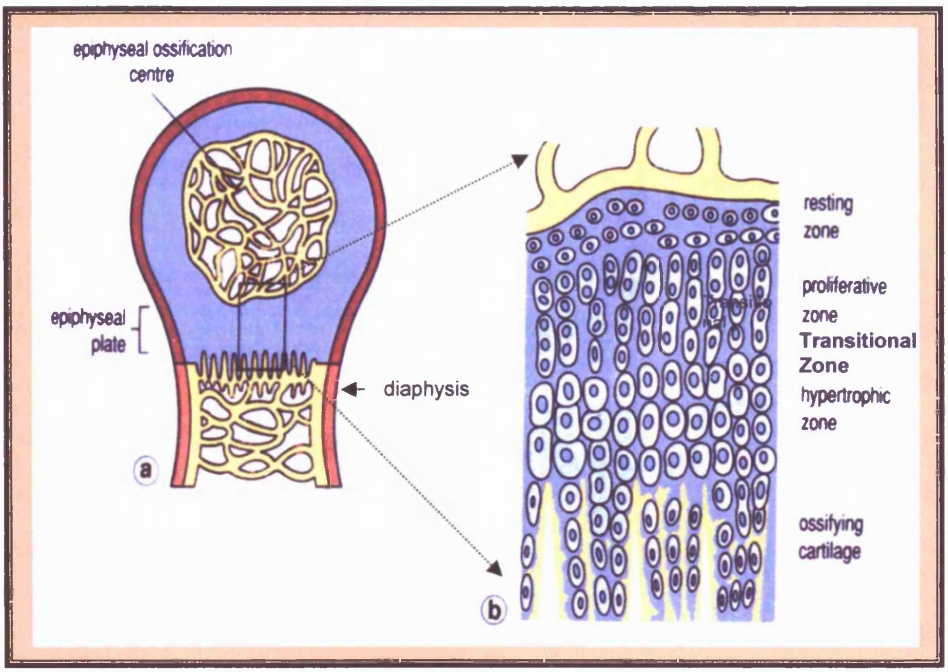


Figure 1.3: The Growth Plate.

(a) Diagram showing the initial enlargement of the secondary ossification centre within the epiphysis. The epiphyseal plate is located between the secondary ossification centre at the top and the diaphysis at the bottom.

(b) Diagram showing a magnified view of a section of the epiphyseal plate (box). Chondrocytes in the epiphyseal plate proliferate in columns towards the diaphysis, becoming hypertrophied as they deposit a hypertrophic matrix. The matrix becomes mineralised before osteoblasts deposit osteoid on the calcified cartilage (Taken from Steven and Lowe, 1996).

1.2.3 Prehypertrophic Zone

The chondrocytes in this zone appear oval and some are still actively dividing. However, some cells in this zone have started to differentiate but have not yet acquired the hypertrophic morphology. The extracellular matrix produced by these transitional chondrocytes is compositionally and biochemically different from that of the previous two zones (Hunziker *et al.,* 1987; Veronica *et al.,* 2005).

1.2.4 Hypertrophic Zone

The chondrocytes of the hypertrophic zone are larger than those in the prehypertrophic zone and secrete extracellular matrix rich in collagen type X and reduced amounts of types II and IX collagen (Provot and Schipani, 2005). A high level of alkaline phosphatase has also been found to associate with these cells, suggesting an active role in mineralisation of the cartilage matrix during endochondral ossification (Schimd *et al.,* 1985; Shum, 2003; Goldring *et al.,* 2006).

1.2.5 Ossifying Zone/Mineralised Cartilage

One of the most important processes is mineralisation that begins after chondrocyte differentiation. Mineralisation is considered to be a prerequisite to osteoprogenitor invasion (Figure 1.4). The major mineral phase is hydroxyapatite (HAP), [$Ca_{10}(PO_4)_6(OH)_2$] (Figure 1.4) which is a member of the family of apatite minerals. However, biological minerals are not pure hydroxyapatite and many other constituents are also included such as carbonate, citrate, magnesium and sodium (Russell *et al.,* 1986). Also this is the zone where the hypertrophic chondrocytes undergo cell degeneration and apoptosis (Cheung *et al.,* 2003). On the other hand, the fate of these cells is currently a topic of much debate. Some evidence has suggested that some hypertrophic chondrocytes in this zone can trans-differentiate into osteoblasts (Roach, 2004). However, this observation has not been substantiated by other researchers. It has been documented that a number of morphological, physiological and biochemical changes take place in this zone (Russell *et al.,* 1986).

It has been observed that terminal hypertrophic chondrocytes occur as condensed cells with their plasma membrane attached asymmetrically to the last transverse septum (Cheung *et al.,* 2003). Morphological and biochemical evidence of apoptosis includes retraction from the pericellular matrix, cytoplasmic and nuclear condensation, and DNA fragmentation. Gibson *et al* (1997) proposed that in the avian system, cell commitment to hypertrophy itself could be the initiator of apoptosis. An increasing number of proteins are known to regulate chondrocyte apoptosis (Chung, 2004; Shum *et al.,* 2003). Expression of the apoptosis inhibitor, *bcl-2* is up-regulated in proliferating and maturing chondrocytes, while expression of the apoptosis inducer, *bax,* increases progressively towards the hypertrophic zone of the growth plate. In the same region vascularisation begins by the migration of vascular endothelial cells from the marrow cavity. Vascularisation within the growth plate allows the supply of hormones and nutrient to the growth plate. Recent studies have shown that many factors are associated with the vascularisation and reabsorption of cartilage such as VEGF, IGF-1, EGF, and PDGF-A) (Gerber *et al.,* 1999; Provot and Schipani, 2005). It has also been demonstrated that osteoprogenitors can migrate to the ossifying zone and differentiate into mature osteoblasts which lay down new bone matrix (Gibson, 1998).

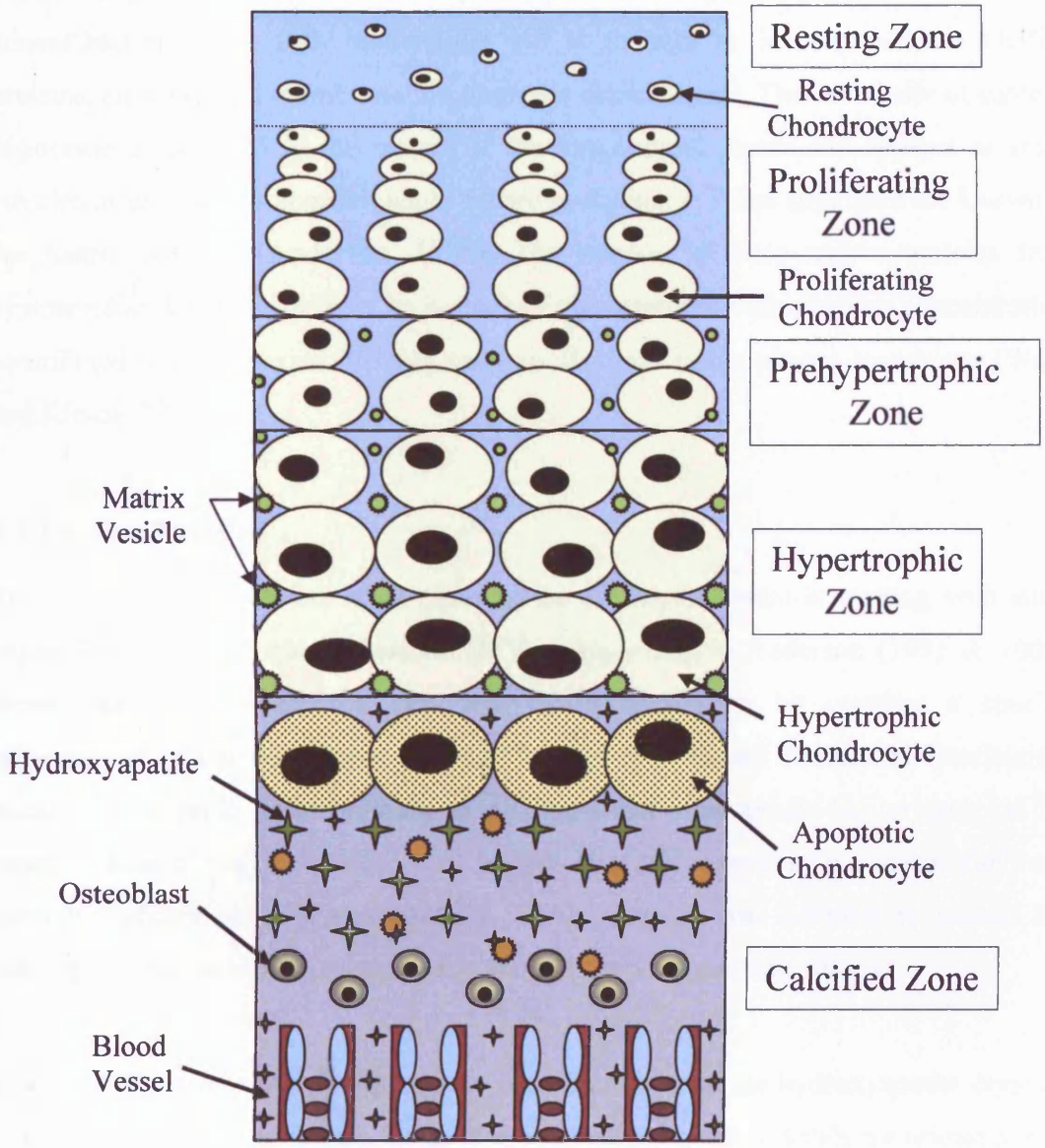


Figure 1.4: A schematic diagram of a longitudinal section of the epiphyseal growth plate. Morphological regions and cell types are highlighted.

1.3 Mineralisation of the Growth Plate

1.3.1 Deposition of Minerals

According to Johnstone *et al* (2000) these minerals are deposited primarily in the extracellular matrix that is specifically localised between the adjacent columns of the prehypertrophic and hypertrophic chondrocytes in the growth plate. The process of mineralisation is not fully understood but is thought to involve calcium binding proteins, enzymes and membrane ion transport mechanisms. The initial site of calcium deposition is confined to the matrix of the longitudinal septae and appears as small vesicles or particles of approximately 50 nm in diameter. These structures are known as the matrix vesicles (Anderson, 1995). The release of these matrix vesicles from hypertrophic chondrocytes may be a result of increased cytosolic calcium concentration, contributed to by annexins forming calcium channels in the plasma membrane (Wang and Kirsch, 2002).

1.3.2 Matrix Vesicles

The mineralisation process takes place in the extracellular matrix starting with small organelles known as matrix vesicles (MVs). According to Anderson (1995 & 2003), these organelles initiate the first step of mineralization by creating a specific environment where deposition of initial amorphous mineral complexes (nucleation) occurs. As a result, hydroxyapatite is produced and form needle-like crystals on the inner surface of the MV membrane. In fact, the MVs serve as a site for Ca^{2+} and inorganic phosphate (Pi) accumulation. These elements are essential to sustain the nucleation process and to propagate the mineralisation (Anderson, 2003).

The second step of mineralisation starts with the release of the hydroxyapatite crystals. After reaching a critical length, the needle-like hydroxyapatite crystals are released from the MVs into the extracellular matrix. These crystals serve as a template for the formation of crystalline arrays, leading to tissue calcification (Anderson, 2003). As Balcerzak *et al* (2003) has speculated, the mechanism by which the hydroxyapatite crystals are propagated, may affect the MV membrane fluidity. The mineral formation occurs between the matrix vesicles. The crystals then grow towards the vesicles and

eventually enter their membrane. The outward growth of the crystals depends on the levels of calcium and phosphate in the extra-vesicular matrix which should allow continued nucleation of the hydroxyapatite (Anderson, 2003). In addition, Wu *et al* (1991) suggested that the outward propagation of the crystals results in mineralisation of the type II and type X collagen pericellular matrix. Matrix vesicles isolated from chicken growth plate cartilage were found to contain MMP-2,-9 and -13 (D'Angelo *et al.,* 2001). These vesicles also contain TGF-β whose reaction is activated by MMP-13 (D'Angelo *et al.,* 2001). In a later study made by Maeda *et al.,* (2002), MMP-3 found in the matrix vesicles was implicated in the release of the large latent complex from TGF-β1.

1.3.3 Annexins

The annexins are a structurally conserved family of proteins characterized by reversible Ca^{2+} dependent intracellular membrane binding. Annexins comprise a large family with more than 100 sequenced proteins from over 45 species (Wang *et al.,* 2005). There are twelve members of the annexin family have been identified. Three were identified in MVs. These members are annexins II, V and VI. Annexin can be associated with both outer and inner leaflets of the MV membrane. Annexins affect membrane stability in a Ca^{2+} dependent manner. In addition, they could be involved in the Ca^{2+} transport, as ion channels inserted within the MV membrane (Balcerzak *et al.,* 2003). Furthermore, Kirsch *et al* (2000) have suggested that type X collagen is able to bind to annexin V and that it would anchor matrix vesicles in the extracellular matrix and also activate the annexin V channel properties. These finding suggest that annexin V may not only form calcium channels in growth plate chondrocytes and matrix vesicles but may also mediate cell-vesicle-matrix interaction (Wang *et al.,* 2005).

1.3.5 Proteoglycans

Proteoglycans are thought to play a role in the calcification process, but the nature of that role is still unknown. Perhaps, aggrecan binds calcium via their chondroitin sulfate chains in vivo and the reaction could promote hydroxyapatite formation. Proteoglycans seem to promote calcification and this hypothesis is supported by the observation that calcification is not always initiated within matrix vesicles but rather in discrete focal sites containing aggrecan and chondrocalcin (a glycoprotein derived from the C-terminal propeptide of type II collagen). The ability of chondrocalcin to bind to annexin V and accumulate in calcifying cartilage has been noted by Kielty and Grant (1992). However, a contradictory report of in vitro studies on hydroxyapatite formation suggests that proteoglycans are inhibitors rather than promoters of the calcification (Hunter, 1991).

1.4 Extracellular Matrix (ECM)

The extracellular matrix (ECM) is a complex structure surrounding and supporting various types of cells within connective tissues. The ECM is composed of three major classes of molecules. These are: Collagen and Elastin, specialized glycoproteins (fibrillin, fibronectin and laminin) and proteoglycans which are all composed of a protein core with long chains of repeating disaccharide units named glycosaminoglycans (GAGs) (Schmal *et al.,* 2006).

Collagen fibres strengthen and help organize the matrix, while the elastin is fibres give its resiliency. The adhesive glycoproteins help cells to attach to the extracellular matrix. For example, fibronectin promotes the attachment of fibroblasts and other cells to the ECM via extracellular domains of members of the integrin family, and laminin promotes the attachment of epithelial cells to the basal lamina, again via the extracellular domains of integrins.

1.4.1 Components of the Extracellular Matrix

1.4.1.1 The Collagen Family

This family of collagens include major proteins of the bone and cartilage extra cellular matrix and is essential for the determination of size, shape and strength of these tissues. Twenty seven members are found to belong to this super-family of proteins which can be grouped according to their molecular structures and the supra-molecular assemblies which they adopt. Apart from showing a variety of structures, they have different biological roles and are expressed in different tissues (Canty and Kadler 2005). These collagens are listed in (Table 1.1).

Collagen type	Localisation	Supermolecular assembly
Type I	Skin, Tendon, Bone and Dentin	Fibrillar collagen
Type II	Cartilage	Fibrillar collagen
Type III	Skin, Muscles, Blood vessels	Fibrillar collagen
Type IV	Basement membrane associated collagen	Short chain collagen, hexagonal network
Type V	Fetal tissue, Placenta and Interstitial tissues	Fibrillar collagen
Type VI	Ubiquitous	Beaded filaments
Type VII	Dermo-epidermal junction	Anchoring fibrils
Type VIII	Basement membrane associated collagen	Short chain collagen, hexagonal network
Type IX	Cartilage, Vitreous Humour	Fibril-associated collagen
Type X	Hypertrophic cartilage	Short chain collagen, hexagonal network
Type XI	Cartilage	Fibril-associated collagen
Type XII	Embryonic skin and tendons	Fibril-associated collagen
Type XIII	Epidermal cell-matrix	Type II membrane protein
Type XIV	Fetal skin and tendons	Fibril-associated collagen

Type XV	Basement membrane associated collagen	Multiplexin, supermolecular assembly unknown
Type XVI	Associated with cartilage hypertrophic	Fibril-associated collagen
Type XVII	Membrane collagen	Type II membrane protein
Type XVIII	Basement membrane associated collagen	Multiplexin, supermolecular assembly unknown
Type XIX	Epithelial basement membrane zone and muscle cells	Fibril-associated collagen
Type XX	Corneal epithelium	Fibril-associated collagen
Type XXI	Vascular walls and fetal development	Fibril-associated collagen
Type XXII	Tissue junctions	Fibril-associated collagen
Type XXIII	Membrane collagen	Type II membrane protein
Type XXIV	Bone and cornea	Fibrillar collagen
Type XXV	Membrane collagen	Type II membrane protein
Type XXVI	Ovary, testis	Supermolecular assembly unknown
Type XXVII	Cartilage	Fibrillar collagen

Table 1.1: A table summarising the different members composition structure and tissue distributions of the collagen family members (Ricard-Blum and Ruggiero, 2005).

All collagens are trimeric molecules composed of three α chains. The α chains are defined as polypeptides that are essentially repeats of the amino acid triplet, Glycine-X-Y in which the position of X is frequently occupied by proline and that of Y is frequently by 4-hydroxyproline (Prochop and Kivirikko, 1995). The unique primary structure of the α-chain allows the three chains in a collagen molecule to intertwine to form the typical collagen triple helix. The sizes of α chains in the family vary from 600 to 3000 amino acids and some contain non-collagenous (non Gly-X-Y) interruptions within and on the ends of sequences. Some of these collagens also have motifs that show homology with other extracellular matrix proteins (Brown and Timpl, 1995).

The most prevalent collagens in tissues are the fibrillar collagens these include types I, II, III, V, XI, XXIV and XXVII. In each α-chain in their triple helical domain, there are about 1000 amino acids (Kietly *et al.,* 2002). Type I collagen fibrils are arranged into parallel bundles in tissues providing physical strength to the tissues under extensive forces, such as bone and tendons. Those fibrils that are formed by type II collagen are narrower than the type I and they are the major collagen components of cartilaginous tissues and vitreous humour. Elastic tissues such as the skin, intestine and aorta also contain Type III collagen fibrils. Type XXVII collagen has recently been assigned to the fibrillar collagen family (Boot-Handford *et al.,* 2003).

As described by Myllyharju and Kivirikko (2001), the biosynthesis of fibrillar collagens consists of a series of post-translational events. α-chains are synthesised as a pre-procollagen chain containing an N-terminal signal peptide, and a N and C-terminal propeptide linked by the triple helical domain. First of all, the pre-procollagen chains having a signal peptide are translocated into lumen of the rough endoplasmic reticulum (RER). According to Hendershot *et al* (2000), formation of the collagen triple helix occurs in the C-N direction and requires modification within the polypeptide chain. The melting temperature of the triple helix correlates with the extent of hydroxylation of proline residues and with the physiological temperature of an organism. Hydroxyproline coordinates with water molecules within the triple helix of collagen increasing the H-bonding between α-chains increasing the thermal stability of the triple helical molecule. The importance of hydroxylation is demonstrated by the disease scurvy that results from

deficiency of vitamin C. Some lysine residues are also hydroxylated by a family of lysyl hydroxylases. Hydroxylysine can be further modified by the addition of galactose and glucose moieties. Hydroxylysyl residues are further modified to form extracellular collagen cross-links essential for the mechanical properties of collagen fibril. The control mechanisms of collagen molecular and fibril assembly are not fully understood. An evidence suggests that in the embryonic fibroblast at least, fibril assembly occurs inside the cell and then secreted through cellular invaginations termed fibropositors (Canty and Kadler 2005).

Contrary to the fibrillar collagens, the non-fibrillar types undergo completely different post-transtionla events. Some collagens have large non-collagenous domains at either end of their triple helix and these domains are not removed by proteinases. These include: IX, XII, XIV, XVI, XIX, XXI and XXII. Other types of collagen require the addition of either the N-linked oligosaccharides or glycosaminoglycans (GAG) chains. The triple helical domains of some collagens (eg. type XIII and XVII) are assembled from the N-terminal to the C-terminal (Myllyharju and Kivirikko, 2001).

1.4.1.2 Cartilage Collagens

1.4.1.2.1 Type II Collagen

Cartilage contains predominantly type II collagen which is composed of three identical α 1(II) chains that wind into the characteristic triple helix (Kielty and Grant, 2002). Type II collagen forms heterotypic fibrils with the lesser abundant type IX and XI collagens. Figure (1.5) shows that the interior of these fibrils contains type XI collagen whereas its exterior is associated with type IX. These fibrils show highly ordered arrangements of regular three-dimensional network throughout the cartilage matrix, thus giving support to the ECM as well as interaction sites for other ECM components.

1.4.1.2.2 Type IX Collagen

Type IX collagen is a heterotrimer which has a molecular configuration of [α1 (IX) α2 (IX) α3 (IX)]. The three chains are products of three distinct genes (Eyre and Wu, 1995). There are short and long forms of type IX collagen depending on the presence or

absence of a large globular domain (NC4) at the amino terminal of the α1 chain. The short form of type IX collagen is found to be dominant in the hypertrophic cartilage of the growth plate whereas the long form is present mainly in articular cartilage. The expression of these different forms is tissue specific and also developmentally regulated (Olesen, 1992). The α2 chain of type IX collagen can also bear a chondroitin sulfate chain which allows the collagen to be classified as a proteoglycan.

1.4.1.2.3 Type XI Collagen

Type XI collagen is a heterotrimer consisting of the following chains: α1 (XI), α2 (XI) and α3 (XI). The α1 and α2 are products of two separate genes. The α3 is coded by the same gene COL2A1 of type II collagen, but according to Burgeson (1982), it undergoes different post-translational hydroxylation and glycosylation. The mutated genes of heterotypic fibrils have been found to cause a variety of diseases with variable degree of severity (Mundlos *et al.,* 1997). Mutations in the COL2A1, COL9A2 and the COL11A2 gene loci can lead to achondrogenesis type II, multiple epiphyseal dysplasia and a Stickler-like-dysplasia, respectively (Horton, 1996).

1.4.1.2.4 Type VI Collagen

Chondrocytes in the pericellular capsule known as chondron, are surrounded by a network of microfibrils of type VI collagen, which have an abundant amount of cell binding motif (RGD) sequences (Poole, 1988). In the growth plate, type VI collagen has been observed in the close vicinity of the resting as well as the early proliferating chondrocytes, but the staining was found to diffuse gradually and in many cases was absent at the end of the proliferating zone. According to Sherwin *et al* (1999) the pericellular staining of type VI collagen was not detected around the hypertrophic chondrocytes of the growth plate.

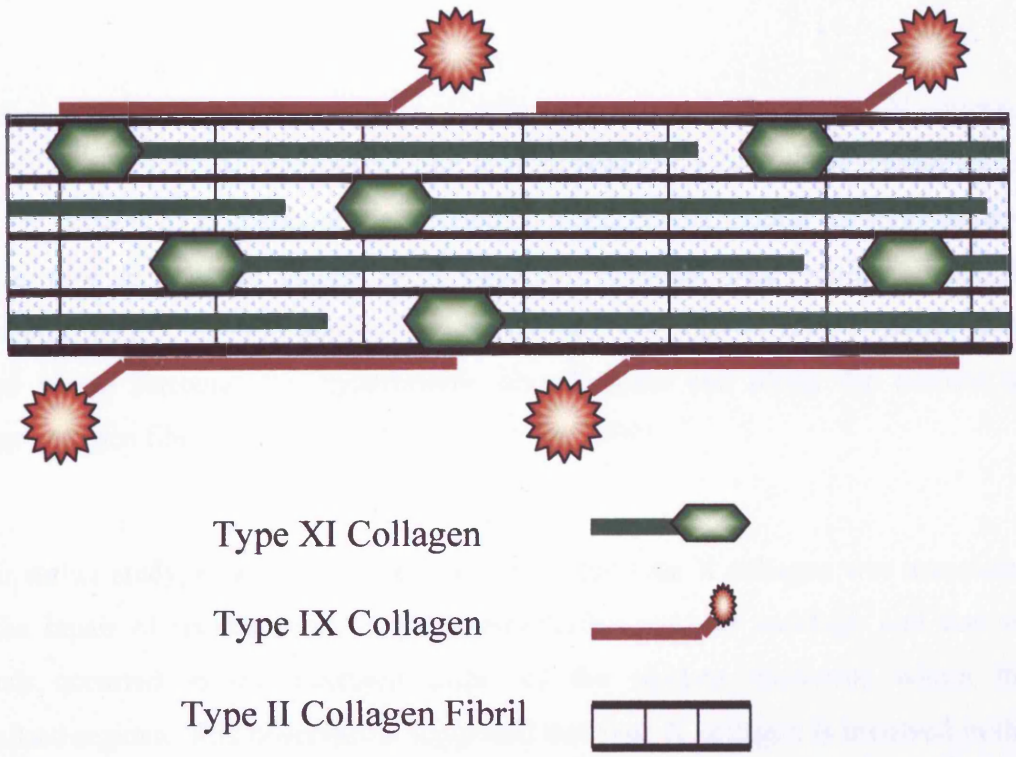


Figure 1.5: A diagram representing schematically the arrangement of collagen molecules in the hypertrophic fibrils in cartilage.

1.4.1.2.5 Type X Collagen

Collagen type X was first isolated from culture medium of chick chondrocytes undergoing hypertrophy and it was described as a short chain (SC) collagen (Schmid *et al.,* 1983; Kielty *et al.,* 1985), because the molecule is a homotrimer composed of three 59 kD α1(X) chains. The human type X molecule has three domains: (1) a short 38 amino acids non-helical region at the amino-terminus known as the non-collagenous domain 2 (NC2); (2) a domain of 161 amino acids at the carboxyl-terminus (NC1); and (3) a helical domain of 463 amino acids (Kielty *et al.,* 1985).

According to Schmid and Linsenmayer (1990); Jacenko *et al* (2001), type X collagen has a very restricted tissue distribution, and is synthesized by hypertrophic chondrocytes during the process of endochondral ossification. Furthermore, within the hypertrophic cartilage, type X collagen occurs in at least two different super molecular forms. Immunoelectron microscopic studies have shown that type X forms mats of filamentous material which surround the hypertrophic chondrocytes and along the surface of cartilage collagen fibrils (Schmid and Linsenmayer, 1990).

In their earlier study, Grant *et al* (1987) indicated that type X collagen was associated with the repair of fracture and human osteoarthritic articular cartilage and that its synthesis occurred in the fractured callus of the chicken humourus within the mineralised regions. This observation suggested that type X collagen is involved in the formation of new bone throughout development. The extracellular deposition of type X collagen in the hypertrophic cells of the middle zone of osteoarthritic articular cartilage has been demonstrated immunohistologically. The synthesis of type X collagen in suspension culture of chondrocytes freshly isolated from osteoarthritic cartilage has provided evidence to suggest that type X collagen may be involved in the pathology of osteoarthritis in the regions where articular cartilage is calcified (Von der Mark *et al.,* 1992).

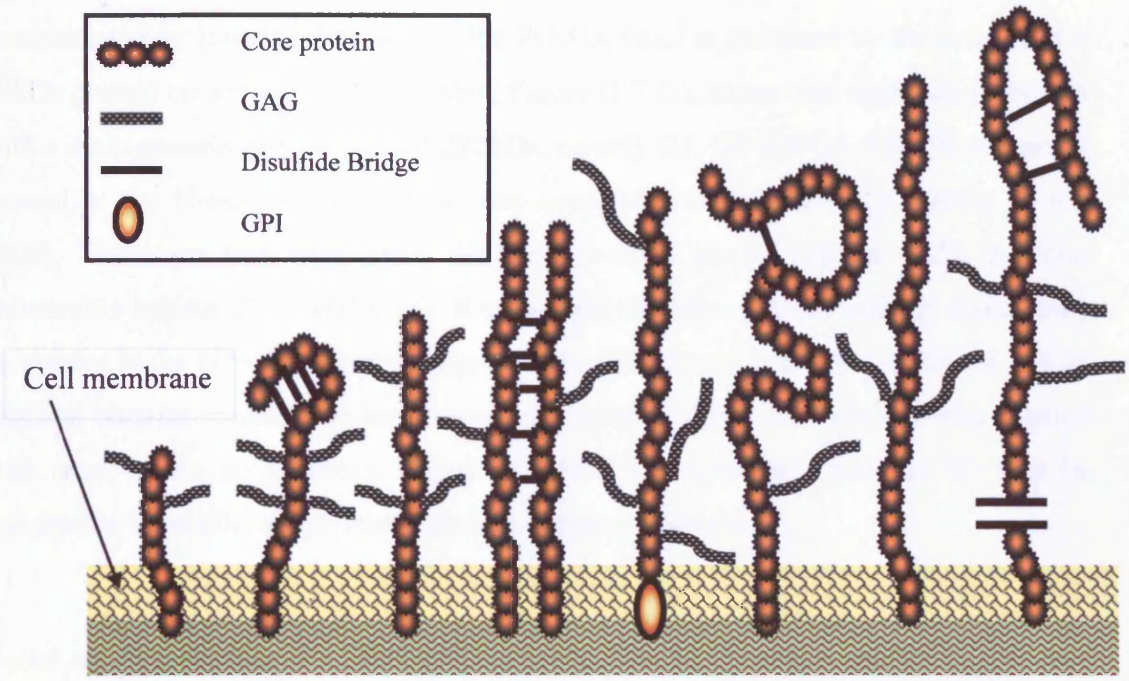
Contrary to the report that the expression of type X collagen is restricted to hypertrophic chondrocytes of the growth plate, the study by Rucklidge *et al* (1996) has shown that the X collagen is immunolocalised to the surface of articular cartilage of

human, pig and rat (Rucklidge *et al.,* 1996). A small amount of type X collagen is also detected in the proliferating zone of the growth plate (Jacenko, 2001). Type X collagen has been recently localised in other regions. It has been found in the mineralised fibro cartilage at the ligament-bone interface of the bovine medial collateral ligament (Niyibizi *et al.,* 1996). Furthermore, Roberts *et al* (1998) has reported that type X collagen is expressed in the degenerating human intervertebral discs. The above observations clearly suggest that the expression of type X collagen is not restricted to the hypertrophic zone of the growth plate, and that it can be detected in other tissues.

The actual function of type X collagen is not well understood. On the one hand, type X collagen is claimed to be associated with vascularisation (Schimd *et al.,* 1985). On the other hand, it is thought to be involved in mineralisation of the matrix (Kielty and Grant, 1992). Furthermore, Wilson *et al* (2002) have shown that mutation of the type X collagen gene was involved in the pathogenesis of the inherited human skeletal disorder, Metaphyseal Chondroplasia type Schmid. Type X collagen null mice have also been shown to develop hematopoietic abnormalities and Jacenko *et al* (2002) and Shum L and Nuckolls (2002) suggested that the type X collagen deposition is important in establishment of bone marrow for normal hematopoiesis of the hypertrophic cartilage.

1.4.1.3 Proteoglycans

Proteoglycans are specialised glycoproteins which contain a large proportion of glycan relative to the amino acid content of the molecule. A proteoglycan is made up of a core protein to which long chains of repeating disaccharides- glycosaminoglycan (GAGs) are attached (see Figure 1.6).



Name:	invariant Chain	CD44	syndecan-1	CSF-1	glypican	thrombo -modulin	betaglycan	NG2
GAG:	CS	CS/HS	HS/CS	CS	HS	CS	HS/CS	CS

Figure 1.6: Structure of some cell surface proteoglycans. Some have disulfide bridges, Syndecan-1, CD44 and betaglycan may carry both heparan sulfate (HS) and chondroition sulfate (CS) GAG chain. (Adapted from Elenius and Jalkanen, 1994)

1.4.1.4 Aggrecan

Aggrecan is a member of the proteoglycan family that forms multimolecular complexes with hyaluronan. The ECM of the cartilage is rich in this protein. As shown in Figure 1.7A, the aggregates of proteoglycans are formed as a result of the multiple binding of proteoglycans to a hyaluronan chain (HA) on the globular protein core. As demonstrated by Hardingham (1984), the PG-HA bond is stabilised by the linkage of a 40kDa protein core (Figure 1.7 B). Also, Figure (1.7 D), shows that aggrecan is formed with a multi-domain protein core of 220kDa, namely G1, G2 and G3. The G1 domain is located at the N-terminus and binds non-covalently to hyaluronan (Caterson *et al.*, 2000). There are two attachment domains, a short keratan sulfate (KS) and two chondroitin sulfates (CS1 and CS2). It is thought that over 100 CS and KS chains may be present in the GAG attachment regions. The G3 domain is at the C-terminus and its function remains obscure. It has properties similar to those of lectin and may interact with other matrix components. Roughley (2001) has speculated that the G3 may be involved in intracellular trafficking during aggrecan synthesis.

Based on its molecular characteristics, aggrecan has an important role to play in the development of cartilage. According to Iozzo (1998), each aggrecan monomer occupies a large hydrodynamic volume in this tissue and the water is displaced from individual monomers when subjected to compressive forces. Iozzo (1998) further described that the swelling of the tissue is dissipated readily when the compressive forces are removed and water molecules are drawn back into the tissue. Mundlos (1994) reported that aggrecan in the growth plate showed a distribution similar to type II collagen and that the highest rate of expression was noted in chondrocytes of the proliferating and upper hypertrophic zones. However, the expression of aggrecan is down regulated when the chondrocytes undergo hypertrophy (Wai, 1998).

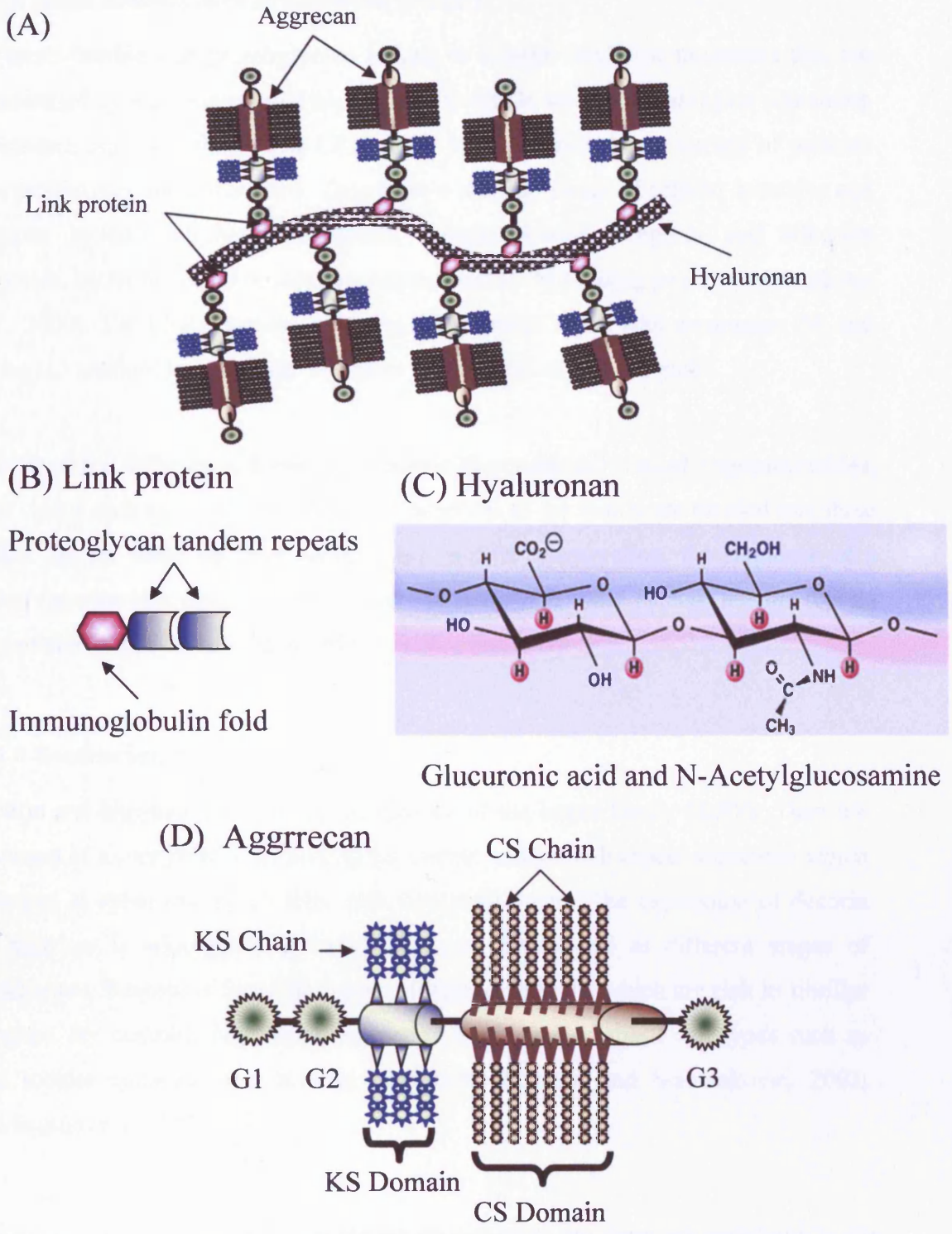


Figure 1.7: (A) representing the cartilage proteoglycan aggregate of high molecular weight and its components; (B) Domains of link protein; (C) Hyaluronan disaccharide repeat; (D) different domains of aggrecan monomer and sites of glycosaminoglycans (GAGs) attachment. G1, G2 and G3 are globular domains.

1.4.1.5 Small Leucine Rich Protoglycan (SLRP)

The small leucine rich proteoglycans belong to a large family of molecules that are characterised by a protein core of approximately 40kDa with a central region containing leucine-rich repeats (LRRs). The LRRs have been discovered in a variety of proteins from prokaryotes and eukaryotes. They have a wide spectrum of cellular locations and functions including hormone receptors, tyrosine kinase receptors, cell adhesion molecules, bacterial virulence factors, enzymes and ECM-binding proteins (Mastushima *et al.,* 2000). The LRRs contain twenty to thirty amino acids, with asparagine (N) and leucine (L) residues in conserved positions in the (LxxLxLxxNxL) motif.

Their structural differences depend on whether they contain N-linked oligosaccharides, GAG chains such as CS, DS and KS. The members of the family are divided into three distinct classes based on their evolutionary protein conservation, the sequence of a distinct cysteine-rich region at the N-terminal, and the number of their leucine repeats and genomic organisation (Iozzo, 1999)

1.4.1.6 Decorin and Biglycan

Decorin and biglycan fall into class I division of the larger family SLRPs. They are composed of a core protein containing 10 tandem leucine rich repeat sequences which is flanked at either end by cysteine rich disulphide loops. The expression of decorin and biglycan is widespread in many types of tissues and at different stages of development. Decorin is found in tissues of human embryos which are rich in fibrillar collagens. By contrast, biglycan is expressed in more specialised cell types such as renal tubular epithelia, and skeletal myofibers (Bosman and Stamenkovic, 2003; Waddington *et al.,* 2003).

Decorin and biglycan are found in bovine growth plate but there are variations in the profiles of their distribution and expression (Alini and Roughley, 2001). Decorin was found predominantly in the proliferating zone, and its expression was down-regulated in the hypertrophic zone. However, biglycan was found throughout the growth plate with a decrease in the intensity of expression being noticed in the proliferative and

hypertrophic zones. Biglycan was later found to be associated with the mineralisation of cartilagenous matrix following a study using the developing epiphyseal cartilage of 10-day old Wistar rats (Takagi *et al.*, 2000; Mochida *et al.*, 2003).

The targeted deletion of genes encoding for decorin and biglycan within mice has provided a lot of valuable evidence for the role of these macromolecules in bone formation in vivo. The most striking effect on mineralised tissues was observed in the biglycan knockouts. Most notably, these mice were seen to develop an osteoporotic phenotype, failing to achieve peak bone mass due to decreased bone formation with significantly shorter femurs (Ameye and Young, 2002). Also it has been shown that the decrease in bone mass was more severe and developed earlier than the single biglycan knockout, suggesting the effects of decorin and biglycan are synergistic within bone (Waddington *et al.*, 2003)

1.4.1.7 Perlecan

Perlecan was first found in basement membrane and identified as a heparan sulfate proteoglycan (Iozzo, 1994). Perlecan is present in the extracellular matrix of cartilage and has both chondroitin sulfate (CS) and heparan sulfate (HS) chains (Hassell *et al.*, 2002; Smith and Hassell, 2006). In cartilage, perlecan is essential for the maintenance of integrity of the matrix and the binding to proteins such as fibulin and fibronectin to form scaffolding network between the chondrocytes in order to stabilse the cartilage (Hassell *et al.*, 2002). Perlecan also has a role in cell attachment to basement membrane (BM), where it binds to other BM components such as laminin and collagen IV (Iozzo, 1994; Smith and Hassell, 2006). Earlier studies localised perlecan in the pericellular matrix of avian, human and murine articular and growth plate cartilage cells. The perlecan expression has been observed in cartilage during murine embryogenesis (Gomes *et al.*, 2002). These studies demonstrated that perlecan expression was intense in cartilaginous tissues especially those undergoing endochondral ossification (Melrose *et al.*, 2002). The ability of this proteoglycan to bind key growth factors as well as cell surface components suggests that it helps coordinate proper cartilage growth and development.

1.4.1.8 Syndecan

The syndecans are a family of transmembrane heparan sulfate (HS) proteoglycans and chondroitin sulfate (CS) which is implicated in the binding of extracellular matrix components and growth factors. Syndecans bind a variety of molecules via their heparan sulfate chains and act as receptor or co-receptors molecules. There are 4 known members of the syndecan family (syndecan-1,-2,-3 and -4) (Figure 1.8). The syndecans are ubiquitous components of the plasma membranes of cells, act as receptors for the extracellular matrix, and are involved in intracellular communications (Elenius and Jalkanen, 1994; Fuki *et al.,* 1997).

1.4.1.8.1 Syndecan-3

The expression of Syndecan-3 is found in the distal mesenchymal cells of the limb bud that undergoes proliferation and as a result of the outgrowth of the apical ectodermal ridge (AER), which is one of the regions that control the limb bud development (Dealy *et al.,* 1997). Furthermore, according to Crossely *et al* (1996); Vogel *et al* (1996); Kosher (1998), this apical ectodermal ridge is induced by the mesoderm and is also involved in the limb bud formation, a process that appears to be mediated at least in part by members of the FGF family of growth factor.

Syndecan-3 is transiently expressed during the onset of the limb cartilage differentiation in vitro (Gould *et al.,* 1992; Fisher *et al.,* 2005). During this process, the mesenchymal cells become closely juxtaposed and undergo cell-cell. Recent studies have indicated that syndecan-3 is intimately associated with chondrocyte proliferation and plays an important role in regulating this process. *In situ* hybridisation data have shown that syndecan-3 gene expression is associated with proliferating chondrocytes and the pattern of its expression changes according to the topographical redistribution of these cells with increasing age of skeletal elements (Shimazu *et al.,* 1996; Kirsch *et al.,* 2002; Fisher *et al.,* 2005; Tkachenko *et al.,* 2005)

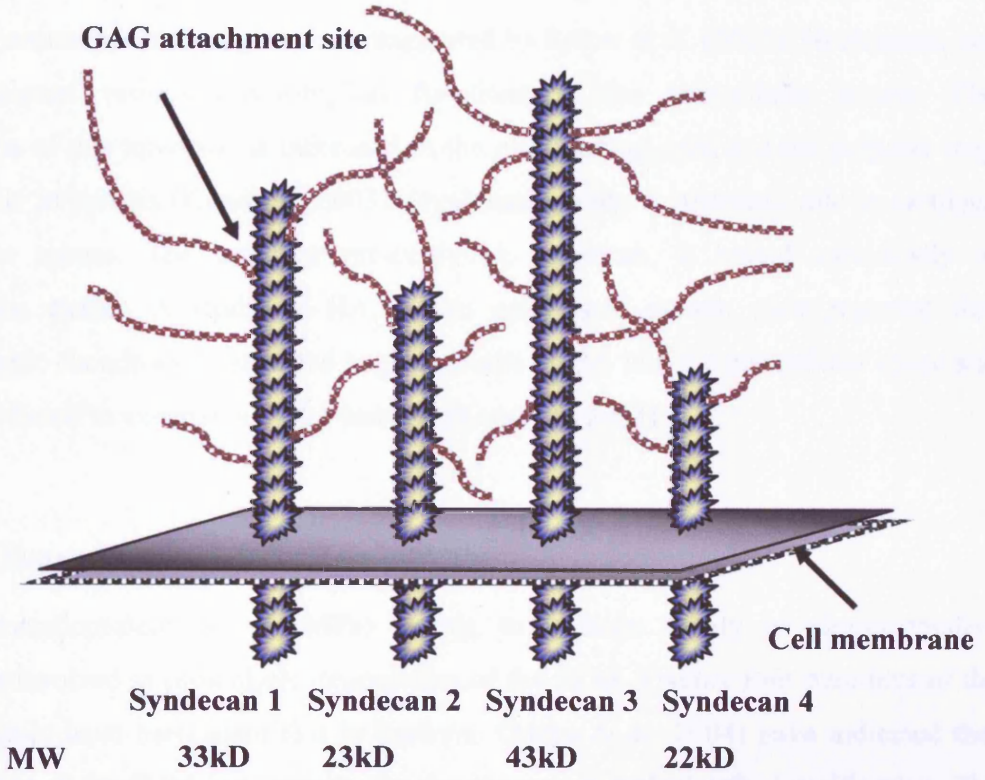


Figure 1.8: Diagram is showing the structure and molecular weight of syndecan-1,-2,-3 and -4 (Adapted from Tkachenko *et al.,* 2005)

1.4.1.9 Hyaluronan (HA)

Hyaluronan (hyaluronic acid) is a high molecular-mass polysaccharide found in the extracellular matrix ECM. About 1-10% of the cartilage glycosaminoglycans are hyaluronan. It is synthesized in the plasma membrane of fibroblast and other cells (Kundson, 2003). Its structure is the simplest of all glycosaminoglycans. Hyaluronan is an unsulfated glycosaminoglycan, made of repeating disaccharide units of β-1, 4-glucuronic acid-β-1, 3-N-acetyl-D-glucosamine, (Figure 1.7 C) and is critical for normal mammalian embryogenesis as suggested by Spicer *et al* (2002). Hyaluronan has been assigned various physiological functions in the intercellular matrix. The production of this molecule is increased in the proliferating cells and the polymer may play a role in mitosis (Kundson, 2003). Hyaluronan plays a structural role in cartilage and other tissues. The cartilage proteoglycan, aggrecan, is bound specifically to hyaluronan chains. A study of HA in the epiphyseal growth plate reported that hypertrophic chondrocytes secreted large amounts of HA into the pericellular space and this contributed to expansion of the lacunae (Knudson, 2003).

1.4.1.10 Matrix Metalloproteinnases (MMPs)

Matrix metalloproteinnases (MMPs) belong to a large family of zinc-dependent proteases involved in proteolytic degradation of the ECM. Twenty four members of the MMP family have been identified in humans. Ortega *et al* (2004) have indicated that degradation of the ECM is necessary for the process of endochondral ossification. The important role of MMPs in endochondral ossification has been pointed out in recent studies using deficient MMP-9, MMP-13 or membrane type 1 MMP (MT1-MMP) deficient mice (Ortega *et al.,* 2004).

MMP-13 is exclusively expressed in hypertrophic chondrocytes and osteoblasts during the development of skeletal tissues. In the hypertrophic and calcified cartilage matrix, MMP-13 degrades type X collagen and aggrecan (D'Angelo *et al.,* 2001). It has been found that a synthetic inhibitor of MMP-13 not only stops the MMP-13 activity in the culture of growth plate chondrocytes but also inhibits mineralisation. This suggests that matrix resorption is essential for mineralisation to occur. According to Wu *et al.,*

(2002), a lack of degradation products leads to suppression of MMP-13 and type X collagen gene expression and this emphasises the important link between hypertrophy and matrix resorption.

Another important member of the MMP family is MMP-9. It has been demonstrated that MMP-9 is a key regulator of growth plate angiogenesis and apoptosis of the hypertrophic chondrocytes (Vu *et al.,* 1998). Active MMP-9 is located at the sites of the matrix resorption where vascular invasion occurs; its expression is found in osteoclasts, endothelial cells and bone marrow stormal cells. The events such as hypertrophic chondrocyte apoptosis, vascularisation and ossification are delayed in homozygous MMP-9 null mice. This delay leads to an abnormal accumulation of hypertrophic cartilage. MMP-9 is thought to take part in the release of angiogenic factors from the hypertrophic cartilage ECM during endochondral ossification (Wu *et al.,* 2002).

1.5 Factors that Regulate Endochondral Ossification

A balance of a large number of factors is required for normal growth and development in the skeleton (Hering, 1999). These factors include TGF-β, BMP, Ihh, PTHrP, Sox9, FGF, CTGF and IGF (Hering, 1999). Also some systemic hormones, vitamins, cytokines were found to have important roles in cartilage formation (Moussad and Brigstock, 2000; Olsen *et al.,* 2000).

1.5.1 Transforming Growth Factor-β (TGF-β)

There are two sub classes of transforming growth factors: TGF-α and TGF-β. These proteins were first isolated from tumour extracts (Graff *et al.,* 1997). Amongst these two subclasses, TGF-α is the smaller protein with a single polypeptide chain of 5.6kDa and shows epidermal growth factor (EGF) like activities with the ability to compete with EGF. On the other hand, the TGF-β has two identical polypeptides linked by disulphide bonds with a size of 25kDa. The subclass TGF-β has three main isoforms (TGF-β1, β2 and β3) which are known to be produced by chondrocytes and osteoblasts and are

highly concentrated in skeletal tissues (Bierie *et al.,* 2006; Machide *et al.,* 2005; Alvarez *et al.,* 2002).

1.5.1.1 Expression Sites of TGF-β

In the growth plate, TGF-β1 expression is restricted to the proliferative and upper hypertrophic zones whereas TGF-β2 is found throughout the cartilage zones with the highest intensity of expression detected in the hypertrophic and mineralised zones. Meanwhile, TGF-β3 is found in the proliferative and hypertrophic zones (Moses *et al.,* 1996).

1.5.1.2 Functional Roles of TGF-β

The transforming growth factor-β (TGF-β) super-family of paracrine and autocrine signalling molecules is known to regulate the process of all embryonic developmental mechanisms (Wrana *et al.,* 1994). Janssens *et al* (2005) suggested that TFG-β1, β2 and β3) are involved in the regulation of bone and its turnover. Moreover, it has been suggested that the transforming growth factor-β is able to regulate the proliferation and the activity of most types of vertebrate cells (Gumienny and Padgett, 2002).

TGFs have been shown to stimulate a number of biological processes such as bone resorption and cell proliferation (Ji *et al.,* 1996). The function of TGF-β is to regulate chondrocyte and osteoblast proliferation and differentiation (Thorp *et al.,* 1992) and may be involved in chondrocyte hypertrophy. The histological changes associated with chondrocyte maturation are well defined and some workers have recently reported that chondrocytes undergoing differentiation and hypertrophy express high level of TGF-β (Thorp *et al.,* 1992; Joyce *et al.,* 1990). The active form of TGF-β is found primarily in differentiating and hypertrophic chondrocytes (Joyce *et al.,* 1990; Thorp, 1992). The activity of TFG-β3 is associated with the processes that result in chondrocyte differentiation. As pointed out by Gumienny and Padgett (2002), disruption of TGF-β often leads to the development of cancer and other diseases; in particular, TGF-β3 was

found to be reduced in the prehypertrophic chondrocytes in some skeletal diseases (Farquharson, 1996).

Transforming growth factor-β is known to be involved in endochondral ossification in association with other known factors such as BMPs, PTHrP and Sox9, and in particular in the regulation of chondrocyte differentiation (Gumienny and Padgett, 2002). Interplay between TGF-β and PTHrP has been demonstrated by Serra R and Chang C (2003) to regulate hypertrophic differentiation in embryonic mouse metatarsal organ culture. According to Alvarez *et al* (2002) TGF-β2 acts as a signal relay between Ihh and PTHrP in the regulation of cartilage hypertrophic differentiation. Included within the TGF-β super family are a subgroup known as the Bone morphogenetic proteins (BMPs), which are described below.

1.5.2 Bone Morphogenetic Protein (BMP)

Bone morphogenetic proteins (BMPs) belong to a conserved sub-family of the super-family of TGF-β and have important functions in many biological tissues. BMP and TGF-β gene family include at least 43 members (Boden, 2005). BMPs were originally isolated from bovine bone and defined by their ability to induce *de novo* ectopic bone formation (Boden, 2005). According to Gooch *et al* (2002), they regulate many different biological processes including embryonic pattern formation, cell growth and cell differentiation. BMPs are dimeric molecules with two chains that are joined together by disulphide bonds. Each monomer consists of about 120 amino acids with seven cysteine residues (Reddi, 1998).

Bone morphogenetic proteins work through members of a larger family of serine threonine kinases, including the receptors of transforming growth factor-β. Massague *et al* (1994) believe that these receptors transduce the signals to the nucleus through pivotal interacellular mediator of TGF-β family member protein (Smad). Smad is a member of the MAD-related family of molecules. MAD-related proteins are a recently identified family of intracellular proteins that are thought to be essential components in

the signalling pathways of the serine/threonine kinase receptor of the transforming growth factor beta superfamily The work of Bubnoff and Cho (2001) has identified a number of nuclear cofactors that cooperate with the Smads to regulate specific target genes depending on the cellular response. These members of the TGF-β super family (BMPs) bind to two different types of serine-threonine kinase receptors, Type 1 and Type 2 which are required for signal transduction (Chen *et al.,* 2004). Both have a similar structure with a short extracellular domain, a single transmembrane domain, and conserved cysteine residues. The intracellular domain contains a serine-threonine kinase motif (Kobayashi *et al.,* 2005).

Bone morphogenetic protein initiates the formation of bone and cartilage and regulates chondrocyte differentiation. In addition, BMPs promote and maintain articular cartilage chondrocytes phenotype in cell culture (Nishihare *et al.,* 2003). Analysis of the BMP expression demonstrated that each protein has a unique tissue distribution. Seven related proteins were found, namely BMP-1 to BMP-7. Bone morphogenetic protein -1 has a different structure to the other BMPs and it is thought to activate the others by proteolysis. Moreover, BMP-2 and BMP-4 are expressed during mesenchyme condensation in the early limb development (Kinglsey, 1994; Zou, 1997). Bone morphogenetic protein-2 also appears to induce differentiation of mesenchymal cells into chondrocytes (Tsumaki *et al.,* 2002). It has been shown that BMP-2 leads to endochondral bone formation and other events such as mesenchymal cell infiltration, differentiation of cells into chondrocytes, removal of the cartilage, formation of bone, population of the bone with bone marrow elements and ultimately normal remodelling of the bone (Swarthout, 2002; Wan and Cao, 2005). Bone morphogenetic protein-6 has been shown to be expressed in the hypertrophic and prehypertrophic chondrocytes (Vortkamp, 2001).

The activity of BMP signalling in the embryo can be interrupted by several other secreted proteins including Noggin and Chordin. Biochemical analyses have demonstrated that both Noggin and Chordin bind BMP proteins directly, preventing them from interacting with their receptors (Tardif *et al.,* 2006). It has also been reported

that members of the BMP family are differentially expressed in the developing cartilage elements where Ihh is present. The missexpression of Ihh in the developing cartilage affects the expression pattern of various BMP genes (Pathi *et al.*, 1999).

1.5.3 Indian Hedgehog (Ihh)

Embryonic development is guided by many different molecules which can be grouped into a relatively small number of categories; some of them act as transcription factors, whereas others are intercellular signalling molecules. Some of this latter group affect other cells rather than those that produce them. One of the most important families of embryonic signalling molecules is that of the hedgehog proteins (Fietz *et al.*, 1994; Enomoto-Iwamoto *et al.*, 2000).

Hedgehog has been studied in *Drosophila.* The studies have demonstrated that hedgehog is a segment polarity gene that regulates segmentation and anterior-posterior patterning of the blastoderm (Fietz *et al.*, 1994; Kobayashi *et al.*, 2005). The hedgehog signalling molecules induce specific pattern of differentiation in a variety of tissues and structures in the development of vertebrates. So far three different forms of hedgehog have been identified: (a) Sonic hedgehog (Shh) is expressed in areas of epithelial-mesenchymal interaction such as the posterior aspect of the developing limb bud and the developing notochord; (b) Indian hedgehog (Ihh) is expressed in the gut as well as in the prehypertrophic chondrocytes in the growth plates of long bones; (c) Desert hedgehog (Dhh) is expressed in Schwann and Sertoli cell precursors in mammalian tissues (Mohle and Vani, 1992). The Indian hedgehog gene (Ihh) regulates several aspects of development. Earlier studies seem to suggest that Indian hedgehog plays at least two roles in the vertebrate development. Firstly, it is involved in the development of endoderm. Secondly, it regulates proliferation and differentiation of the chondrocytes in the growth plate via a negative feedback loop. The intercellular communication is mediated by the secreted signalling molecules of parathyroid hormone-related protein (PTHrP) (Maye, 2000; Lai *et al.*, 2005).

Indian hedgehog undergoes autocatalytic internal cleavage, yielding C-terminal and N-terminal peptides (Zhang *et al.,* 2001). The former is then released from the cell surface but the latter still remains associated with it. The C-terminal has been shown to display autoproteolytic and cholesterol transfers activity that results in the cleavage of the full-length protein and covalent attachment of a cholesterol moiety to the newly generated N-terminal domain. The N-terminal however is active in both local and systemic signalling and binds to the Ihh receptor patched (Patch) receptor to mediate downstream Ihh signalling (Stone and Shi, 1996; Zhang *et al.,* 2001). The exact mechanisms, by which Ihh binds to Patched, and the involvement of Smoothened (Smo) in this process, are still unclear (Kalderon, 2000). Smoothened is a protein with seven transmembrane domains that is distantly related to G-protein coupled receptors (GPCR) (King, 2002). Patched has been reported to inhibit Smo activity directly whereas the Ihh binding may induce a conformational change within the Patched/Smo complex. This activity thus liberates Smo from the repressive influence of Patched, resulting in downstream signalling (Stone and Shi, 1996; Incardona *et al.,* 1998). It has also been suggested that Patched may increase expression of Smo without directly interacting with Smo but through a diffusible intermediate molecule (Taipale *et al.,* 2002). On the other hand, it has been observed that the binding of Ihh to Patched can alter the activity of this molecule, thereby resulting in the loss of the expressive effect of Patched on Smo activity. Further evidence has suggested that the hedgehog signalling pathway may be blocked by steroidal alkaloid compound, Cyclopamine (see Figure 1.9). (Clifford and McMahon, 1997; Cooper *et al.,* 1998; Incardona *et al.,* 1998 and Kim, 1998 and Taipale *et al.,* 2002 and Kayed *et al.,* 2003). Previous studies have shown that during the endochondral bone formation, Ihh is a key molecule responsible for the regulation of chondrocyte maturation (Vortkamp, *et al.,* 1996, 1998; St-Jacques *et al.,* 1999; Lia *et al.,* 2005). As suggested by Pathi *et al* (1999); MacLean *et al* (2005), Ihh achieves its regulatory functions by inducing a series of downstream factors which include its receptor such as patched (Patched).

During the maturation of chondrocytes, Ihh expression is restricted to the lower region of the prehypertrophic zone. This biological function of Ihh promotes chondrocyte proliferation through PTHrP signalling mechanisms (McMahon *et al.,* 2003; Vortkamp,

1996; Hammerschmidt, 1997). A number of recent studies have indicated that disrupting the Ihh mechanism in mice leads to a decrease in the chondrocyte proliferation and changes in the expression of PTHrP, hence resulting in an abnormal positioning of hypertrophic chondrocytes close to the prearticular cartilage surface (Farquharson *et al.,* 2001). Mutation in Ihh may inhibit binding to its receptor and this kind of inhibition results in the shortening of the digits in the syndrome brachydactyly type A-1 (Chung *et al.,* 2000; Shum *et al.,* 2003; Maclean *et al.,* 2005).

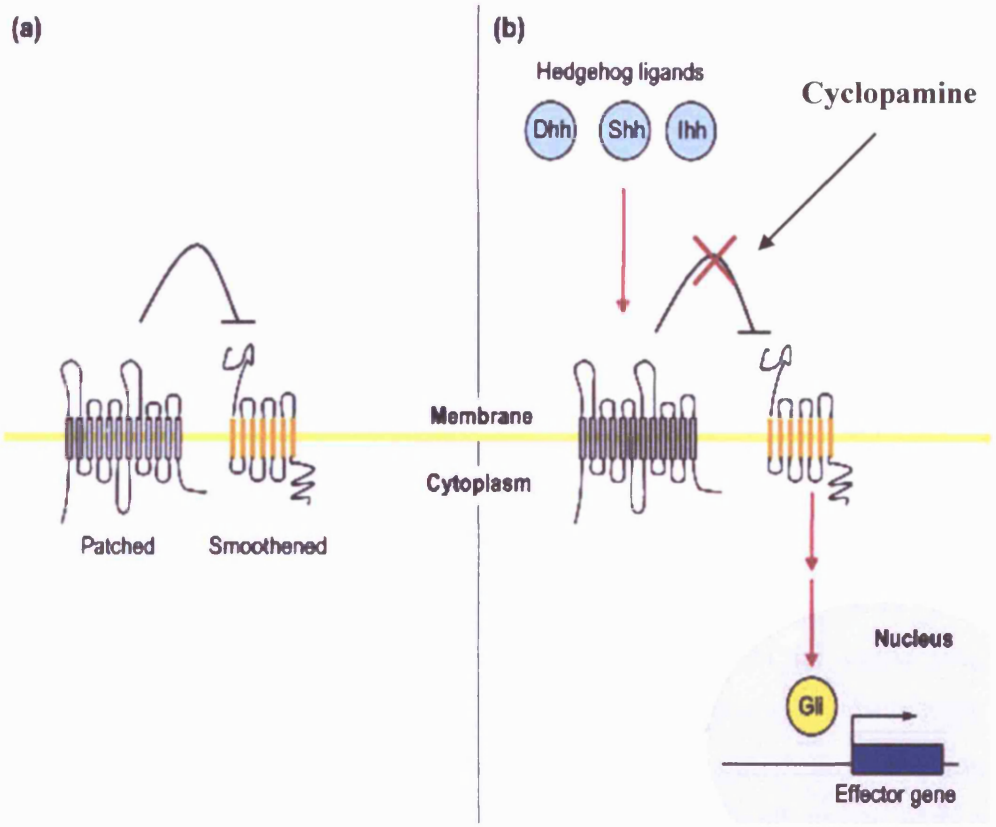


Figure 1.9: Diagram of a generalized Hh-Gli signalling pathway derived from knowledge in model systems. The zinc-finger protein Ci (Cubitus interruptus, a member of the Gli family) is cleaved proteolytically to a repressor which supresses expression of Hh target genes. The binding of Hh with Patched blocks the cleavage of Ci and it acts as a transcriptional activator (Adapted from Weitzman, 2002).

Ectopic expression of Ihh has been shown to affect the normal development of the cartilage growth plate, thus leading to abnormal bone formation (Jüppner, 2000; Razzaque *et al.*, 2005). The expression of Ihh also induces that of Patched (Patched) and Gli, which are known to be associated with Ihh signalling mechanism (Vortkamp, 1996). Patched and Gli are expressed in the perichondrium adjacent to the transitional zone of the growth plate where Ihh is expressed.

As suggested by Long *et al* (1998), the expression of Ihh, stimulates its receptors in the perichondrium and as a result increase the production of PTHrP in the periarticular perichondrium. The PTHrP then binds receptors on chondrocytes in the prehypertrophic zone above the location where Ihh is expressed. When these cells become mature (or hypertrophic) they cease to express Ihh (Figure 1.10). Furthermore, it has been demonstrated that surgical removal of the perichondrium leads to an increased rate of cell proliferation and an extended hypertrophic zone and this observation suggests that this tissue may play an important role in the control of chondrocyte differentiation. This process will be described in detail later (1.5.4).

Some studies have reported that Ihh-null mutants exhibit a severe reduction in skeletal growth, resulting in long bones that are only one-fifth to one-third of the normal length at birth (St-Jacques *et al.*, 1999). Moreover, chondrocyte differentiation in these mice is profoundly disturbed, as indicated by an initial delay in hypertrophy followed by rapid appositional hypertrophy throughout the bulk of cartilage. Ihh-null mouse embryos display a 50% reduction in chondrocyte proliferation (St-Jacques *et al.*, 1999). On the other hand, it has been found that some members of the BMP family are expressed in the perichondrium flanking the Ihh expression domain suggesting a potential interaction with the Ihh/PTHrP signalling pathway (Zou, 1997). For example BMP 2 and BMP 4 are expressed in the perichondrium of developing cartilage (Shum *et al.*, 2003). Experiments in the chick limb indicated that expression of both molecules can be up-regulated by miss-expression of Ihh (Pathi *et al.*, 1999).

1.5.3.1 Ihh/PTHrP Interaction with FGF

It has been recently reported that FGF signals interact with the Ihh/PTHrP and the BMP signalling pathways to regulate chondrocyte development (Figure 1.11). As previously described, Ihh expressed in the prehypertrophic chondrocytes induces the expression of various BMPs in the adjacent perichondrium/periosteum and proliferating chondrocytes (Minina *et al.*, 2001). Indian hedgehog and BMP signals act in parallel to induce chondrocyte proliferation, whereas FGF signalling inhibits chondrocyte proliferation independently of Hh signalling system. Ihh, in addition, regulates the expression of PTHrP in the periarticular region; the expression level of PTHrP signalling determines the distance from the joint where the onset of hypertrophic differentiation takes place, thereby indirectly regulating the chondrocytes that are undergo proliferate. Chondrocytes that are released from the expression level of PTHrP signalling, react to BMP signals with the up-regulation of Ihh expression. In contrast, FGF signals act as antagonists of BMP signalling and negatively regulate Ihh expression. By acting upstream of Ihh, the balance of FGF and BMP signals regulates the distance from the joint region where the hypertrophic differentiation takes place. Furthermore, when FGF signalling accelerates the rate of terminal hypertrophic differentiation, BMPs have been shown to suppress this process. By simultaneously regulating proliferation, Ihh expression, and the rate of terminal hypertrophic differentiation, therefore, the balance of FGF and BMP signals seems to control the process of hypertrophic differentiation to the proliferation rate through Ihh/PTHrP negative feedback loop (Figure 1.10) (Minina *et al.*, 2002).

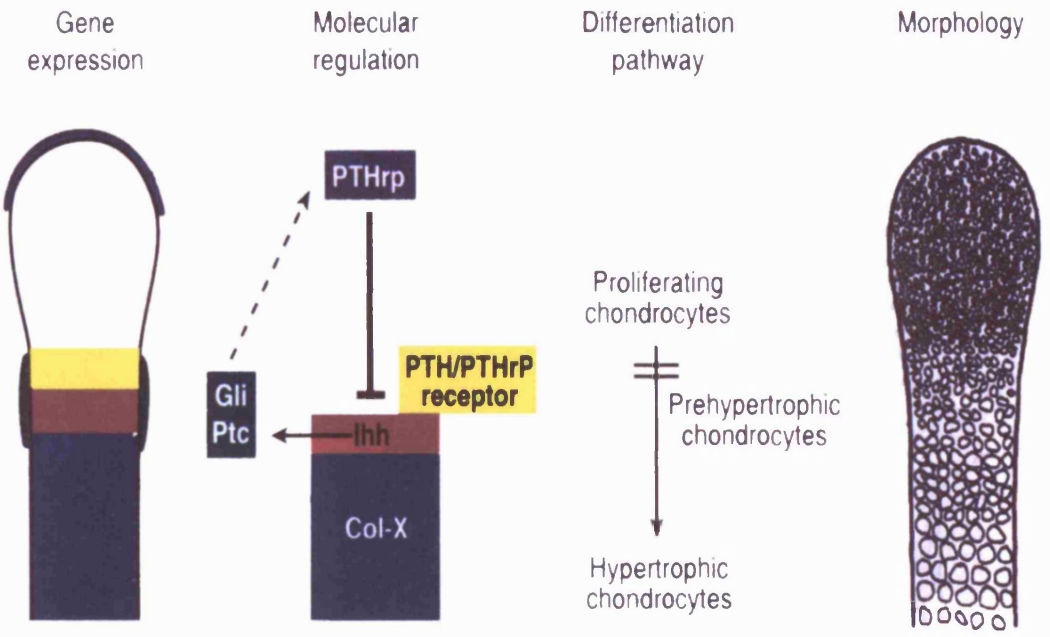


Figure 1.10: The Indian Hedgehog and PTHrP negative feedback loop.
(Taken from Vortkamp *et al.*, 1996)

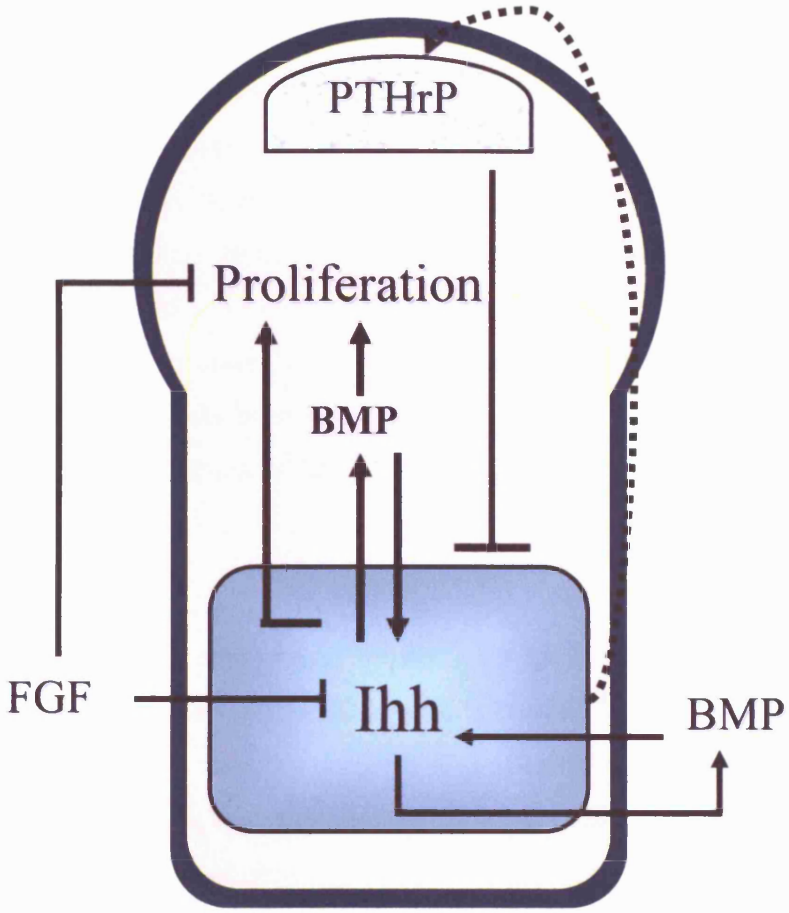


Figure 1.11: Diagram showing the interaction of FGF, BMP and Ihh/PTHrP signalling pathway in the growth plate (Adapted from Minina *et al.*, 2002).

1.5.4 Parathyroid Hormone Related Protein (PTHrP)

Parathyroid hormone–related protein (PTHrP) is an autocrine/paracrine regulator of chondrocyte differentiation was originally identified as a pathogenic factor for malignancy–associated hypocalcaemia (Martin *et al.,* 1988). According to van Donkelaar *et al* (2006), PTHrP is considered as a physiological paracrine factor regulating cell proliferation and differentiation in many tissues during fetal and postnatal growth. It has been shown that the amino terminal region of PTHrP is homologous with the amino terminal of the domain of parathyroid hormone (PTH) which is an 84 amino acid polypeptide hormone (Chung *et al.,* 2000). This hormone functions as a major mediator of bone remodelling and an essential regulator of calcium homeostasis (Chung *et al.,* 2000). Both PTH and PTHrP indirectly activate osteoclasts, hence resulting in an increased bone resorption (Swarthout *et al.,* 2002). Some studies have indicated that both bind to class I PTH/PTHrP receptor found in the avian growth plate (Jüppner 1991). Also, it has been shown that PTHrP is a negative regulator of chondrocyte terminal differentiation in longitudinal bone growth (Vortkamp *et al.,* 1996; Farquharson *et al.,* 2001).

In the growth plate, PTHrP is synthesized by the cells in the periarticular cartilage region and adjacent perichondrial cell near the ends of bones. However, PTH/PTHrP receptor mRNA is expressed in the chondrocytes immediately below the periarticular region, mostly in the proliferative region where the cells form columns and at high level in prehypertrophic chondrocytes (Kobayashi *et al.,* 2002; Lee, 1996; MacLean *et al.,* 2005). An *in situ* hybridization study has demonstrated that PTH/PTHrP receptor is expressed by mature chondrocytes that express type II collagen, but not by hypertrophic chondrocytes expressing type X collagen in fetal and 4-weeks old rat femur (Figure 1.12) (Lee *et al.,* 1996).

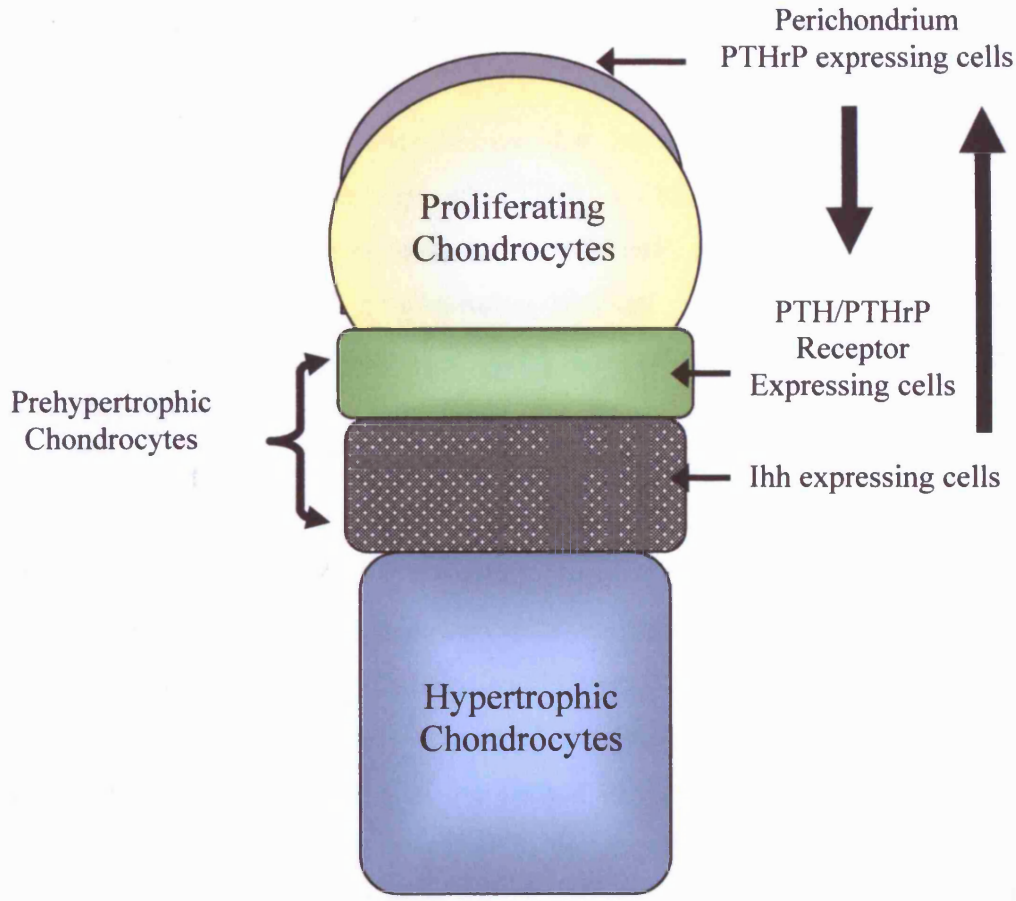


Figure 1.12: Diagram showing the expression regions of Ihh/PTHrP and PTH/PTHrP receptors in the growth plate. PTH/PTHrP is expressed by the chondrocytes in periarticular surface, and their receptor is expressed in the upper region of the prehypertrophic zone. Ihh is expressed by the cells in the lower region of the prehypertrophic region.

PTHrP has been demonstrated to be a potent mitogen as well as an inhibitor of chondrocyte alkaline phosphatase (ALP) activity. PTHrP is known to slow down the rate of differentiation of prehypertrophic within the growth plate (Vortkamp *et al.*, 1996; Farquharson, 2001).

In embryogenesis, it has been suggested that PTHrP acts as an endogenous inducer of parietal endoderm differentiation (Medill *et al.*, 2001) and that it is expressed in response to Ihh, which mediates its action through the membrane receptor 'Patched' (Patched). Recently, it has been shown that bone shows abnormality during its development when the activity of PTHrP is blocked. The differentiation of chondrocytes is accelerated in mice that lack PTHrP, and the bones show premature ossification throughout the endochondral process. Similar abnormalities occur in animals that lack PTH1 receptor which plays a central role in chondrocyte proliferation and differentiation and which mediates the autocrine/paracrine actions of PTHrP. Jüppner (2000); Kobayashi *et al* (2002) have reported that a lack of PTHrP gene leads to quick death after birth with striking abnormality of endochondral bone development. However, an increase in PTHrP signalling also results in abnormalities of the growth plate. PTHrP is expressed in a wide variety of adult and embryonic cell types, including osteoblasts and chondrocytes (Broadus and Stewart, 1994; Suva, 1987; Lee, 1995). As described above, during the development of endochondral bones PTHrP is expressed in the periarticular perichondrium on the surface of the epiphyseal plate. Many studies have indicated that PTHrP is involved in the regulation of bone formation. Recent genetic knockout experiments have demonstrated that knocking out the PTHrP gene leads to an increase in the endochondral bone formation. In the PTHrP knock out epiphyses reveal diminished resting and proliferating zones but accelerated chondrocyte maturation and apoptosis (Lanske, 1996). Moreover, transgenic mice lacking either PTHrP or PTH/PTHrP receptors show evidence of dwarfism resulting from accelerated differentiation and premature hypertrophy. Also, the over expression of PTHrP in mouse growth plate has been shown to exhibit dwarfism (Schipani *et al.*, 1996; Ballock and O'Keefe, 2003).

Some studies suggest that the development of chicken tibial dyschondroplasia (TD) is associated with over expression of PTHrP (Farquharson *et al.,* 2001). In the earlier studies made by Lee *et al* (1996); Vortkamp *et al* (1996) PTHrP expression was detected throughout the proliferative and hypertrophic zones of the juvenile chick growth plate but this expression was much less obvious in the embryonic tissue. In addition, van der Eerden *et al* (2000) have reported that PTHrP is distributed throughout the rat growth plate and suggested that the expression pattern of PTHrP differs markedly at the embryonic and post- stage of growth plate development. PTHrP expression was found to be normal in the chondrocytes of the growth plate and reduced within the lesion of tibial dyschondroplasia (TD) itself (Ben-Bassat *et al.,* 1999).

1.5.5 Sox 9

Sox proteins belong to super family of DNA-binding proteins. In normal cartilage development, transcription factors are required to interact with other regulatory factors. This interaction is necessary to control the multi-step process of gene expression that takes place at different stages of endochondral ossification (Bell *et al.,* 2000; Lefebvre *et al.,* 2005). A number of transcriptions factors have been under investigation for two decades, namely those of Sox family which are known to be associated with the cartilage development. The Sox gene family plays a critical role in the development of a wide variety of tissues during embryogenesis. Twenty-seven mammalian Sox genes have so far been identified (Kulyk *et al.,* 2000).

A member of the most rapidly growing family of Sox is Sox 9 which has been demonstrated to be involved in the regulation of embryogenesis (Marshall and Harley, 2000). This transcription factor has been shown to be involved in the process of chondrocyte differentiation and chondroprogenitor condensation in the early stage of cartilage formation, but not in hypertrophic chondrocytes (Shibata *et al.,* 2006). Sox 9 has the ability to activate the transcription of some regulatory genes. The transcription factor is required for expression of a number of chondrocyte specific proteins such as type II, type IX, type XI collagens and aggrecan. It has been shown that blocking Sox 9 gene expression influences chondrocyte differentiation. This indicates that Sox 9 plays

an important role in the regulation and the synthesis of extracellular matrix. Also, Sox 9 mutation has been demonstrated to cause human skeletal malformation syndrome campomelic dysplasia (de Combrugge *et al.,* 2000;).

It is known that mutation of Sox 9 leads to the human genetic disease called Campomelic Dysplasia (CD), and is characterized by the hypoplasia of most skeletal elements that are derived by endochondral ossification and is caused by heterozygous mutations in the Sox 9 gene (Wagner *et al.,* 1999). The disease is characterised by bowing and angulations of the long bone as a result of the deformation of the cartilage (Lefebvre *et al.,* 1998). The knockout experiments of Sox 9 using mice have indicated that the mutant mesenchymal cells do not differentiate into chondrocytes, and as a result do not form cartilage (Marshall and Harley, 2000).

1.5.6 Fibroblast Growth factor (FGF)

Fibroblast growth factors (FGFs) regulate cell proliferation, migration and cell differentiation. They comprise a large family of polypeptide growth factors found in all organisms. The FGF family includes at least 13 members, which have been found to be highly conserved in both gene structure and amino-acid sequence among species (Hering, 1999; Govindarjan *et al.,* 2006). FGFs and their receptors (FGFRs) play important roles in skeletal development and bone formation, by regulating the expression of various genes involved in osteoprogenitor cell replication, osteoblast differentiation and apoptosis. In addition FGFs have been identified to be expressed in many physiological and pathological processes such as embryonic development, cell survival, and malignant transformation (Marie, 2003).

In the early stage of skeletal development, a number of FGFs have been reported to be expressed locally. Fibroblast growth factor-2 and FGF9 were found to be expressed in mesenchymal cells and osteoblasts. Fibroblast growth factor-2 molecule has been demonstrated to be produced by mature osteoblasts and is eventually stored in the extracellular matrix in the growth plate. Recent studies have indicated that over expression of FGF2 induced abnormal long bone formation. In addition, FGF2 has been

shown to be involved in the regulation of marrow stormal cells in the long bone which eventually induce osteoblast differentiation and matrix mineralisation. Hence, FGF signalling is required for the proper biological process of long bone formation (Marie, 2003; Bioilly *et al.*, 2000).

Fibroblast growth factor receptor-3 is one of the high affinity receptors for the FGF ligands. Ligand binding to FGFRs is facilitated by heparan sulfate proteoglycan (HSPG) and specified by alternative splicing of the Ig-like extracellular domain. Fibroblast growth factor receptor-3 is expressed adjacent to FGF18 in developing cartilage and bone, suggesting that it may mediate some of the biological effects of this ligand in the developing skeleton. In human, activating mutation in FGFR3 results in chondrodysplatic disorder that are characterised by a reduction in proliferating chondrocytes and defective bone formation (Muenke and Schell, 1995). These disorders have been replicated in mice expressing transgenes encoding the activating mutation in the cartilage. Conversely, mice homozygous for targeted inactivation of FGFR3 develop severe kyphosis and skeletal overgrowth, which has been attributed in part to increased proliferation of chondrocytes and reduced turnover of mature cartilage into bone at the chondroosseous junction (Amizuka *et al.*, 2004).

1.5.7 Small Acidic Protein (SAP)

In our laboratory, a novel chicken gene was isolated from embryonic cartilage using differential display PCR (Webster, 1999). This gene was found to be homologous to the mRNA of a gene that encodes a small acidic protein in the *Coturinx japonica* (Japanese quail), accession number U37722 (Gong, 1997). It has been reported that the small acidic protein (SAP) is upregulated in the proliferative chondrocytes of the embryonic chick sternum (Webster, 1999). This gene has also been shown to be expressed by cells from brain, eye, heart, liver, kidney and skeletal muscle of quail tissues (Gong, 1997). The sequence of this gene is short in quail, chick and human, with an mRNA of 758 nucleotides with a poly –A tail (Webster, 1999). The mRNA and peptide sequence are highly conserved between species (Figure 1.14).

The chick SAP mRNA sequence (Figure 1.13) is 92% and 60% identical to the quail and human SAP sequences respectively. The predicted amino acid sequences of the chick SAP are 95% and 74% identical to the quail and human forms respectively (Figure 1.14).

The quail SAP has distinct acidic and basic regions (Figure 1.16) and in the chick, the acidic amino acids are concentrated within the carboxyl terminus of the protein from approximately residue 105 to residue 158, which may point to a protein-binding domain. The basic region is located near to the amino terminus and the SAP protein has no signal peptide (Webster, 1999). The secondary structure of SAP was predicted to be of a basic-helix-loop-helix , with 5α - helical regions, 6 or 7 looping regions and one short β-sheet (Webster 1999), (Figure 1.15). From the predicted secondary structure, SAP appears to have distinct DNA and protein binding regions. Therefore, this protein may function as a transcription factor regulating transcription of genes that are involved in mediating chondrocyte proliferation or differentiation (Webster, 1999).

5′

```
        *         20          *         40          *
TCGGCACAAGCGGCCATGAGCTCGGCCCGGGAGTCGCAGGCTCGCCACGG :  50

       60          *         80          *        100
CCTCAAGCGAGCGGCCTCTCCCGACGGCTCCGGCAGCTGGCAGGCGGCGG : 100

        *        120          *        140          *
ACCTCGGCAACGAGGAGAGGAAGCAGAAGTTCCTGAGGCTGATGGGCGCG : 150

      160          *        180          *        200
GGGAAGAAAGAACATACTGGCCGCCTTGTTATCGGAGACCACAGATCAAC : 200

        *        220          *        240          *
CTCTCACTTCAGGACAGGGGAAGAAGACAAGAAAATGAATGAAGAACTGG : 250

      260          *        280          *        300
AGTCTCAGTACCAGCAGAGCATGGACAGCACGATGTCTGGACGAAACCGG : 300

        *        320          *        340          *
CGCCATTGTGGACTGGGTTTCAGTGAGTTTCAGGAAGGTGAAGAGGAGGC : 350

      360          *        380          *        400
AGCTGGGCATTCCTCTGACCATGAGAGTTCAGAGGACTCCGAAAGTGGCT : 400

        *        420          *        440          *
CTGATTCAAAGCAAGATGAATCTGCAGAGGAACTGCAAGCTGCTGAAACA : 450

      460          *        480          *        500
CACGATGAAGCTGCGGTCCCAGAAACCAAAAAGGAAGCAAAAAGCAATTA : 500

        *        520          *        540          *
TAAAATGATGTTTGTTAAAGCCAGTGGTTCATAACTGCAGATGTAACAGC : 550

      560          *        580          *        600
TTTGGATTTAAAGTACAGTAAGCCTTATTGTTACAGTGCAAAGTGGAGGA : 600

        *        620          *        640          *
CTGCTACAGCACAAATCTTTGTATTATGATTTTAAAACCCCCCTGCTAGA : 650

      660          *        680          *        700
TGACAAAAAGTAGTGTTTGCTTTCAGTTGCCGTGGGCACAGTGAGATTCC : 700

        *        720          *        740          *
TGGTTTTTGTAAAGTGTATAAAATAATGGATGAAGAAATAAATTCTTTGT : 750

      760          *
TCCTGCAAAAAAAAAAAAA : 770
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3′

Figure 1.13: the sequence of the chick small acidic protein RNA. Assession number: CR353822.

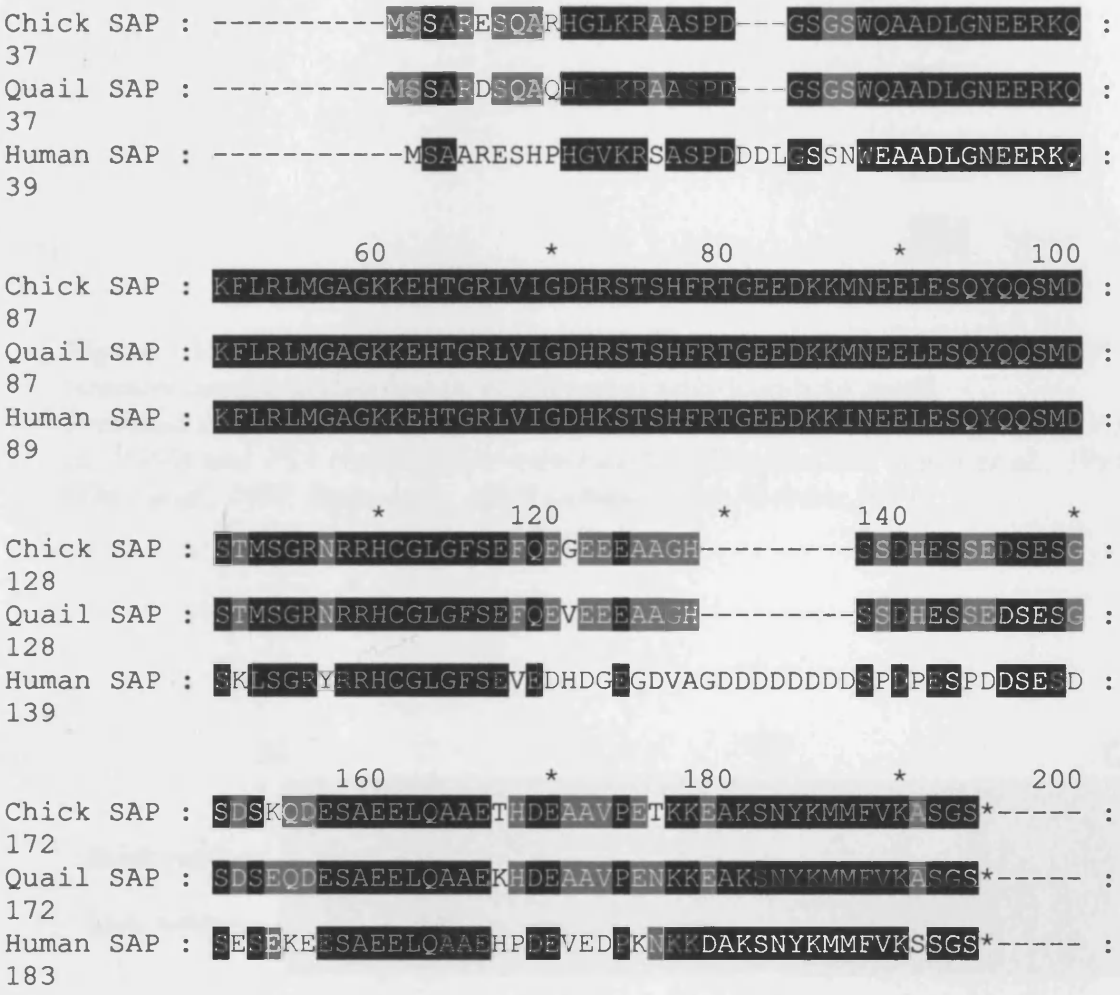


Figure 1.14: Amino acid sequence homologies of chick, quail and human small acidic protein peptides. Black shading indicates identity between all three, and grey shading indicates homology between two. Chick and quail share 95% amino acid identity, chick and human share 72% identity, and quail and human share 74% identity. Prepared using GeneDoc software (KarlNicholas; www.cris.com/~ketchup/genedoc.shtml) Adapted from (Webster, 1999).

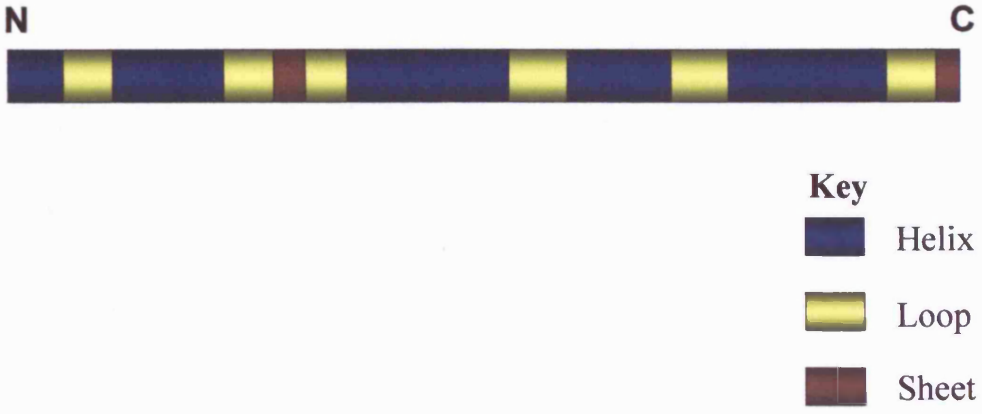


Figure 1.15: Predicted secondary structure of the quail small acidic protein. The structure consists predominantly of a repeated helix-loop-helix motif.
Predicted using NN-predict software (McClelland and Rumelhart, 1988; Kneller et al., 1990) and PSA (http://bmerc-www.bu.edu/psa/request.htm. Stultz et al., 1993; White et al., 1994; Stultz et al., 1997) (Adapted from Webster,1999).

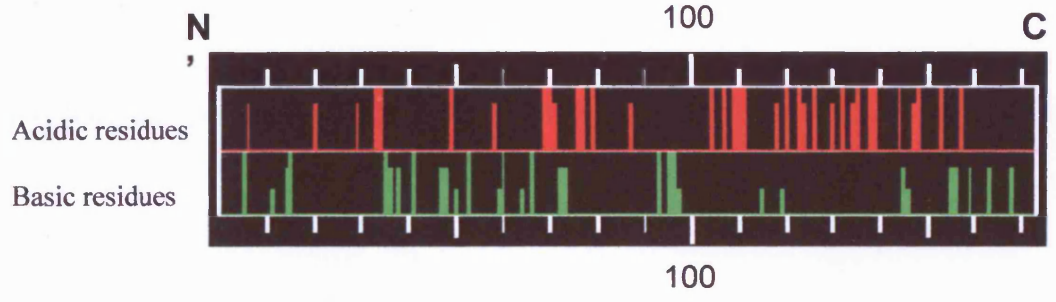


Figure 1.16: The acidic and basic amino acids within the quail small acidic protein. The acidic residues **(red)** are grouped in the carboxyl end of the protein and a predominantly basic region **(green)** exists in the amino half.
Prepared using DNA Strider software (Christian Marck, Service de Biochimie, Département de Biologie, Institut de Recherche Fondamentale, CEA, France). (Adapted from Webster, 1999).

1.8 Aims and Objectives of the Present Project

During a recent study performed in our laboratory, a novel gene was found in the embryonic chick sternum (Webster, 1999) which is a highly likely candidate gene for the regulation of chondrocyte maturation in endochondral ossification. This gene is homologous to a small acidic protein found by Gong *et al* (1997) in the Japaneese quail (*Coturinx japonica*). It is hypothesised that SAP is a nuclear protein which may act as a transcription factor for genes that are involved in mediating the process of proliferation and differentiation of chondrocytes during EO.

1.8.1 Aims

The main aim of the present study is to determine whether SAP plays a role in the differentiation of chondrocytes either independently or in association with Ihh and PTHrP.

1.8.2 Objectives

The current work utilising both, chick sternal and tibial growth plate cartilage is designed to achieve a set of objectives:

(1) To ascertain that SAP is differentially expressed in the growth plate.

(2) To investigate any possible link between SAP expression and expression of the Ihh/PTHrP feedback loop in cartilage.

(3) To investigate the biological consequences of SAP miss-expression in hypertrophic chondrocytes.

CHAPTER 2

Investigation of the expression of SAP in embryonic chick cartilage by *In Situ* Hybridisation

2 Introduction

The chondrocytes within the growth plate go through a series of distinctive developmental stages that include proliferation, maturation and hypertrophy. During these important stages these chondrocytes show variations in their gene expression. For instance, type X collagen and indian hedgehog have been known to regulate the process of endochondral ossification. It has been reported that type X collagen is expressed by the hypertrophic chondrocytes (Safarali *et al.,* 2001; Shen, 2004). In contrast, Indian hedgehog (Ihh) has been shown to be expressed by transitional, prehypertrophic chondrocytes in the prehypertrophic zone (transition zone) of the growth plate but not by the hypertrophic chondrocytes. This gene (Ihh) has been reported to be involved with other genes to block further differentiation of proliferating chondrocytes to hypertrophy (Seth *et al.,* 2000; Lia *et al.,* 2005). According to Vortkamp *et al* (1996), the missexpression of Ihh shows a failure of chondrocyte hypertrophy.

In this study, Type X collagen and Ihh have been used as markers for prehypertrophic and hypertrophic chondrocytes respectively. *In situ* hybridisation was used to localize the expression of these regulatory genes in the sternum and tibia of 17-day chick embryo. The main aim of this series of experiments was to detect the mRNA of Small Acidic Protein (SAP) in these tissues. In this study I localized the SAP mRNA in the chick growth plate and the embryonic sternum using *in situ* hybridisation, and the gene was found to be upregulated in the transition zone of the growth plate and downregulated in the hypertrophic zone. Also this gene was found to be upregulated in the caudal region of the chick sternum and downregulated in the cephalic region in the same tissue. The differential display PCR result showed that this gene (SAP) appears to be upregulated in the proliferative chondrocyte region of the embryonic chick sternum and downregulated in the hypertrophic region in the same tissue.

The aim of the present study is to utilise the currently accepted knowledge in the expression patterns of cell differentiation markers such as Ihh, type X collagen to study Small acidic protein (SAP) in the sternum and tibia of 17 days embryonic chick since these tissues contain different phenotypes of chondrocytes. *In situ* hybridisation has been used in this investigation.

2.1 Materials & Methods

In Situ Hybridisation techniques were performed in this project to localise the mRNA of Small Acidic Protein (SAP), Type X collagen and Ihh using Digoxigenin–labelled sense and antisense riboprobes specific for chicken SAP, Type X and Ihh mRNAs.

2.1.1 Preparation of APES-Coated Slides

Slides were sequentially washed in Lipsol for 30 minutes, 2% (w/v) SDS (Sigma) for 30 minutes and twice in running distilled water for 10 minutes to remove the SDS. These slides were then washed in 95% (v/v) industrial methylated spirit (IMS) for 5 minutes and left to dry in a fume cupboard. They were then dipped in fresh acetone for 2 minutes and in 2% (v/v) 3-aminopropyltriethyloxysilane (APES, Sigma) in fresh acetone for 2 minutes and then rinsed with acetone for 30 seconds and finally allowed to dry in the fume cupboard for 15-30 minutes.

2.1.2 Preparation of Embryonic Chick Sterna and tibia

For *in situ* hybridisations, the entire sternum of a 17-day chick embryo was dissected free using two pairs of sharp forceps and fixed in 4% (w/v) paraformaldehye (Sigma) in 100mM PBS (Sigma) for 6 hours at room temperature. The sternum was then dissected into separate regions as illustrated in (Figure 2.1). Following fixation, the sternum was dehydrated through a series of increasing concentrations of ethanol (50, 70, 80, 90 and 100 % v/v) in Diethylpyrocarbonate (DEPC, Sigma) treated water. The tissues were then cleared in xylene for 15 minutes. The fixed tissues were embedded in pure paraffin wax using a LEICA TP 1050 histology tissue processor. The embedded samples were sectioned with a LKB 2218 Historange microtome at a thickness of 8μm. The sections were then adhered onto APES-coated slides, dewaxed in xylene twice for 15 minutes and then rehydrated through a series of decreasing concentrations of ethanol. The sections were stained with a number of different histological stains including haematoxylin and eosin; Alcian blue; Masson's trichrome (celestine blue B, Mayer's haematoxylin, ponceau/acid fuchsin, 1% (v/v) phosphomolybic acid, and light green). The sections were mounted under cover slips with DPX (Sigma) mountant and examined under a LEITZ DM RB microscope.

The tibia of 17-days chick embryos were fixed in 4 % (v/v) Paraformaldehye in 100mM PBS for 12 hours and decalcified in 10% (w/v) EDTA (Sigma) solution for 5-7 days. The tibia were dehydrated through a series of increasing concentrations of ethanol in DEPC treated water, cleared in xylene and embedded in paraffin wax. Sections were cut at thickness of 8μm on a LKB Historange microtome. The sections were adhered onto freshly APES coated slides, dewaxed in two changes of pure xylene (each for 15 minutes), rehydrated through a series of decreasing concentrations of ethanol and processed for histological studies according to the methods mentioned in the previous section.

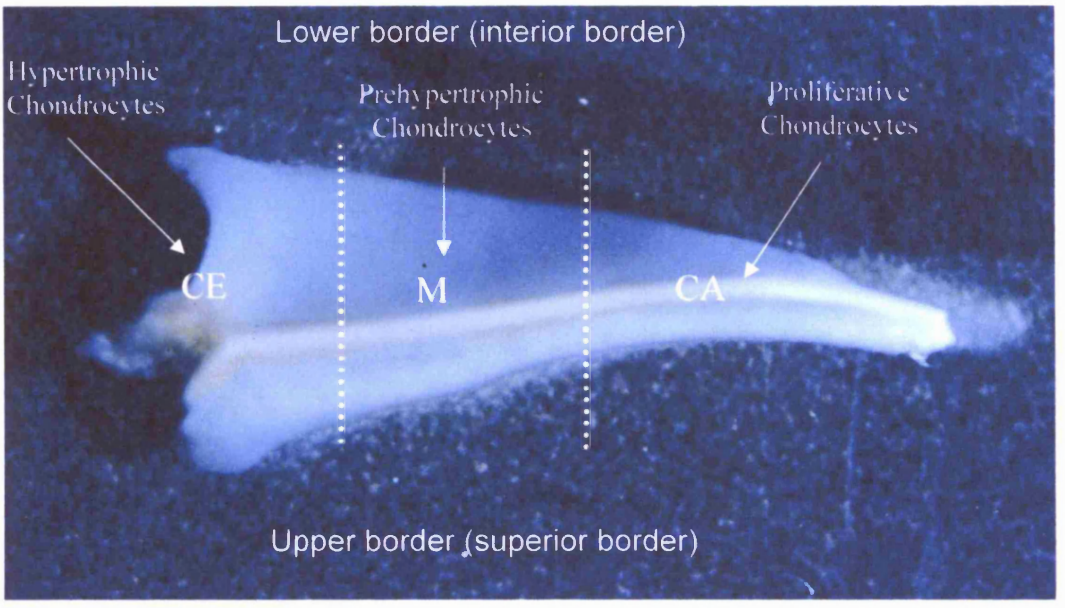


Figure 2.1: Photograph of a 17-day chick embryo sternum taken with a dissection microscope showing the different regions of the tissue used in the preparations of chondrocyte culture (Adapted from Webster, thesis 1999).

(CA) Caudal region contains proliferative chondrocytes.

(M) Middle region contains prehypertrophic chondrocytes.

(CE) Cephalic region contains hypertrophic chondrocytes.

2.1.3 *In Situ* Hybridisation

2.1.3.1 Preparation of *In Situ* Hybridisation Slides

In order to maintain an RNase and DNase free environment, all solutions used in this study were prepared with 0.1% (v/v) DEPC treated water followed by autoclaving. Also all the glassware was baked overnight at a temperature of at least 200°C.

The prepared sections of the sternum and tibia were dewaxed in two changes of fresh xylene for 15 minutes, and then rehydrated through a series of decreasing concentrations of ethanol, 100%, 95%, 90%, 80% and 70% (v/v), each for 5 minutes. The fixed tissues were immersed in two changes of DEPC dH_2O each for 5 minutes, and then incubated at 37°C with 20μg/ml Proteinase K (Sigma) in TE buffer (10mM Tris/ 1mM EDTA, pH8.0) for 45 minutes. The slides were post-fixed in 4% (v/v) Paraformaldehye in 100mM PBS for 5 minutes and washed in two changes of DEPC dH_2O each for 5 minutes. The slides were rinsed twice with freshly prepared 0.1M TEA (5.3ml of TEA 7M in 400 ml DEPC, 20ml of 0.4M HCl /0.25% (v/v) acetic anhydride (Sigma) each time for 5 minutes to reduce non-specific binding of probes and then immersed in 5x SSC for 15 minutes.

2.1.3.2 Preparation of Sense and Antisense Riboprobes for Chicken Small Acidic Protein (SAP), Type X Collagen and Indian Hedgehog (Ihh)

Riboprobes of SAP, Type X and Ihh were prepared by transferring the corresponding cDNA, which have been generated using RT-PCR into pGEM-T™ vector (Promega) using the following protocol:

2.1.4 Reverse Transcription –Polymerase Chain Reaction RT-PCR

The SAP, Type X and Ihh cDNAs were amplified by oligonucleotide primers using RT-PCR to specific regions of chicken SAP, Type X and Ihh sequences. These primers were designed using Bio/Oligo software and are shown in Table 2.1. The RNA used for RT-PCR was isolated from the cells of 17-day old embryonic chick sterna. The sternum of 17 day chick embryo was dissected and the chondrocytes were isolated by digesting the tissue with (0.25mM trypsin/collagenase). The sternal chondrocytes were washed twice with DMEM (Invitrogen) 10% FCS (Gibco). The RNA of these chondrocytes was

isolated using RNeasy kit (Qiagen). The RT-PCR was used to make cDNA from the chondrocyte RNA according to the method described in Chapter 3.

Primers		Sequence 5′ to 3′	Fragment size (bp)	Accession numbers
Ihh	Forward	$^{5'}$CAT CAT CTT CAA GGA CGA GGA GAA C $^{3'}$	224	1493841
	Reverse	$^{5'}$TAC TTG TTG CGG TCC CTG TCT GAC$^{3'}$		
SAP	Forward	$^{5'}$GCC TTG TTA TCG GAG ACC ACA G$^{3'}$	360	7533037
	Reverse	$^{5'}$TTT GGT TTC TGG GAC CGC AG$^{3'}$		
Type X collagen	Forward	$^{5'}$AGC AGG AGC AAA TCA AGC$^{3'}$	340	2829294
	Reverse	^{5}CAG AGG AAT AGA GAC CAT^{3}		

Table 2.1: The forward and reverse sequences of the primers used for the preparation of the riboprobes of chick Ihh, SAP and Type X collagen.

2.1.5 Purification of PCR Product from Agarose Gel

The PCR products were purified from the agarose gel using the GenElute Minus EtBr Spin columns kit (Sigma) as follows:

The PCR products (bands) were cut out from the agarose gel and placed into a 1.5ml RNase DNase free tube. The GenElute Minus EtBr spin columns were placed into RNase/DNase free collection tubes. The spin columns were pre-washed by adding 100µl of 1X TE and then centrifuged at maximum speed (13,000 rpm) for 10 seconds. The elutes were discarded and the excised bands were loaded onto the pre-washed spin columns. The spin columns were then centrifuged at 13,000 rpm for 10 minutes, and the purified DNA collected in fresh RNase/DNase free collection tubes. The collected DNA was precipitated by adding 0.1 volumes of autoclaved 3M sodium acetate to one volume of the recovered DNA solutions. Then 2.5 volumes of absolute ethanol were added to the DNA solution and the mixtures incubated at room temperature for 2 hours. The mixtures were centrifuged at 13,000 rpm for 15 minutes and the DNA pellets washed with 70% ethanol diluted with freshly prepared DEPC treated water. The DNA pellets were resuspended in either 30µl of RNase/DNase free Sigma distilled water or DEPC

treated dH_2O water. The eluated cDNA (1μl) of each different sample were diluted with 1ml of DEPC dH_2O. The cDNA absorbance was measured by a spectrophotometer at A_{260}. Then the concentration was calculated according to the equation below:

$$[DNA] = 50 \times (A_{260}) \ \mu g/ml$$

2.1.6 Ligation of cDNAs Using pGEM®-T Vector

The ligation of SAP, Type X and Ihh cDNA using the pGEM®-T easy vectors kit (Qiagen) were prepared as follows:

The pGEM®-T vector circle map and sequence are shown in Figure 2.2. The tube containing the pGEM®-T vector and that with the control insert DNA were briefly centrifuged and placed on ice. The ligation reaction mixtures were set up as shown in Table 2.2. To give a maximum number of transformants, three different standard reactions for the three different genes were prepared using the following concentrations of the PCR products (SAP, Type X and Ihh cDNA) 50ng, 100ng and 150ng. The following calculation was used to convert molar ratio to mass ratio:

$$\frac{\text{ng of vector} \times \text{kb size of inserts}}{\text{kb size of insert}} \times \text{Molar ratio} \frac{\text{Insert}}{\text{Vector}} = \text{ng of insert}$$

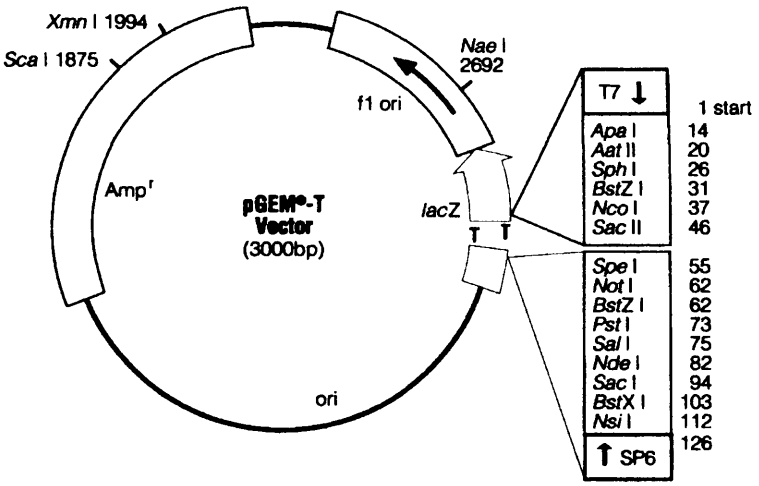
As it has been recommended by the manufacturers for high ligation efficiency, all different reactions were mixed thoroughly by pipetting 3-5 times and the reactions were incubated overnight at 4°C. The ligation reactions were used for transformation with XL-1 blue *E.Coli*

	Standard Reaction	Positive Control	Background Control
2X rapid ligation buffer, T7 DNA ligase	5μl	5μl	5μl
pGEM-T vector (50ng)	1μl	1μl	1μl
PCR Product	X μl *	-	-
Control insert DNA	-	2μl	-
T4 DNA ligase (3weiss units/μl)	1μl	1μl	1μl
Deionized water to final volume of	10μl	10μl	10μl

X μl* (Molar ratio of PCR product: vector)

Table 2.2: Standard protocol for the pGEM®-T ligation

(a)



(b)

pGEM®-T Vector

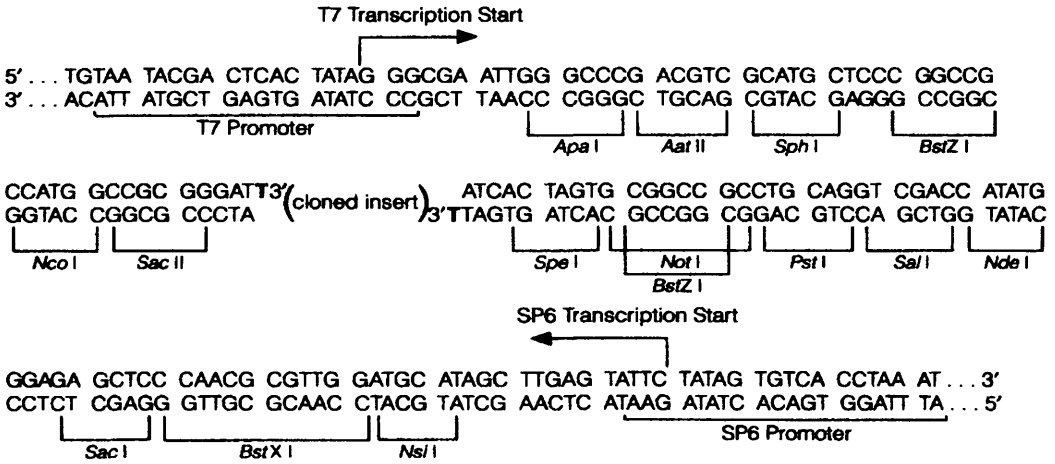


Figure 2.2: (a) pGEM®-T vector circle map and sequence reference points (b) The promoter and multiple cloning sequence of pGEM®-T vector (taken from Promega, 2004).

2.1.7 Preparation of Ultra-Competent *E.Coli* (XL-1 Blue strain) Cells

The XL-1 competent cells used in this project were prepared according to the methods described by *Inoue* and *Okayama* (1990):

The XL-1 cells were inoculated in 5ml LB (Sigma) medium and the culture was incubated overnight at 37°C. An aliquot (3ml) of the overnight grown culture was inoculated in 250ml of SOB medium as shown in Table 2.3. The culture was incubated at 37°C with continuous shaking (280 rpm) for 3-5 hours during which 1ml aliquots were taken every hour from the culture to check their absorbances. As soon as the optical density (OD_{600}) reached ≤ 0.6, the cell suspension culture was taken out and placed on ice for 15 minutes. The culture was then centrifuged at 2500g in a Sigma 3K-10 centrifuge for 10 minutes at 4°C. The cells were resuspended gently in 80ml of ice cold transformation buffer (TB) and placed on ice for 10 minutes (Table 2.3). The suspended cells were centrifuged once again at 2500g for 10 minutes at 4°C. The resulting pellets were resuspended gently in 20ml of ice cold TB for freezing the competent XL-1, then 1.4 ml DMSO (Sigma) was added to a final concentration of 7%. The cell suspension was placed on ice for 10 minutes and then 0.5ml aliquot was transferred into 1.5ml pre-chilled sterilised tubes. The tubes containing the competent XL-1 cells were dipped into liquid nitrogen and stored at –80°C for future use.

500ml of SOB medium		**500 ml of Transformation buffer (TB)**	
2% (w/v) Bacto Tryptone	10 g	10mM Pipes	1.89g
0.5% (w/v) Yeast extract	2.5g	15mM $CaCl_2$	1.10g
10mM NaCl	0.292g	250mM KCl	9.30g
2.5mM KCl	0.093g	55mM $MnCl_2$	5.44g
10mM $MgCl_2$	1.0165g	* Autoclave the solution and then add the $MnCl_2$	
10mM $MgSO_4$	0.602g		
Adjust pH at 6.7-7.0		Adjust pH at 6.7 with 5M KOH prior to adding the $MnCl_2$	

Table 2.3: Preparations of SOB medium and transformation buffer

2.1.8 Transformation of pGEM®-T Vectors into XL-1 Blue

High-efficiency competent cells (XL-1 blue *E.Coli* strain) were used for the transformation of p-GEM®-T vectors containing SAP, Type X and Ihh cDNA. The XL-1 blue competent cells were thawed on ice for 15 minutes. All tubes containing the ligation reactions were centrifuged to collect contents at the bottom of the tubes. The competent cells were mixed gently by flicking the tube 4-5 times, and 150µl of these cells was added into each individual ligation tube and mixed gently. The tubes were placed on ice for 20 minutes, followed by a heat-shock incubation in a pre-warmed water bath without shaking for exactly 45 seconds at 50°C. The tubes were returned immediately to ice and left for 10 minutes. SOC medium (200µl) was added into each tube containing the cells which were transformed with ligation reactions; these tubes were incubated at room temperature for 25 minutes. The content of each tube was spread individually on the top of *Luria broth* (LB) ampicillin (Sigma) /IPTG (Promega) /X-Gal (Promega) agar plates and incubated at room temperature for 30 minutes, and then overnight at 37°C.

2.1.9 Preparation of LB Broth Plates with Ampicillin

LB agar powder (15g) was dissolved in 500ml of dH_2O and autoclaved. The LB medium was left to cool at room temperature for 30-45 minutes until the temperature dropped to approximately 40°C. Ampicillin was added into the LB medium at a final concentration of 100µg/ml. Tetracycline (Gibco) was also added into the medium at a final concentration of 2.5µg/ml. LB medium (15ml) was poured into each plate. The plates then were left at room temperature for 1 hour. The surface of the agar in the plates was pre-coated with 100µl of IPTG and 20µl of X-Gal (50mg/ml).

2.1.10 Isolation of Plasmids from the Transformed XL-1

The isolation of the plasmids was done using the mini prep kit (Qiagen). White colonies of XL-1 cells were selected for the isolation of plasmids containing SAP, Type X and Ihh cDNA. Some of these colonies were selected and inoculated in 5ml of LB-broth containing Ampicillin (100µg/ml). The cultures were grown overnight at 37°C with continuous shaking (250 rpm), after which 1.5 ml of each culture was transferred into a sterile DNase/RNase free tube. The remaining cultures of the three different

plasmids were frozen in liquid nitrogen and then stored at -80°C for future use. All prepared tubes containing the cells were centrifuged in a microcentrifuge for 5 minutes at 10,000 rpm. The resulting supernatant in each tube was discarded and the bacterial pellet resuspended in 250µl of P1 buffer. P2 buffer (250µl) was added gently into the tubes which were then inverted 4-5 times to obtain a well mixed solution. Buffer N3 (350µl) was added into the solution, and the tubes were inverted immediately 4-6 times and centrifuged for 10 minutes at 13.000 rpm. The supernatants were pipetted onto QIAprep spin columns and centrifuged for 60 seconds. The flow-through was discarded. The QIAprep spin columns were washed by adding 0.5 ml of PB buffer and centrifuged for 60 seconds. The resulting flow-through was again discarded and the QIAprep spin columns were washed by adding 0.75 ml of PE buffer and centrifuged at full speed (13,000 rpm) for 60 seconds. The same procedure was repeated again at the same speed to remove residual wash buffer. The QIAprep spin columns were placed into DNase/RNase free 1.5 ml microcentrifuge tubes, and then the DNA was eluted by adding 40µl of EB buffer (10nM Tris-Cl, pH 8.5) to the centre of each QIAprep spin columns. The columns were left on the bench surface at room temperature for 1-2 minutes. They were then centrifuged at maximum speed 13,000 rpm for 1 minute.

The DNA concentration was measured by using a spectrophotometer. The DNA elutes of SAP, Type X and Ihh were then used for riboprobe preparations. In order to check that the insert was correct 1µl of plasmid DNA containing SAP, Type X and Ihh insert were separately diluted in 10 µl of dH_2O (DEPC) and then they were used as a template for PCR reaction.

2.1.11 Restriction Digestion of SAP, Type X and Ihh Plasmids

Some of the purified plasmids from the three different inserts were linearised (Figure 2.3) as follows:

A variable amount of the SAP, Type X and Ihh DNA plasmids were cut separately using the appropriate restriction enzymes with the optimum supplied buffers. These enzymes cut just a few base pairs beyond the sequence from both sides of the insert. This amount was calculated according to the absorbance reading obtained from the DNA/RNA spectrophotometer. The amount of each individual plasmid was calculated

to give a final concentration of 2-3 μg in each reaction. The two different restriction enzymes were used individually with each different plasmid (SAP, Type X and Ihh) as follows:

dH_2O (DEPC or Sigma)	11.8μl
Restriction enzyme Buffer D (Promega)	2μl
BSA, Acetylated (1mg/ml)	0.2μl
Enzyme ***SalI*** or ***NcoI*** (10U/μl) (Promega)	1μl
Plasmid (DNA) 2-3μg	5μl
Total	**20μl**

Table 2. 4: Standard protocol of endonuclease restriction/digestion of the isolated plasmid

The samples were mixed individually and incubated for 2-3 hours at 37°C. After the incubation, 5μl of each sample were mixed with 2μl of the loading dye and run on 1% agarose gel. The gel was stained in ethidum bromide (EB, Sigma) for 30 minutes and visualised under UV light.

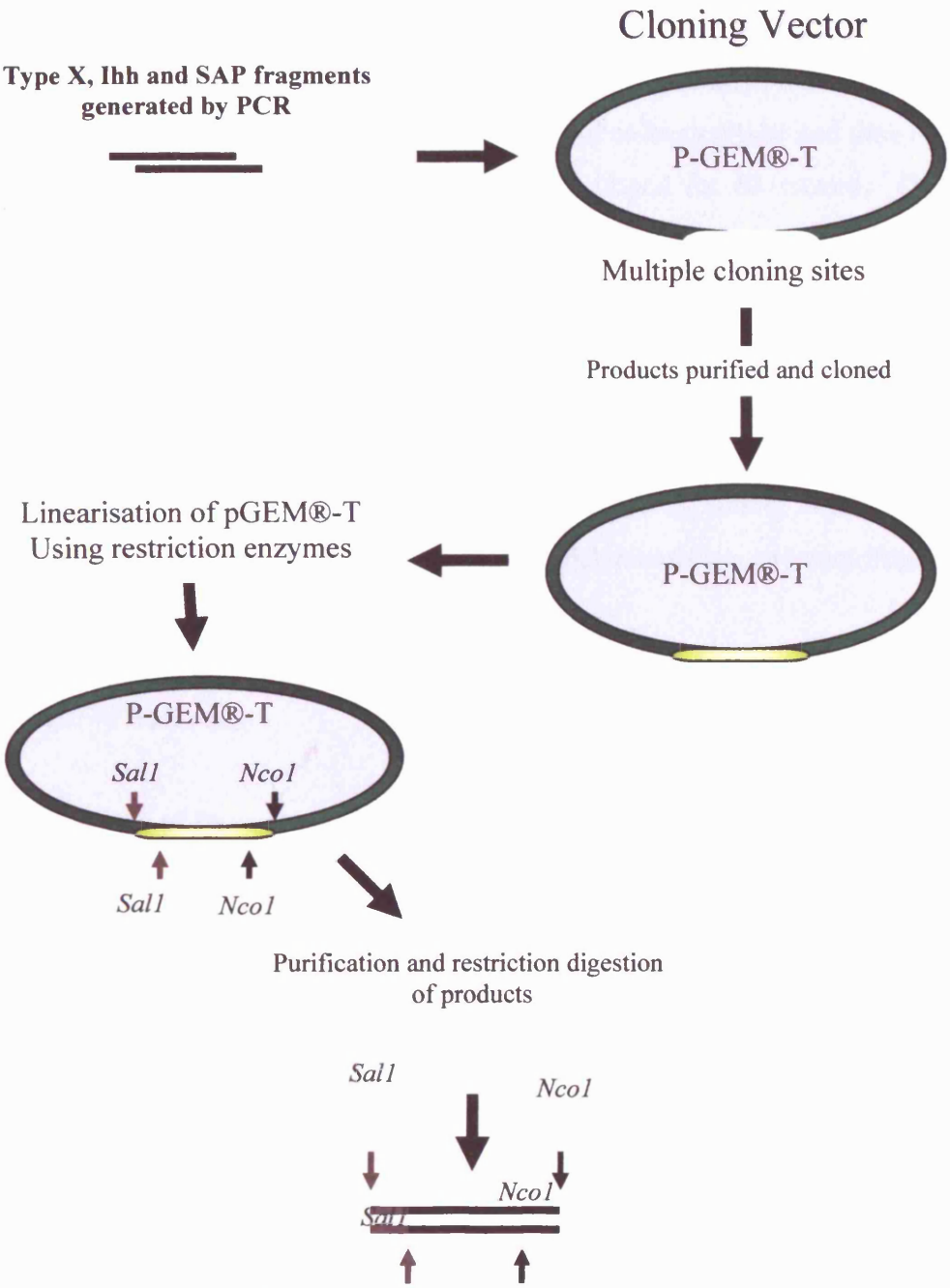


Figure 2.3: Schematic diagram of protocol for cloning and linearization of type X collagen, Ihh and SAP inserts into p-GEMT-T vector and restriction digestion of the inserts using *Sal*I and *Nco*I.

2.1.12 Purification of SAP, Type X and Ihh Inserts from the Cut pGEM®-T Vector Using QIAquick PCR Purification Kit

The following protocol was Adapted for the preparation:

All the solutions used in this preparation are supplied in the QIAquick PCR purification kit (Qiagen), five volumes of buffer PB were added to 1 volume of the DNA elute. The QIAquick spin column was placed in a provided 2 ml collection tube and then the DNA was applied into the QIAquick columns and centrifuged for 60 seconds. The flow-through was discarded and the QIAquick columns were placed back into the same collection tubes. The columns were washed by adding 750µl of PE buffer and centrifuged for 60 seconds. The flow-through was discarded and the columns were placed back into the same collection tubes. The columns were centrifuged at the maximum speed (13,000 rpm) for 1 minute. The QIAquick columns were placed into new 1.5 ml microcentrifuge tubes. The DNA was eluted by adding 50µl of EB buffer (10mM Tris-Cl, pH8.5) to the centre of the QIAquick membrane and centrifuged at full speed for 1 minute.

The DNA concentration was measured using the DNA/RNA spectrophotometer at the wavelength of A_{260}/A_{280}.

2.1.13 Labelling of linearised plasmids with Digoxigenin

The purified plasmids (SAP, Type X and Ihh), which were previously cut as described earlier with *Nco1* and *Sal1,* were labelled using Digoxigenin kit (Roche) as follows:

Digoxigenin (DIG) labelled riboprobes were synthesised from cloned PCR products and the DIG-labelled RNA were transcribed from SP6-T7 RNA polymerase promoter regions as recommended by the manufacturer.

The cloned sequences within the pGEM-T™ vector were transcribed separately with T7 and SP6 RNA polymerase resulting in either the sense or antisense probes depending on the orientation of the cloned DNA insert. The two different linearised plasmids which were cut individually, one with *Nco*1 (Promega) and the other with *Sal*1 (Promega) of the three different plasmids (SAP, Type X and Ihh), were labelled as follows:

	Plasmid cut with *Nco1*	Plasmid cut with *Sal1*
Plasmid (1µg/µl)	Different amount	Different amount
10X DIG labelling mix	2µl	2µl
5X Transcription buffer	4µl	4µl
DTT (100mM)	2µl	2µl
RNase inhibitor (Promega)	0.5µl	0.5µl
RNA Polymerase	**Sp6** 1µl	**T7** 1µl
DEPC dH2O	–	–
Total	**20 µl**	**20µl**

Table 2.5: Standard protocol of labelling the riboprobe with Digoxigenin

All tubes containing different reactions were incubated for 2-3 hours at 37°C. During the incubation 1µl of the RNA polymerase (Sp6 or T7, Promega) was added. The DNA template of the plasmids was removed by adding 3µl of DNase into each sample. The samples were mixed and centrifuged briefly and incubated for 20 minutes at 37°C. The reactions were stopped by adding 10µl of 0.2M EDTA. The mixtures were left at room temperature for 2 minutes. The RNA was precipitated by adding 2.5µl of 4M Lithium chloride (LiCl) followed by 75 µl of 100% ethanol. The mixtures were centrifuged at maximum speed (13.000 rpm) for 5 minutes to precipitate the RNA pellets. The RNA pellets were washed gently with 75µl of ice cold 70% ethanol. The pellets were centrifuged at full speed again for 5 minutes. The supernatants were removed from all the samples and the RNA pellets were left to dry at room temperature. The riboprobe

pellets were resuspended in 20µl of DEPC dH_2O and stored at -20°C. From the serial dilutions (1:10, 1:100 and 1:1000) of the prepared riboprobes 1 µl was spotted onto Hybond-Nylon membrane (Amersham,UK) which was then incubated with alkaline phosphatase conjugated DIG antibody and alkaline phosphatase activity was detected using nitroblue tetrazolium (NBT)/5-bromo-4-choro-3-indolyl phosphate(BCIP), (Roche) as described in detail in section 2.1.14

2.1.14 Blotting and Detection of DIG Labelled Riboprobes

Five different solutions were prepared in 20 ml universal tubes:

The Hybond-Nylon membrane was dipped into in 2ml blocking solution (100mM Tris-HCl/150mM NaCl pH7.5 and 1% blocking agent) for 2 minutes. The membrane was then transferred into 2ml blocking solution with 1µl of Anti-DIG-AP antibody for 3 minutes. The membrane was dipped again in the blocking solution for 1 minute. The membrane was washed with 2ml of washing buffer (10mM Maleic acid/15mM NaCl pH 7.5 and 0.3% Tween-20) for 1 minute. The membrane was immersed into 2ml of detection buffer (10mM Tris-HCl/150mM NaCl pH9.5) for 1 minute. The riboprobe spots were developed by dipping the membrane into 2ml of freshly prepared NBT/BCIP solution (Promega) (2ml detection buffer, 9ml NBT and 7ml BCIP) for 5 to 60 minutes, depending on the quality of the riboprobe preparation. The intensity of DIG labelling of antisense and sense riboprobe was compared, and the lowest concentration of the riboprobes that gave a reasonable signal was used at the hybridisation step.

2.1.15 Hybridisation

The probes were diluted 100x with hybridisation mixture (50% v/v formamide (Sigma) 10% dextran sulfate (Sigma), 5x SSC) and the resulting solution was mixed by pipetting 3-5 times. Aliquots (60µl) of the diluted probes were applied to each slide containing the tissue section. The slides were sealed under hybridisation plastic cover slips (Hybrislip, Sigma) and incubated overnight at 47°C in a humid box saturated with 5x SSC / 50% formamide (v/v).

2.1.15.1 Stringency Wash

Following the overnight incubation, the hybridisation cover slips were floated off in 2x SSC at room temperature for 30 minutes. The slides were taken through a series of stringency washes for 1 hour at different salt concentrations and temperatures as follows:

2 x SSC at 55°C and 1x SSC at 55°C in a Hybaid Omnislide wash module to remove non-specifically bound probes. The sections were then equilibrated in (100mM Tris-HCl / 150mM NaCl, pH7.5). Alkaline phosphatase conjugated anti-DIG-antibody (200ml of 1:100 dilution) in 100mM Tris-HCl /NaCl pH 7.5 in 5% (w/v) bovine serum albumin (BSA) (Sigma) was added onto each slide and incubated in a humid black box at room temperature for 1 hour. The slides were washed twice with Tris/NaCl, pH7.5 to remove unbound antibodies and then in 100mM Tris-HCl /100mM NaCl / 50mM $MgCl_2$, pH9.5 for 10 minutes; 1ml of the 50x diluted substrate (500ml Tris / NaCl/ $MgCl_2$, pH9.5, 2.25µl NBT 75mg/ml, 1.75µl BCIP 50mg/ml and 2.5µl of 1M Levamisole (Sigma) was applied onto each slide.

All the slides were left in the dark box for 2-5 hours. The colour reaction was stopped in TE buffer pH8.0 for 15 minutes. The slides were rinsed in running tap water for 2 hours and mounted under aqua-mount (Sigma). Photomicrographs were taken with a Leica DM RB photomicroscope fitted with a WILD MPS48 photographic system.

2.2 Results

2.2.1 Confirmation of the Insertions of Chick Type X Collagen, Indian Hedgehog and SAP cDNAs in pGEMT® Cloning Vectors Using Restriction Enzyme Digests

The size of the cloned PCR products was confirmed by restriction digestion and electrophoresis with both the positive controls and molecular markers. The procedures are described in the 'Materials and Methods' section. Briefly, 1μl of purified plasmids containing the appropriate inserts were cut at the appropriate restriction sites using NcoI & SalI. After the incubation, 5μl of each sample were mixed with 2μl of the loading dye and run on 1% (v/v) agarose gel. Following the staining in ethidum bromide (EB) for 30 minutes, the gel was then visualised under UV light. The results are shown in Figure. 2.4 Type X, Ihh and SAP are found to have the following respective size: 340bp (Lane 3; Plate A), 224bp (Lane 3; Plate B) and 360 bp (Lane 3; Plate C). The lengths of each inserted fragments (type X, Ihh and SAP) were PCR with the corresponding primers. The PCR products were matched the exact size of the primers used (Figure 2.4) Lane 4.

2.2.2 Immunoblotting of Type X, Ihh and SAP Riboprobe

After the preparations of the various riboprobes, the sense and anti-sense riboprobes of Type X collagen, Ihh and SAP were blotted on Hybond-Nylon membrane. A series of dilution (1:10, 1:100 and 1:100) of riboprobes were used to determine the optimum concentration for use in the in situ hybridisation experiments. The probes were labelled with alkaline phosphatase conjugated Digoxigenin (DIG) antibody and the product was detected with NBT/BCIP. The results indicate that 1:100 dilution is the minimum concentration for detection of both Ihh (Figure. 2.6) and SAP (Figure. 2.7) whereas the type X collagen riboprobe requires a 1:10 dilution (Figure. 2.5).

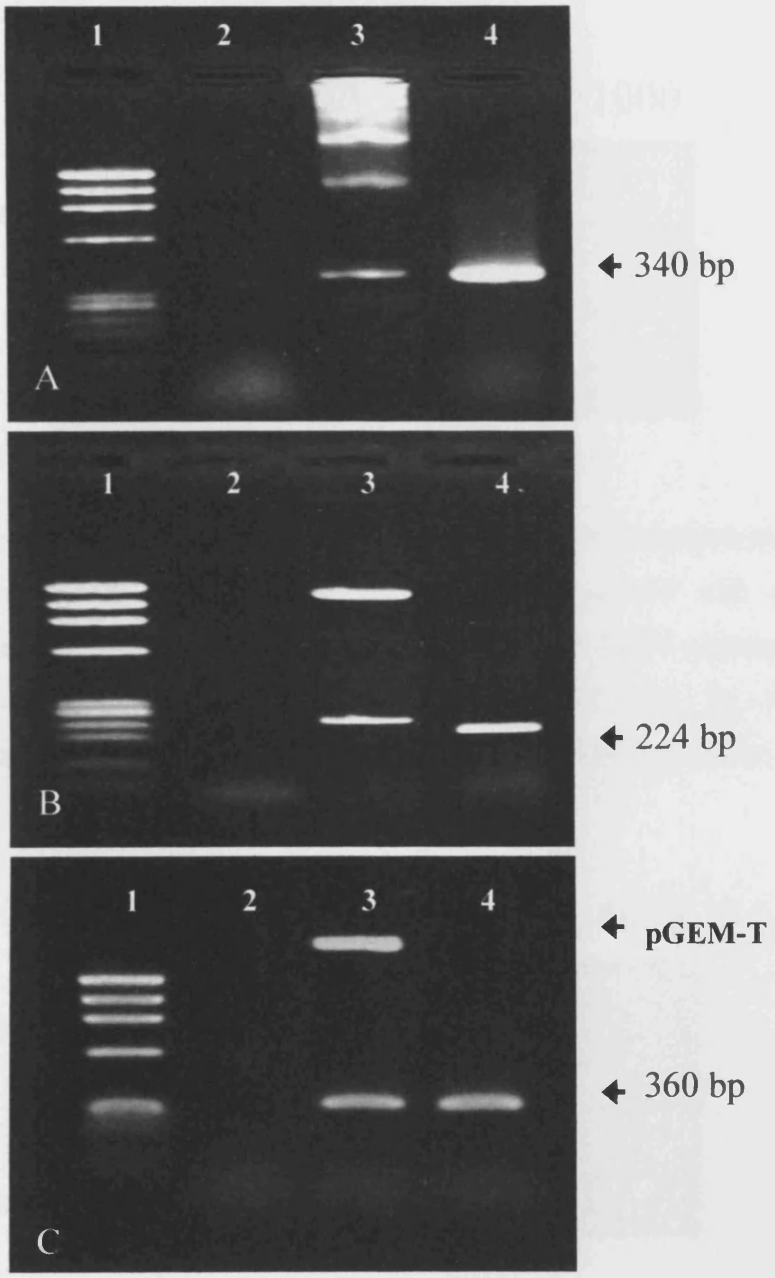


Figure 2.4: Agarose gel (1% w/v) analysis of p-GEM-T plasmids following restriction digestion with *SalI*/*Nco*I restriction enzyme. **(A)** p-GEM-T containing the type X collagen cDNA insert; **(B)** p-GEMT containing the Ihh insert; **(C)** p-GEM-T containing the SAP insert. Lane 1: -174 *Hae* III DNA ladder. Lane 2: dH_2O control. Lane3: digested plasmid showing the appropriate insert that has the same electrophoresis mobilities/size as the standard PCR products. Lane 4: PCR product contains the cDNA (inserts) with the flanking sequences of the plasmid.

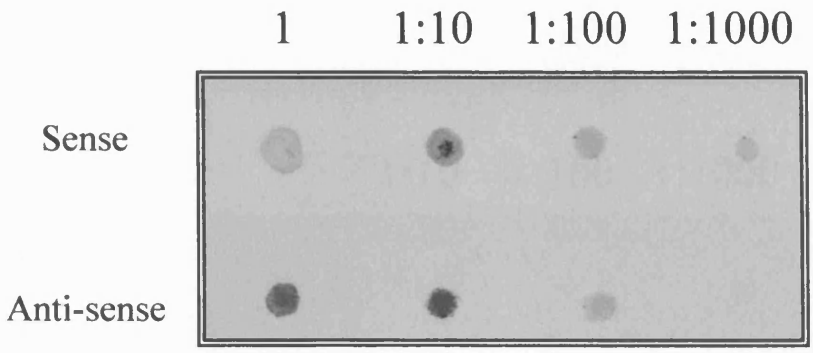


Figure 2.5: Serial dilutions of chick Type X collagen Digoxigenin-labelled sense and anti-sense riboprobes blotted onto Hybond-Nylon membrane and visualized with an alkaline phosphatase conjugated Digoxigenin antibody and NBT/BCIP for 45 minutes. In both sense and anti-sense, spots are visible until about 1:100 dilution. *In situ* hybridisation was performed using 1:10 dilution of both probes in hybridisation buffer.

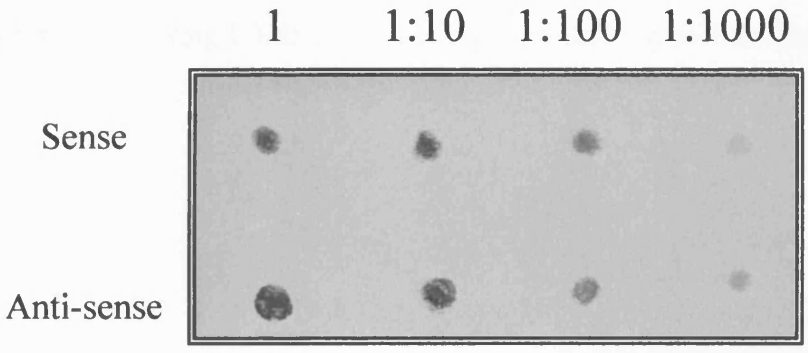


Figure 2.6: Serial dilutions of chick Indian hedgehog Digoxigenin-labelled sense and anti-sense riboprobes blotted onto Hybond-Nylon membrane and visualized with an alkaline phosphatase conjugated Digoxigenin antibody and NBT/BCIP after 1 hour. In both sense and anti-sense spots are visible until about 1:100 dilution. *In situ* hybridisation was performed using 1:100 dilution of both probes in hybridisation buffer.

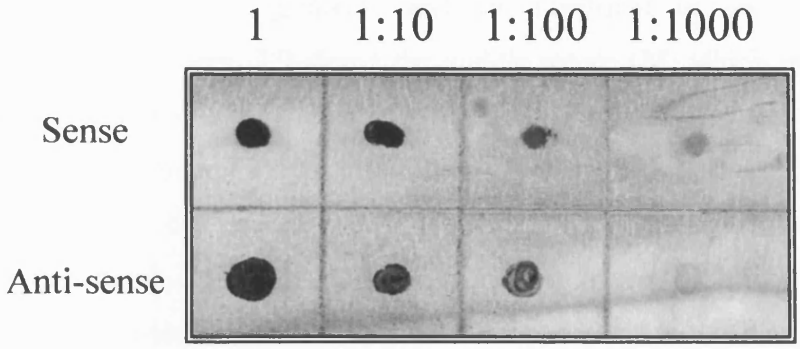


Figure 2.7: Serial dilutions of chick Small acidic protein (SAP) Digoxigenin-labelled sense and anti-sense riboprobes blotted onto Hybond-Nylon membrane and visualized with an alkaline phosphates conjugated Digoxigenin antibody and NBT/BCIP for 3 hours. In both sense and anti-sense spots are visible until about 1:100 dilution. *In situ* hybridisation was performed using 1:100 dilution of both probes in hybridisation buffer.

2.2.3 Histology of 17 Day Old Embryonic Chick Cartilage

Two types of tissue were examined, the sternum and the tibia. These tissues were sectioned longitudinally and sections were stained with Massons' trichrom. Figure. 2.8 shows a micrograph of a section through the caudal region of the sternum. This shows that this is a region packed with cells that appear round and small where some cells appear densely packed cytoplasmic contents and are identified as proliferative chondrocytes Figure. 2.11 A. Figure. 2.9 shows the middle region (M) which is wider than the caudal region. As shown in the higher magnification (Figure. 2.11), the cells in this region appear to have a diamond shape; most of these cells appear elongated (Plate B) as compared to those in the caudal region (Plate A) and they are known as prehypertrophic (or transition) chondrocytes. Moreover, it is interesting to note that many of the cells in the middle region exhibit clear space, the typical effect of cytoplasmic shrinkage and some have two nuclei, showing cell division activity. Figure 2.10 illustrates the third region which is more expanded than the middle region and which consists mostly of cells that have either a flattened shape or a hexagonal shape most (Figure. 2.11 - Plate C). This third region is known as the cephalic region where most of the cells are much larger (Plate C) as compared to those in the other regions, and thus are considered as highly differentiated cells (Hypertrophic chondrocytes).

A longitudinal section of the tibia is shown in Figure. 2.12 highlighting the typical histological morphologies of cells within the epiphysis. The three different zones are easily distinguishable depending on cell density, size and shape: proliferative (PR), prehypertrophic (PH) and hypertrophic (H). The PR zone is occupied by a network of proliferative chondrocytes that appear slender and are arranged in columns (Figure. 2.14 - Plate A). Adjacent to PR, is the prehypertrophic zone is composed of columnar or globular cells. Many cells also have a tadpole-like structure and show sign of enlargement and eventually become round (Figure. 2.14 - Plate B). Some cells also show cytoplasmic shrinking. Close to PH zone towards the diaphysis, the hypertrophic zone is observed and this consists mainly of much larger cells but with a much smaller and condensed cytoplasm. Due to the cytoplasmic shrinkage, large empty spaces are observed within these cells. But it is now known that the spaces are the artefacts of fixation.

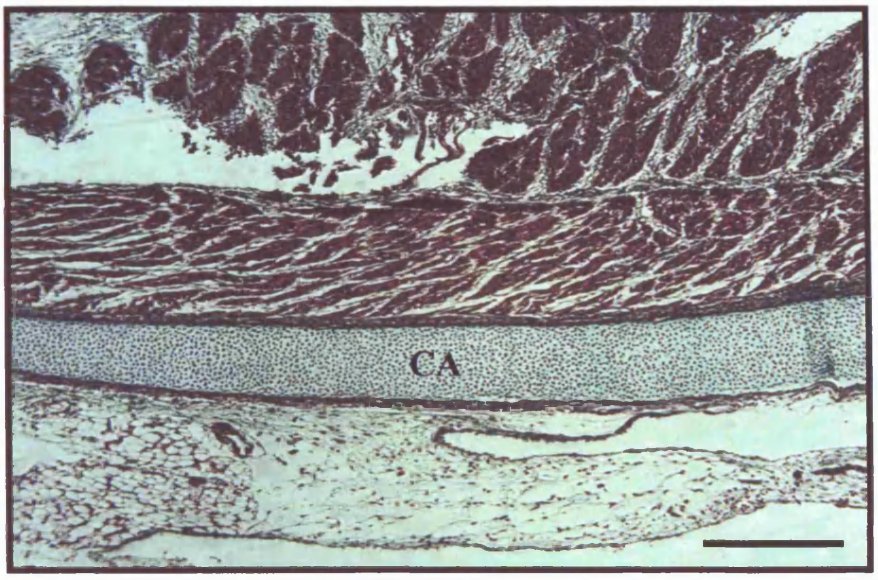


Figure 2.8: A typical photomicrograph of longitudinal section cut from 17-day old embryonic chick sternum. The tissue was histologically prepared as described in the text and the paraffin embedded section was stained with Masson's trichrome. The picture illustrates the caudal region densely packed with proliferative chondrocytes. Scale bar, 200μM.
(**CA**) Caudal region.

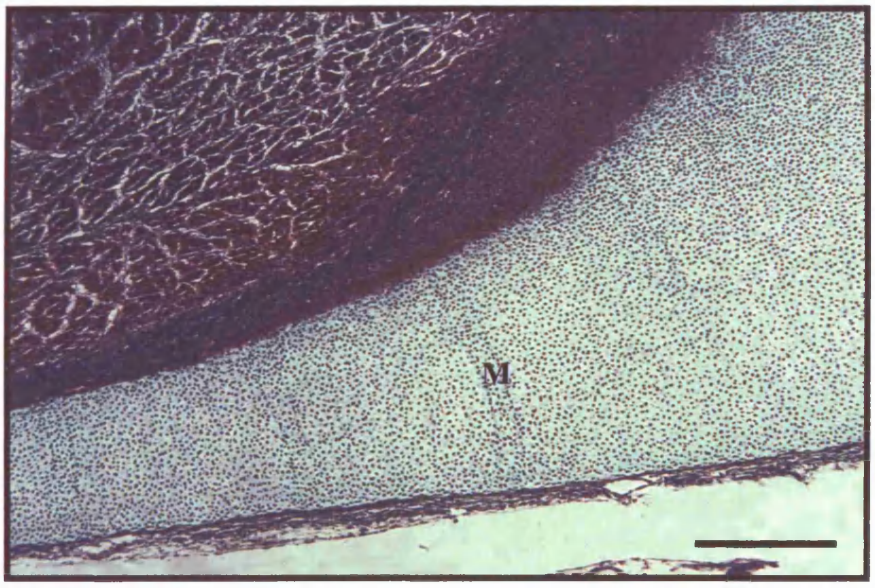


Figure 2.9: The same longitudinal section (as in Figure 2.8) of 17-day old embryonic chick sternum. The tissue was histologically prepared as described in the text and the paraffin embedded section was stained with Masson's trichrome. The photograph reveals the middle region with prehypertrophic chondrocytes. Scale bar, 200μM.
(**M**) Middle region

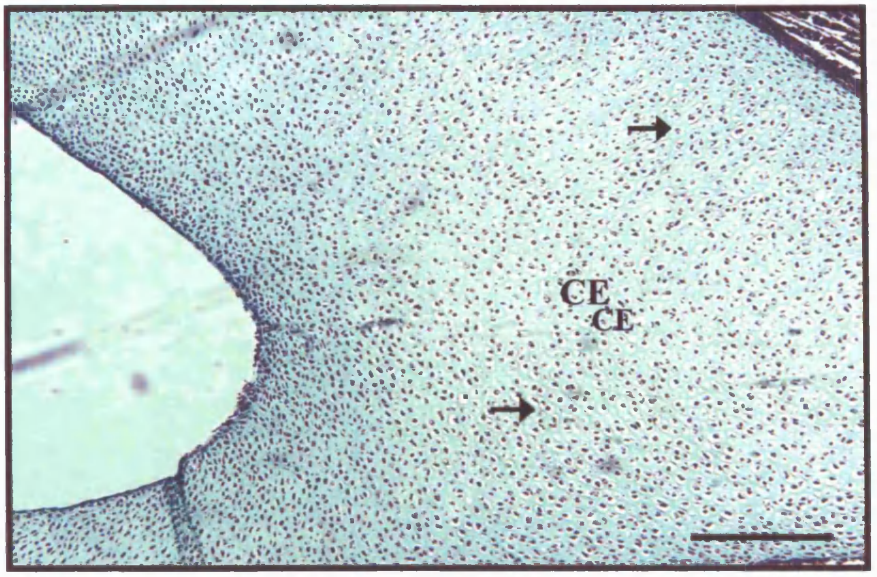


Figure 2.10: A longitudinal section of 17-day old embryonic chick sternum shows the morphology of the hypertrophic chondrocytes in the cephalic region (arrow). The tissue was histologically prepared as described in the text and the paraffin embedded section was stained with Masson's trichrome. Scale bar, 200μM.
(**CE**) Cephalic region

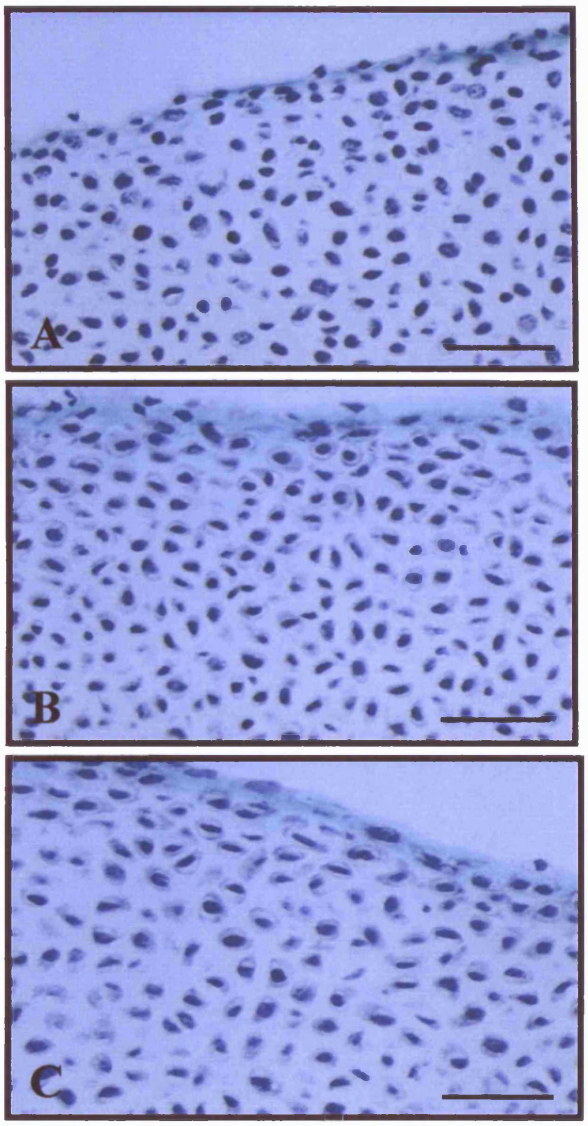


Figure 2.11: Photomicrograph showing the morphologies of cells within the sternum, Masson's trichrome stain. (**A**) Proliferative chondrocytes are round and small. (**B**) Prehypertrophic chondrocytes in the middle region begin to enlarge and become round. (**C**) Hypertrophic chondrocytes in the cephalic region are larger than the other chondrocytes in the caudal and middle regions. Scale bar, 100μM.

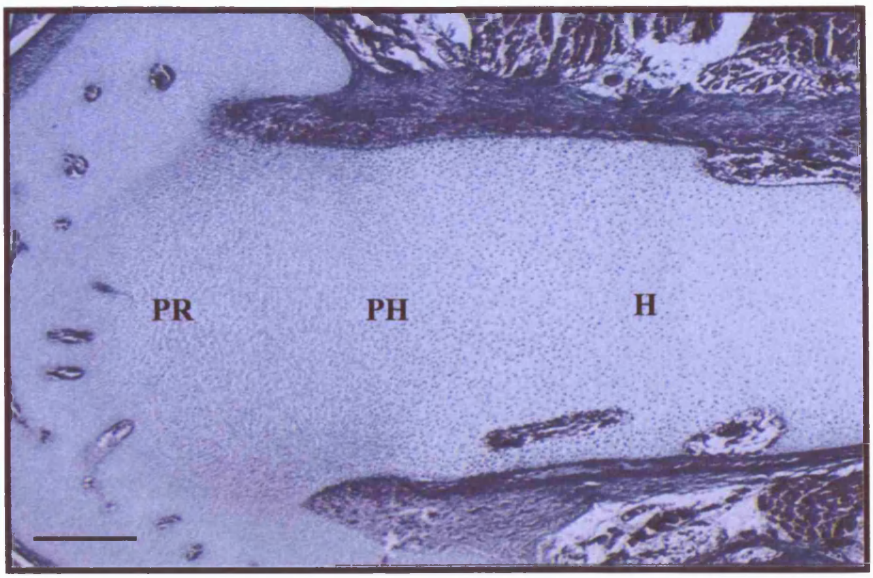


Figure 2.12: A longitudinal section of 17-day old embryonic chick tibia showing the proliferative, prehypertrophic and hypertrophic chondrocytes in their respective zones. The tissue was histologically prepared as described in the text and the paraffin embedded section was stained with Masson's trichrome. Scale bar, 200μM.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

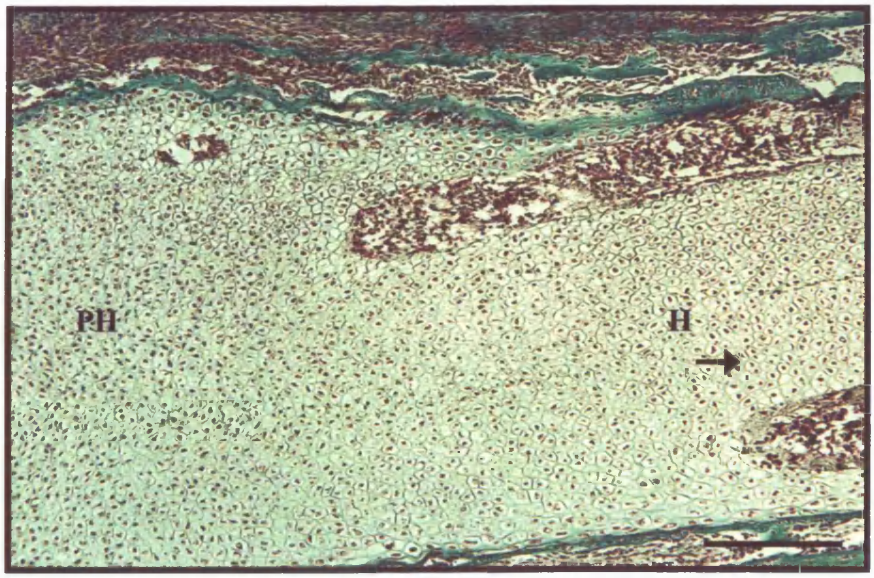


Figure 2.13: A longitudinal section of 17-day old embryonic chick tibia showing the prehypertrophic and hypertrophic chondrocytes (arrow) in their respective zones. The tissue was histologically prepared as described in the text and the paraffin embedded section was stained with Masson's trichrome. Scale bar, 200μM.
(**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

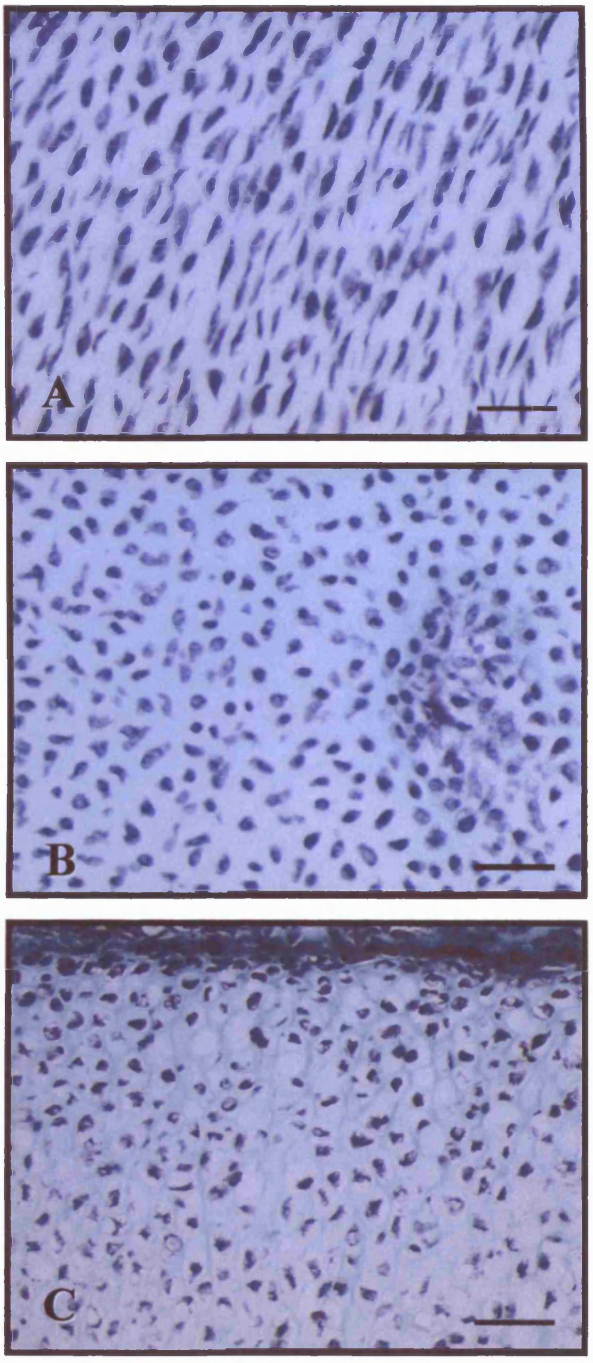


Figure 2.14: Photomicrograph showing the morphologies of cells within the growth plate. Masson's trichrome stain. (**A**) Proliferative chondrocytes are flattened and arranged in columns. (**B**) Prehypertrophic chondrocytes begin to enlarge and become round. (**C**) Hypertrophic chondrocytes are larger than the cells in the other zones. Scale bar 100μM.

2.2.4 *In Situ* hybridisation

2.2.4.1 Type X Collagen

The results of the *in situ* hybridisation with a riboprobe specific to the mRNA of type X collagen show that the gene is expressed by the highly differentiated chondrocytes (hypertrophic) in the normal sternum at different areas of the cephalic region of the 17 day chick sternum (Figure. 2.15). The same observation was detected in the 17-day chick tibia. However, type X collagen is not detected in the proliferative and prehypertrophic chondrocytes in the tibia (Figures. 2.16 & 2.17). The control slides with the sense riboprobe show no positive reaction and this indicates that the expression of type X found in the hypertrophic chondrocytes is indeed specific (Figure. 2.19). Although type X collagen mRNA expression appears to begin in the prehypertrophic zone, higher levels of expression are mainly associated with the cells in the hypertrophic zone. The number of positive cells appears to be decreased distally and in the region of calcified cartilage. The control section that was treated with the hybridisation mixture contained neither anti-sense nor sense showed non-reactive (Figure. 2.20).

2.2.4.2 Indian Hedgehog (Ihh)

As shown in Figure. 2.21, Ihh mRNA is present in the chondrocytes of both the middle and cephalic regions of the sternum. It is noteworthy that the middle region of this tissue is occupied by the prehypertrophic chondrocytes and that the cephalic consists of hypertrophic cells. Furthermore, the intensity of Ihh expression is much higher in the middle than in the cephalic region (Figure. 2.24). The control slides of the sternum and tibia tissues are non-reactive (Figures. 2.22 and 2.23).

In contrast, the in situ hybridisation of the tibia sections indicates that the expression of Ihh is predominant in chondrocytes in the lower region of the prehypertrophic zone (Figure. 2.24) and that the expression becomes less intense in the hypertrophic zone (Figure. 2.25). No reaction is detected in the control sections as shown in Figures. 2.26 and 2.27 and this evidence confirms the specificity of the reaction found in the test sections.

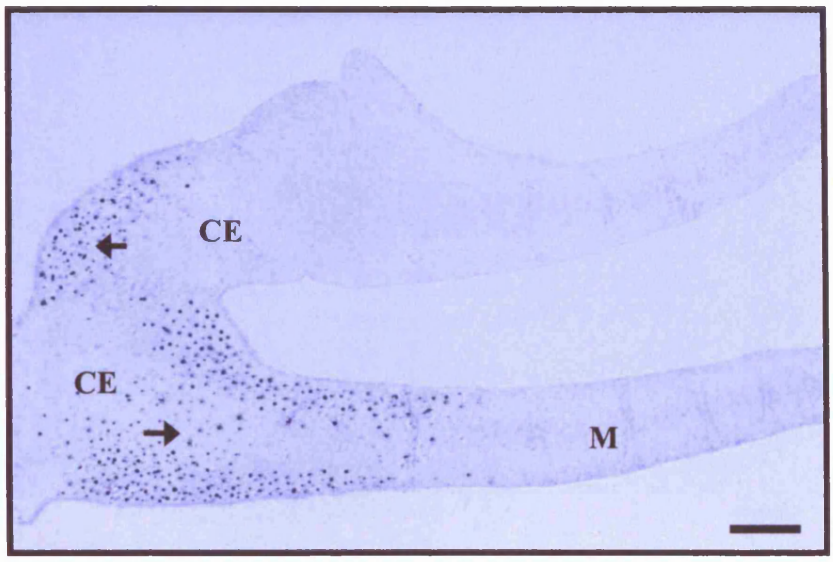


Figure 2.15: *In situ* hybridisation of type X collagen mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled anti-sense riboprobe. Type X collagen is expressed in the hypertrophic chondrocytes in the cephalic region (arrow). Scale bar, 200 μm.
(**M**) Middle region (**CE**) Cephalic region

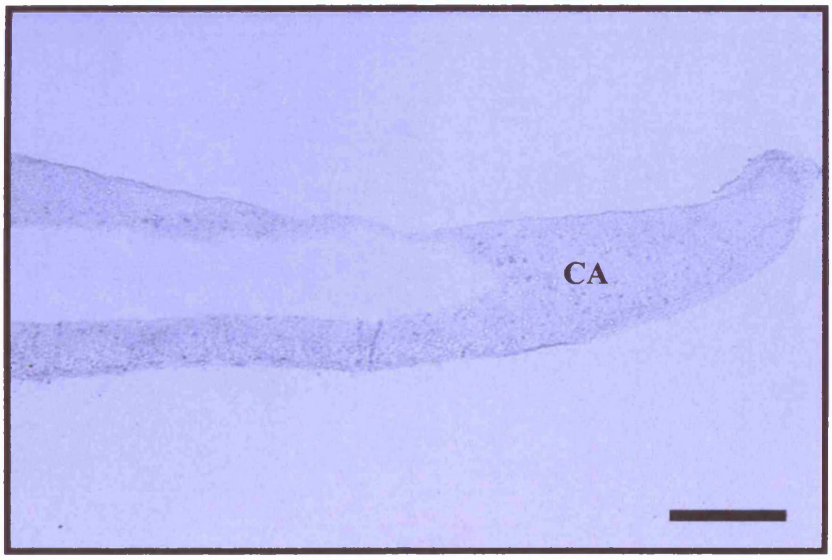


Figure 2.16: *In situ* hybridisation of type X collagen mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled anti-sense riboprobe. The proliferative chondrocytes in the caudal region show no expression of type X collagen. Scale bar, 200 μm.
(**CA**) Caudal region.

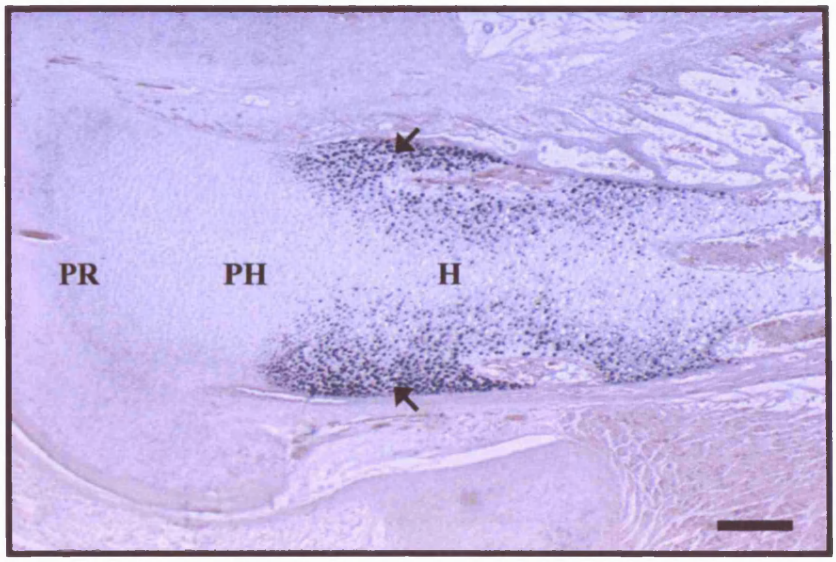


Figure 2.17: *In situ* hybridisation of type X collagen mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled anti-sense riboprobe. The reaction was predominantly observed in the hypertrophic region occupied mainly by chondrocytes (arrow). The expression was obtained using the anti-sense SP6 riboprobe. Scale bar, 200 μm.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

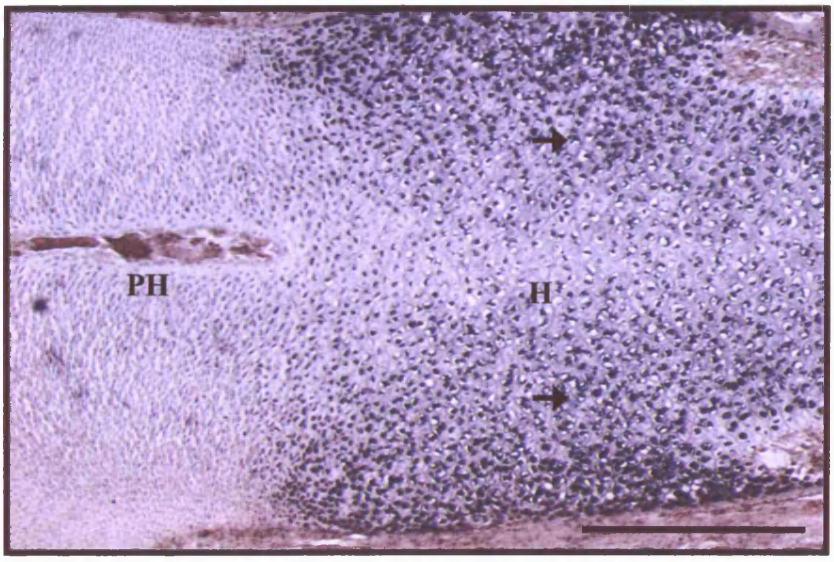


Figure 2.18: *In situ* hybridisation of type X collagen mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled anti-sense riboprobe. A high magnification of the same Figure: 2.17 showing the expression of type X collagen in the hypertrophic region (arrow). Scale bar, 200μm.
(**PH**) Prehypertrophic zone (**H**) Hypertrophic Zone

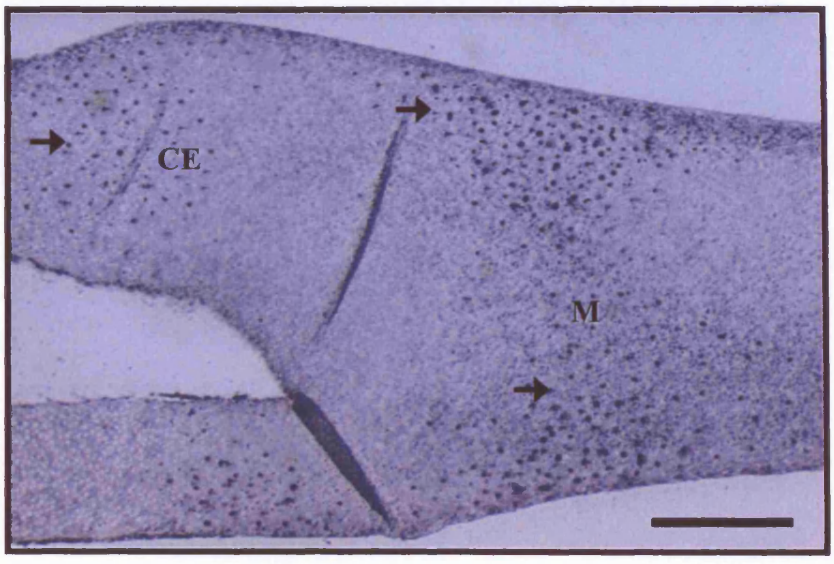


Figure 2.21: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled anti-sense riboprobe. The reaction was mainly observed in the middle region and in the cephalic region (arrow). Scale bar, 200μm.
(**M**) Middle region (**CE**) Cephalic region

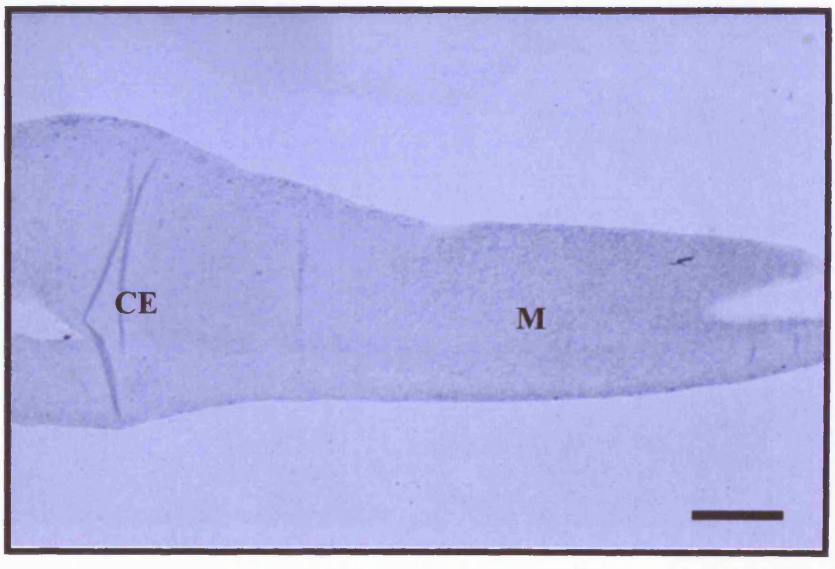


Figure 2.22: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled riboprobe. The section was treated with the sense riboprobe. No reaction of Ihh is observed in any of the regions. Scale bar, 200μm.
(**M**) Middle region (**CE**) Cephalic region

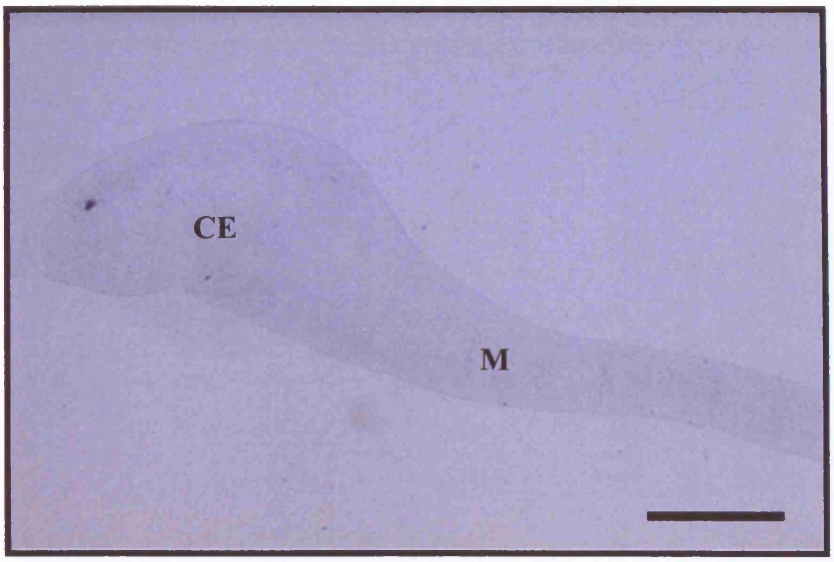


Figure 2.23: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick sternum. Sternum of 17-days embryonic chick was treated with the hybridisation mixture that contained neither anti-sense nor sense riboprobes of Ihh. Scale bar, 200μm.
(**M**) Middle region (**CE**) Cephalic region

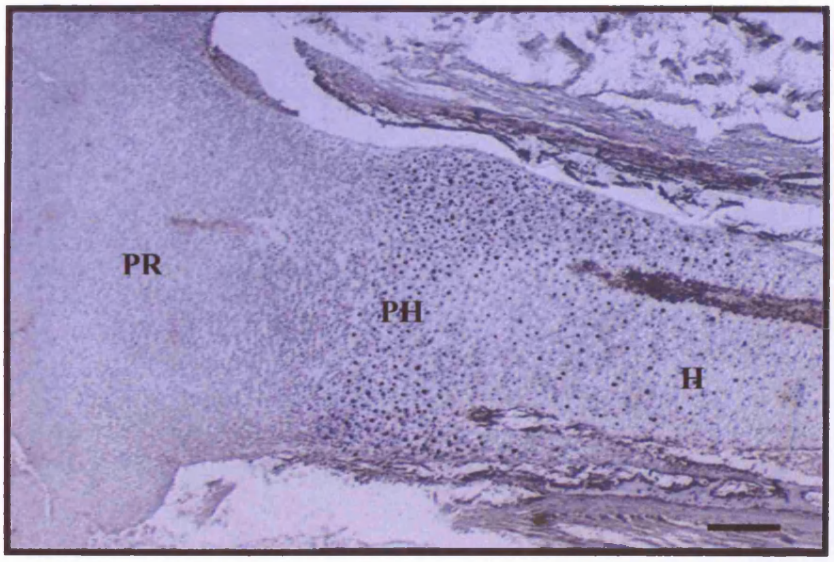


Figure 2.24: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled antisense riboprobe. The reaction was mainly observed in the prehypertrophic region and in certain cells within the hypertrophic region. Scale bar, 200μm.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

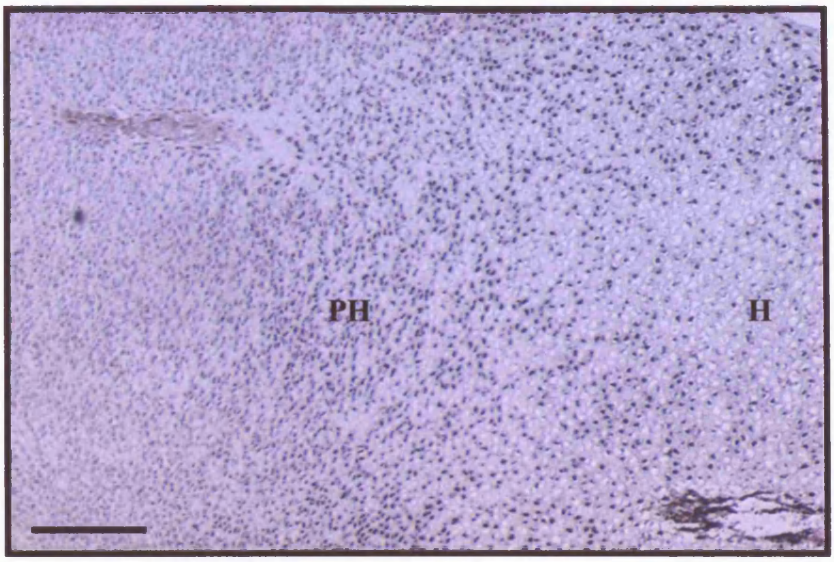


Figure 2.25: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled anti-sense riboprobe. A higher magnification micrograph is showing the expression of Ihh in the prehypertrophic region. Scale bar, 200μm. (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

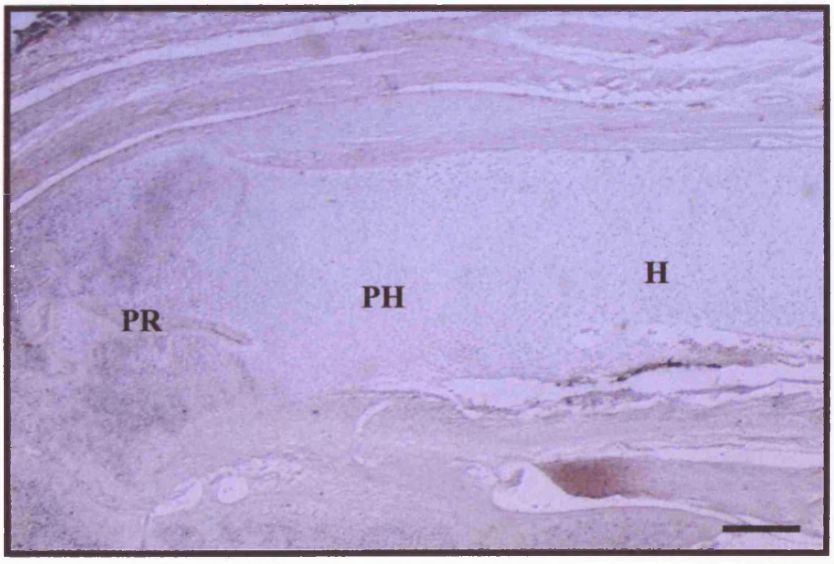


Figure 2.26: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick tibia. The section was treated with the sense riboprobe. No reaction is observed in any of the cartilage zones. Scale bar, 200μm.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

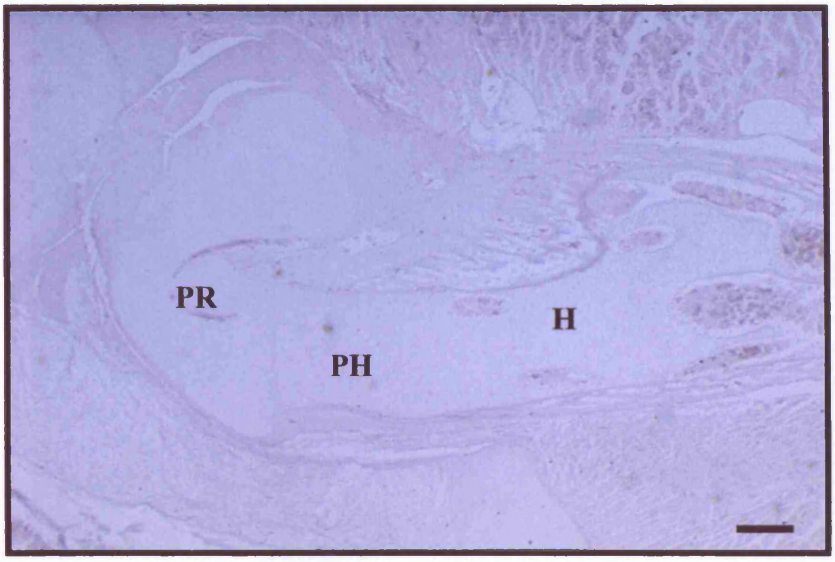


Figure 2.27: *In situ* hybridisation of Ihh mRNA in a longitudinal section of a 17-day embryonic chick tibia of 17-days embryonic chick was treated with the hybridisation mixture that contained neither anti-sense nor sense riboprobes of Ihh. Scale bar, 200μm. (**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

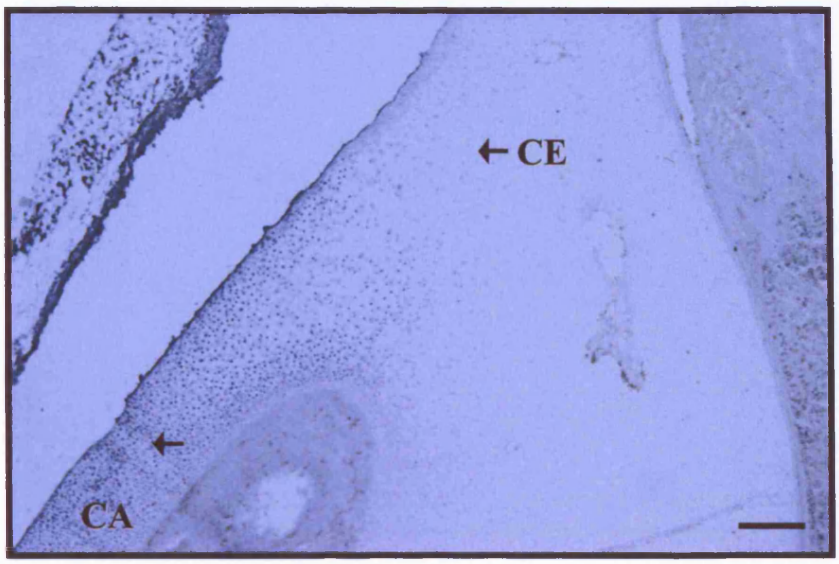


Figure 2.28: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled anti-sense riboprobe. The reaction was intense in the caudal and middle regions and downregulated throughout the cephalic region. Scale bar, 200μm.
(**CA**) Caudal region (**CE**) Cephalic region

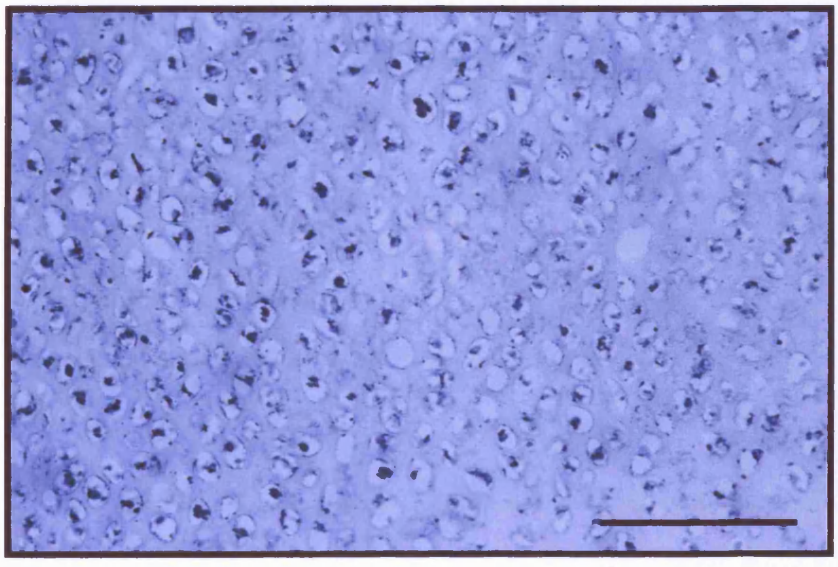


Figure 2.29: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled anti-sense riboprobe. A high magnification of the same reaction shown in Figure 2.28 is showing the expression of SAP in the caudal region of the 17 days chick sternum. Scale bar, 200μM.

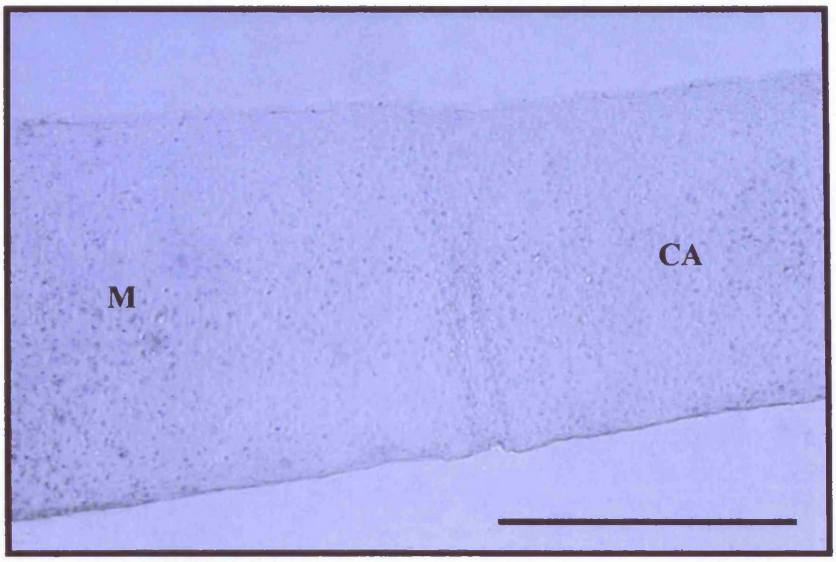


Figure 2.30: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick sternum using Digoxigenin labelled sense riboprobe. The reaction showing no detection of SAP observed in the caudal region and in the middle region. Scale bar, 200μM.
(**CA**) Caudal region. (**M**) Middle region.

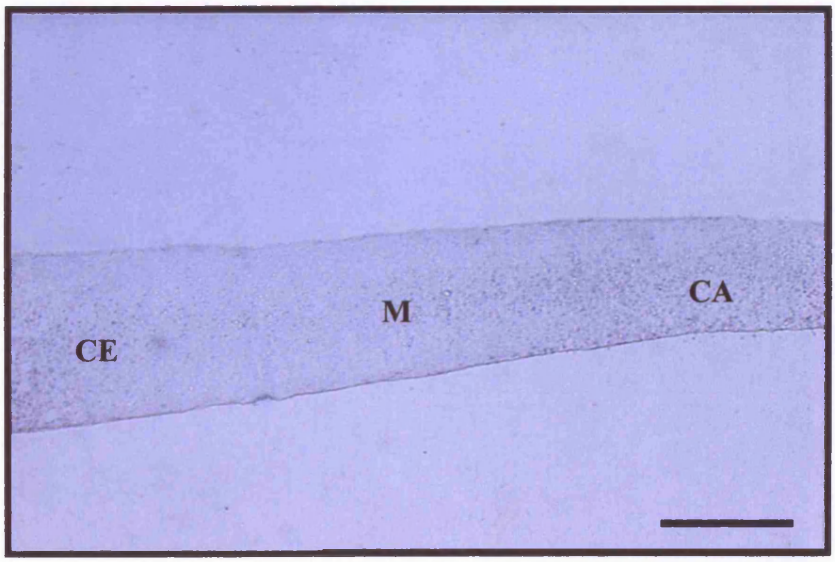


Figure 2.31: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick sternum. Sternum of 17-day embryonic chick was not treated with the hybridisation mixture that contains neither anti-sense nor sense riboprobes of SAP. Scale bar, 200μM.
(**CA**) Caudal region (**M**) Middle region (**CE**) Cephalic region

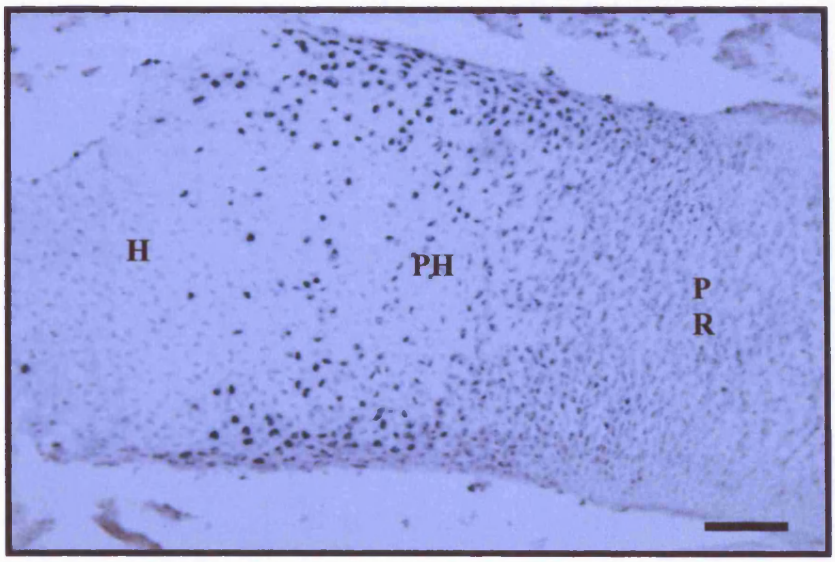


Figure 2.32: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled antisense riboprobe. SAP mRNA was mainly localised in the prehypertrophic region and SAP expression is downregulated throughout the hypertrophic region. Scale bar, 200μM.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

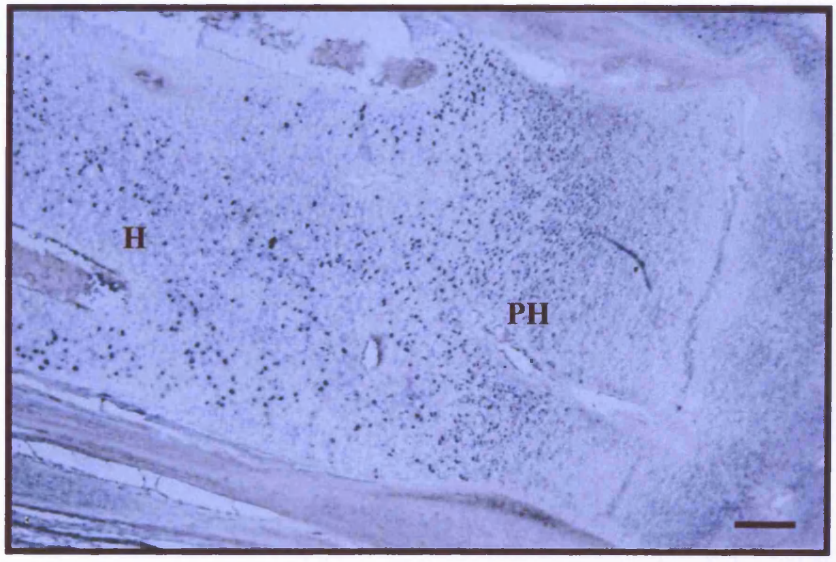


Figure 2.33: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick tibia using Digoxigenin labelled anti-sense riboprobe. The reaction was mainly observed in the prehypertrophic region and in the hypertrophic region.
Scale bar, 200μm.
(**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

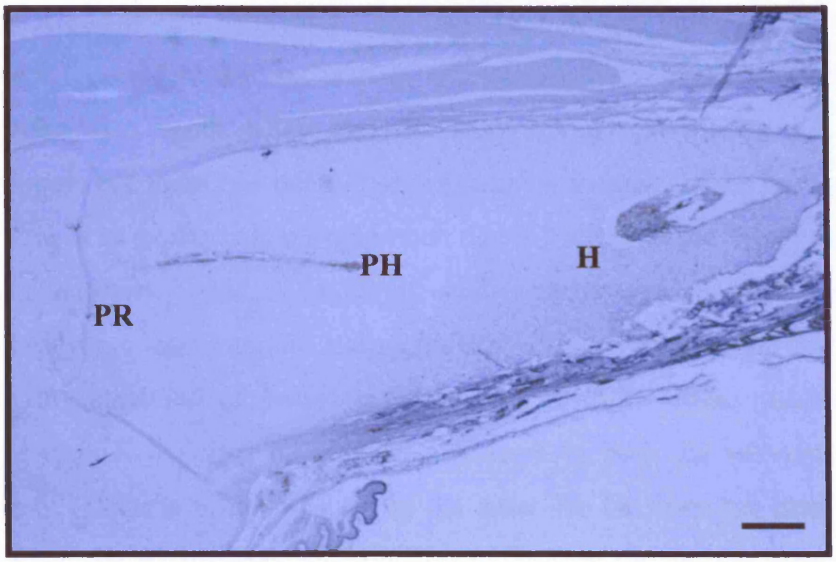


Figure 2.34: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick tibia. Tibia of 17-days embryonic chick was treated with the hybridisation mixture that contained neither anti-sense nor sense riboprobes of SAP. Scale bar, 200μM.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

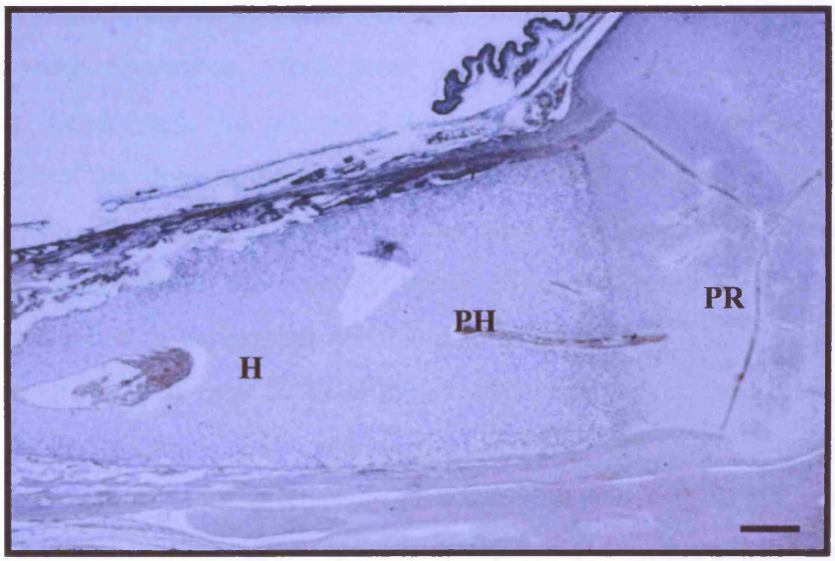


Figure 2.35: *In situ* hybridisation of SAP mRNA in a longitudinal section of a 17-day embryonic chick tibia. Tibia of 17-days embryonic chick treated with the SAP T7 sense riboprobe. No staining is observed in the prehypertrophic and hypertrophic regions. Scale bar, 200μM.
(**PR**) Proliferative zone (**PH**) Prehypertrophic zone (**H**) Hypertrophic zone

2.2.5 Discussion

Collagen type X and Indian hedgehog (Ihh) have been widely studied in recent years since they were reported to be involved in endochondral ossification. A novel gene recently identified as a Small Acidic Protein (SAP) has also been proposed to play a part in this process but there has been little information to substantiate it. The growth plate of the tibia is an embryonic cartilaginous tissue leading to the formation of long bone and has been mostly used in the earlier studies of the endochondral ossification. There is another type of cartilaginous tissue, the embryonic chick sternum that has been used for the investigation of bone development. In most cases, studies on the localisation of type X collagen have been conducted in both the sternum and tibia whereas those of Ihh have been restricted to the latter. So far there has been a lack of information about the localisation and function of SAP in the growth plate cartilage. Moreover, very little is known about the molecular structure of SAP. Earlier, Gong *et al* (1997) identified an avian cDNA encoding a small acidic protein of unknown functions in the quail embryo during development of the ear, using a differential display method.

My aim in this part of my PhD study is to localise type X, Ihh and SAP mRNA in the 17 days old embryonic chick using an *In situ* hybridisation technique with Digoxigenin labelled anti-sense riboprobes. Since there had been attempts made previously to localise Ihh in the sternum, this prompted the present work to include the sternum as well as the tibia of the chick in the investigation to establish the cellular localisation of these three genes. The results confirm that the type X collagen gene is confined to hypertrophic chondrocytes in the cephalic region of normal sternum (Figure. 2.15). In the tibia, higher levels of expression are found to be associated with the hypertrophic zone but low intensity of type X labelling is also observed in the prehypertrophic chondrocyte zone (Figures. 2.17 and 2.18). Unlike the type X gene, Ihh is predominantly expressed in the prehypertrophic chondrocytes of the middle region (M) sternum although some light staining also takes place in the hypertrophic chondrocytes of the cephalic region (CE) of the tissue (Figure. 2.21). In the tibia, the high intensity of expression of Ihh is detected in the lower part of the prehypertrophic zone (PH). The Ihh expression continues throughout this transitional zone where the majority of cells are stained strongly but the reactivity becomes less pronounced throughout the hypertrophic

zone (H) of the same section. With regard to the SAP mRNA, its expression is intense in the caudal and middle regions of the sternum but its labelling intensity is reduced in the cephalic region (Figure. 2.28). A high magnification micrograph shows that the expression of SAP is localised in the cells which have the characteristic features of the prehypertrophic chondrocytes (Figure. 2.29). In the tibia, the same pattern of SAP expression is also observed. The chondrocytes in the prehypertrophic zone are found to be highly expressed SAP, and downregulated throughout the hypertrophic zone (Figures. 2.32 and 2.33).

Most of the earlier studies have found that type X mRNA is expressed specifically by hypertrophic chondrocytes during bone formation (Jacenko *et al.,* 2001)This observation seems to support the view of Thomas *et al* (1991) that type X collagen is one of the best characterized and unique macromolecules which help define the hypertrophic stage of chondrocyte phenotype. This long established view is further strengthened by the results obtained from the present work as well as by those from the earlier studies. The expression of type X collagen has been considered as a characteristic feature of hypertrophic chondrocytes and the transcriptional regulation of this gene has been thought to be dependent on cellular hypertrophy (Chung *et al.,* 1995). Salo *et al* (1996) have successfully localised type X collagen by *in situ* hybridisation and immunohistochemistry methods in mandibular condyle of rats. Their results have shown that type X collagen is expressed by hypertrophic chondrocytes of condyles cartilage. *In situ* hybridisation of new-born rat condylar and angular cartilages undergoing endochondral ossification showed restricted labelling with the type X collagen probe in the hypertrophic chondrocyte layer.

As shown by the present study, the high level of Ihh expression only occurs in the prehypertrophic cells in both sternum and tibia. The results are consistent with the reported observation that the prehypertrophic chondrocytes are responsible for Ihh synthesis (Vortkamp, 1996). The detection of high intensity of Ihh expression in the prehypertrophic cells by the present investigation adds further evidence to support the findings of several previous studies. Indian hedgehog (Ihh) has been reported to be in the developing skeletal elements and in particular in the prehypertrophic chondrocytes

of the growth plate (Bitgood and McMahon, 1995; Koyama *et al.,* 1996; Colnot *et al.,* 2005). Indian hedgehog was expressed not only in cartilaginous growth plate during limb development but also during fracture healing in bone callus. In mice, Ihh has also been shown to be expressed widely within the cartilage of the long bone. However, by birth, it was localised in a zone of postmitotic, prehypertrophic chondrocytes, immediately adjacent to the zone of proliferating chondrocytes (Bitgood and McMahon, 1995; Lanske *et al.,* 1996; St-Jacques *et al.,* 1999; Seth *et al.,* 2000). By contrast, Gritli-Linde *et al* (2001); Yin *et al* (2002) found Ihh in both, proliferative and prehypertrophic zones. On the other hand, Kindblom *et al* (2002) detected Ihh immunoreactivity only in early hypertrophic chondrocytes, but not in either resting or proliferative chondrocytes. Indian hedgehog was primarily expressed by pre and early hypertrophic chondrocytes, and it also signalled to both immature chondrocytes and overlying perichondrial cells (St-Jacques *et al.,* 1999; Vortkamp *et al.,* 1996).

Despite of the wealth of information about the molecular structure and cellular localisation of type X collagen, the precise function of this protein and its role in endochondral ossification still remain unresolved. However, it has been suggested that type X collagen may provide an open temporary matrix permissive to vascular invasion and mineralisation. Previous study has demonstrated that type X collagen is important for compartmentalization of matrix components in the hypertrophic zone of growth plate, providing the proper environment for mineralization and modelling (Barber and Kwan, 1996). Also, type X collagen may function as a scaffold preventing collapse of the matrix, since the proteoglycans and collagen II are degraded in the hypertrophic region of the growth plate (Sutmuller *et al.,* 1997).

On the other hand, the localisation of Ihh in the sternum and tibia sections obtained from the present investigation together with those of the earlier studies support the observation that Ihh is involved in the early stage of chondrocyte differentiation. The reduced staining intensity of Ihh in the hypertrophic zone may be attributed to a process that either down-regulates the expression or inhibit the synthesis of Ihh or both. Many studies have in fact suggested that Ihh plays two important roles in chondrocyte proliferation, a direct stimulator and a regulator of this process. It is thought that first of

all, through its receptor (Patched), Ihh stimulates perichondrial cells to produce PTHrP. Then Ihh co-ordinates with the PTHrP to regulate the rate of chondrocyte proliferation, and the timing for cell differentiation (Long *et al.,* 2001; Karp *et al.,* 2000; St-Jacques *et al.,* 1999; Lanske *et al.,* 1998; Vortkamp *et al.,* 1996).

Earlier studies have also found that the Ihh/PTHrP interaction prevents the cells from reaching maturation. One of the relevant studies was made by Vortkamp *et al* (1999). They remarked that the ectopic expression of Ihh in the chick limb upregulated PTHrP gene expression in the perichondrium whereas the maturation and hypertrophy of chondrocytes in this region were inhibited. These observations have led to the proposal that the role of Ihh in the skeletogenesis is to serve as an inhibitor of chondrocyte maturation via PTHrP. Moreover, some studies have indicated that Ihh and its two putative downstream target genes, Patched (Ptc) (Vortkamp *et al.,* 1998) and Gli (Marigo *et al.,* 1996; Goodrich *et al.,* 1996) are expressed in the precartilaginous condensation of early limb. These findings further suggest that Ihh may recruit the prechondrogenic mesenchymal cells and stimulate their cytodifferentiation at very early stages of skeletogenesis.

The effect of Ihh on chondrocyte proliferation is clearly demonstrated in the study made by Long *et al* (2001). They observed that in the growth plate of Ihh-null mouse embryos, the zone of chondrocyte proliferation preceding the prehypertrophic zone was considerably reduced. They also found that chondrocyte proliferation was less intense in the mouse embryos where the Ihh receptor was conditionally deleted in cartilage (Long *et al.,* 2001). These observations support the view that Ihh is an important regulator of chondrocyte proliferation in the growth plate. As suggested by several workers in later years, Ihh and its putative genes have multiple roles one of which is that they exert influences on the proliferative zone as well as surrounding perichondrial tissues (Chung *et al.,* 2000; Donklelaar and Huiskes, 2006). Studies in both chicken and mouse have established that Ihh controls the onset of chondrocyte hypertrophy primarily via PTHrP (Karp *et al.,* 2000; Lanske *et al.,* 1996). The Ihh knockout studies also reveal that Ihh is a potent positive regulator of chondrocyte proliferation (St-Jacques *et al.,* 1999; Long *et al.,* 2004).

Most studies seem to agree that Ihh is exclusively produced in the prehypertrophic zone. One intriguing thought is that Ihh appears to be able to undergo long-range diffusion from its site of synthesis to reach target cells in other zones and surrounding tissues. During bone elongation, terminal differentiation of growth plate chondrocytes is an important prerequisite for the normal development and repair of the skeleton. Many transcription and soluble growth factors are involved in chondrocyte differentiation and these include insulin-like growth factor-1(Darling and Athanasiou, 2005), transforming growth factor β (Thorp *et al.,* 1992), fibroblast growth factor (Deng *et al.,* 1996; Peters *et al.,* 1993), Ihh (Vortkamp *et al.,* 1996) and PTHrP (Amizuka *et al.,* 1995; Farquharson *et al.,* 2001). Such diversity of controlling molecules suggests that chondrocyte proliferation and differentiation are likely to be mediated through a number of interacting control loops.

Interestingly, there are recent reports that the Ihh/PTHrP interaction takes place in a negative feedback fashion. Some recent studies have found that PTHrP indirectly inhibit Ihh expression. Farquharson *et al* (2001) remarked that in the postembryonic growth plate, post-proliferative chondrocytes secreted Ihh which increased secretion of PTHrP to slowdown hypertrophy and that consequently; the slowing down of hypertrophy will reduce the production of cells that secreted Ihh. The presence of a functional Ihh/PTHrP negative feedback loop has also been reported elsewhere (Yoshida *et al.,* 2001; Kindblom *et al.,* 2002; Karp *et al.,* 2000; Vortkamp, 1999; St-Jacques *et al.,* 1999). One further example of the Ihh/PTHrP negative feedback interaction is that in fetal bone, Ihh stimulates the periarticular cartilage to express PTHrP, which then acts on the prehypertrophic chondrocytes to inhibit cell differentiation (Kindblom *et al.,* 2002; Vortkamp, 1999). As pointed out by Wu *et al* (2001), Ihh is a key molecule that regulates chondrocyte proliferation and differentiation during endochondral bone formation. They found that when Ihh was synthesized nearer to the articular end of the growth plate, PTHrP synthesis was also increased. In this way, Ihh signalled the relative position of prehypertrophic and hypertrophic chondrocytes to the periarticular growth plate. They further explained that PTHrP delayed the transition from proliferation to hypertrophy of growth plate chondrocytes. Through PTHrP, Ihh may control the site at which Ihh also determines the site of formation of bone collar. Chung *et al* (2001) have

suggested that the source of Ihh protein for this signalling might be the Ihh coming from both the prehypertrophic and hypertrophic chondrocytes.

As far as the present work is concerned, the most important observation is that SAP is expressed in the prehypertrophic rather than hypertrophic cells of the embryonic chick. These results may suggest that the protein is involved in the early stages of endochondral ossification, hence the development of the growth plate cartilage. These findings also indicate that the expression pattern of SAP is similar to that of Ihh. Furthermore, the present study confirms the observations made by Webster (1999) that SAP expression is developmentally regulated and differentially expressed during cell hypertrophy. Intense expression of SAP is found to be restricted to the prehypertrophic zone where most cells undergo early differentiation. Although little labelling of SAP is occasionally found in the proliferative zone, the cells that reach full maturation in the hypertrophic zone are completely devoid of SAP mRNA expression. These observations suggest that expression of the SAP gene is specific to the prehypertrophic chondrocytes. Based on these results, SAP may have an important role to play in the chondrocyte differentiation in the cartilage template.

It is generally acknowledged that development of the growth plate involves a genetic cascade, that is, a programmed series of sequential gene activation and inactivation. Previous studies on vertebrate skeleton have also confirmed that genetic analysis of embryonic mutations can identify genes that are involved in the early events of development, for example, axis specification, segmentation, and pattern formation. Many of these genes are transiently expressed during embryonic development and encode transcription factors containing either homeodomains or paired domains. These transcription factors regulate the expression of many as yet unidentified target genes. SAP has a predominantly acidic part which is concentrated within the carboxy domain of the predicted peptide. This implies that SAP may have a protein-binding domain. However, the work of Webster (1999) has shown a high probability that the protein resides within the nucleus. If indeed this is a nuclear protein, it may function as a transcriptional regulator that is able to bind other proteins and up regulate transcription of one or more genes. In recent years, gene expression studies have revealed that the

developing cells have unique patterns of gene expression that restrict some genes and their protein products to specific cells at critical period in the development process. In the context of the present study, cell differentiation may be triggered by molecular signals as a result of transcription activation in chondrocytes. SAP may either regulate directly or act indirectly as a transcription factor regulating the gene(s) that causes the immature cells to undergo differentiation. As its expression is upregulated only in the prehypertrophic and absent in the hypertrophic zone, this indicates that the protein may be a transcription activator involved in the activation of one of those genes that regulate the cell maturation. One can also argue that the gene expressed in the chondrocytes presumably activates the expression of additional target genes required for the regeneration or even repair of the damage if any, hence maintaining the continuity of the growth and development of the cartilage. Molecular analysis of several organ systems has shown that genes that are expressed during development are re-expressed during regeneration. It has been proved that such regeneration not only repopulates the immature cells but also restores its functions. As a wealth of literature has indicated, when the regulatory factors are deficient, the normal growth and development of the cartilage are adversely affected, resulting in skeletal disorders.

The assumed involvement of SAP in cell differentiation indicates that this protein could be one of the important proteins that play a significant role in the maintenance of the normal endochondral ossification. One interesting thought is that by analogy, SAP has shown a similar pattern expression as that of Ihh but not so with Type X collagen. In short, both SAP and Ihh expression only occurs in the prehypertrophic cells whereas type X is condensed in the hypertrophic zone. Ihh plays two important roles, a direct stimulator and a regulator of cell differentiation process. Furthermore, Ihh interacts with PTHrP to achieve these functions. It remains to be seen however whether SAP has combined roles with Ihh/PTHrP.

CHAPTER 3

Studies of the regulatory gene expression and biochemical analyses of extracellular matrix production in chondrocyte micromass culture

3 Introduction

As shown in the previous experiments using *in situ* hybridisation method (Chapter 2), the novel gene known as small acidic protein (SAP) has been successfully localised in the sternum and tibia of the embryonic chick.

The *in situ* hybridisation results have confirmed that the prehypertrophic chondrocytes in the sternum and tibia expressed Indian hedgehog (Ihh). Earlier studies using mouse tibial growth plate have indicated that both Ihh and PTHrP were detected in the prehypertrophic chondrocytes and that they were found to interact with each other in a negative feedback loop (Vortkamp 1996; 1999). On the basis of these observations, it is interesting to find out (1) the expression of SAP in regulation to these of other genes known to be involved in endochondral ossification. (2) If SAP regulates the transcription key genes in endochondral ossification such as Ihh or PTHrP or type X collagen and (3) whether SAP is linked to the Ihh/PTHrP feedback control mechanism.

The aim of the present study is to ascertain if SAP plays a role in the differentiation of chondrocytes. Since SAP and Ihh cohabit in the prehypertrophic chondrocytes, it is interesting to see whether the non expression of SAP results in the up regulation or down regulation of Ihh. Earlier studies have used cyclopamine, a plant steroidal alkaloid, to disrupt cholesterol synthesis (Goodrich *et al.,* 1998). Furthermore, cyclopamine is known to have the ability to inhibit cellular response to Hh signalling by blocking its pathway (Chen *et al.,* 2002). The steroidal nature of cyclopamine and its ability to disrupt cellular response are thought to affect the signalling of Patched, which contains an apparent sterol-sensing domain (Goodrich *et al.,* 1998). Based on this information, the present study used RT-PCR method to determine the effect of cyclopamine on the expression of SAP, Ihh, PTHrP, Patched and type X collagen, all of which a are known to regulate the differentiation of chondrocytes. Its objective is to ascertain (1) whether cyclopamine has an inhibitory effect on the expression of these genes, and (2) whether the inhibition of SAP leads to either down regulation or up regulation of the expression of other genes of interest. Three phenotypes of chondrocytes were included in the study (proliferative, prehypertrophic and

hypertrophy); these were isolated from their respective regions (caudal, middle and cephalic) of the sternum and grown in cultures with or without cyclopamine.

In addition to the gene expression analysis by RT-PCR, the present study also examined the effect of cyclopamine on the production of sulphated glycosaminoglycans (GAGs) and collagen (hydroxyproline assay). The amount of GAGs produced was determined using Dimethylmethlene blue (DMMB) whereas that of hydroxyproline was measured according to the biochemical assay as described in the 'Materials and Methods'.

3.1 Biochemical Analysis of Micromass Culture of Embryonic Chick Chondrocytes

3.1.1 Preparation of Micromass culture

Micromass cultures of 17-day embryonic chick sternal chondrocytes were established as described by Daniels *et al* (1996). 17-day chick sterna were dissected free from the chick bodies and collected in a small Petri dish supplemented with DMEM 10 % FCS medium. The samples were washed three times in DMEM serum free medium. Three regions (caudal, middle and cephalic) were identified and cut out from the sterna. The tissues were individually placed in a separate universal tube containing 5ml of freshly made collagenase and trypsin. The tubes were incubated at 37°C for 1.5 to 2 hours. The contents of each tube were filtered through a 40μm Strainer filter. The flow-through cell suspension was collected in sterilized 15ml tubes which were subsequently centrifuged at 1800 rpm for 5 minutes to pellet the cells. The supernatant was carefully removed and discarded. The resulting cell pellet was resuspended in 5ml of DMEM 10 % FCS medium and then re-centrifuged. The same procedure was repeated three times to ensure complete removal of enzymes. The cell pellet was then resuspended in 5ml of DMEM-Serum free medium and the number of cells counted using a hemacytometer slide. The cell concentration was adjusted to 2×10^7cells/ml (2×10^5 cells/10μl) with DMEM 10 % FCS medium.

The tubes containing the cells were then inverted a number of times to obtain a good homogenous mixture. Aliquots (10μl) of the cell suspension were pipetted out from the tubes and dispensed carefully and slowly in dot form (2×10^5 cells) onto the centre of a culture dish. Care was taken not to introduce air bubbles into the dots, as these could reduce the interaction and the biological contact between cells and the extracellular matrix which might be formed between the chondrocytes in each single dot. After incubating for 6 hours at 37°C in a humidified atmosphere of 5% CO_2, 50 % of the dishes were flooded slowly with 3ml of pre-warmed DMEM 10% FCS from the edge. The other 50 % of dishes were flooded with pre-warmed DMEM 10% FCS containing cyclopamine at a final concentration of 10μg/ml, and incubated as described above. The medium was replaced every day with fresh DMEM 10%FCS medium for normal

culture and DMEM 10% FCS containing Cyclopamine for the treated culture throughout the experiment period (8 days).

For the biochemistry assays the micromass dots of the three different phenotypes of chondrocytes were removed from the surface of the dishes without digestion and collected separately in eppendorf tubes for the assays. Whereas, the micromasses of all three types of chondrocytes (proliferative, prehypertrophic and hypertrophic) which were used for the RT-PCR, were harvested and digested every 24 hour. The total RNA of each different population of chondrocytes was isolated using RNeasy Mini Kit (Qiagen).

3.1.2 Analysis of Sulphated Glycosaminoglycans by DMMB Assay

The Dimethylmethlene blue (DMMB) assay was used to measure the production of sulphated GAGs (i.e chondroitin-4-sulphate) in both micromass dots and that released into the culture media. Dimethylmethlene blue is a metachromatic dye used originally in histochemical detection of sulphated GAGs and then it was developed to be used as an analytical assay. In this assay a reduction of sample volumes was used and analysis was carried out on a 96 well plate.

3.1.2.1 Preparation of Dimethylmethlene Blue (DMMB) Reagent

The GAG measurement procedure was carried out using the methods as described by Little *et al.*, (1999). Dimethylmethlene blue (32mg) was dissolved in 20ml of ethanol at room temperature overnight with stirring. The dissolved DMMB was added to a solution containing 1500 ml of double distilled H_2O. NaOH (1M) (56 ml) and formic acid (98%) (7ml) were added to the DMMB solution, which was then made up to 2 liters with ddH_2O. The solution was stirred for 2 hours. Aliquot (1ml) was taken from the solution and its absorbance was measured at 525 nm wavelength. DMMB binds to the sulphated groups of GAGs to form a chromophore which appears pink. This pink coloration is readily absorbed at the 525nm wavelength. The DMMB dye was stored in a lightproof bottle at room temperature.

3.1.2.2 Digestion of Micromass

The suspensions of the three different phenotypes of chondrocyte were cultured separately as 10 µl dots in 60mm Petri dishes and 15 dots of micromass were cultured in each dish. The micromass culture was incubated at 37°C and at 5% air CO_2 for 6 hours and then 3ml of DMEM 10 % FCS medium were added gently to the culture. The micromass culture was incubated for 8 days and one Petri dish of each phenotype was collected daily and used for the DMMB assay. The medium of the three different phenotypes (Caudal, Middle and Cephalic) were collected separately and stored at-20°C for the assay whereas the micromass dots were washed twice with PBS. The micromass was carefully removed and put in a sterile eppendorf tube. The tubes were immersed in liquid nitrogen and stored at -80°C for DMMB analysis. The frozen micromass dots were digested using the non-specific proteinase papain. Dots were dissolved in 1ml digestion buffer (20mM sodium phosphate pH6.8, 1mM EDTA and 2mM DTT) containing 300µg papain. Samples were incubated at 60°C for 60 minutes and any partially digested samples were incubated for longer. Iodoacetamide was added to give a final concentration of 10mM to alkylate the reactive sulphydryl groups generated through DTT treatment. Samples were further diluted 3-5 times with 50mM Tris/HCl (pH 8.0).

3.1.2.3 Measurement of GAG Content

The standards and samples (40µl) were pipetted gently in triplicate into a 96- well plate and then 200µl of DMMB dye added into each well and mixed gently by pipetting up and down three times. The plate was read immediately using a plate reader at a wavelength of 525 nm; further dilution was required with some of the samples that were outside the standard curve limit. These samples were diluted in their respective buffer. A serial dilution was prepared using whale chondroitin-4-sulphate with standards ranging from 0 to 70µg/ml. All standards were made up using the same buffer as real samples (i.e for conditioned media-DMEM and for micromass extract- dilution buffer).

3.1.3 Analysis of Hydroxyproline Production in the Micromass Culture

This method was used to measure the hydroxyproline content produced in the micromass culture samples.

3.1.3.1 Preparation of Hydroxyproline Reagents

The hydroxyproline measurement procedure was carried out using the method as describes by Woessner (1976).

1) Concentrated HCl.
2) Stock Buffer (500ml) 28.5g sodium acetate trihydrate
   18.75g tri sodium citrate dehydrate
   2.75g Citric acid
   200ml Propan-2-ol

   These chemicals were dissolved in 250ml of ddH_2O first followed by the propan-2-ol, and then it was made up to 500ml with ddH_2O.

3) Diluent (100ml) 100ml propan-2-ol
   50ml ddH_2O

4) Oxidant 0.7g chloramine T
   10 ml ddH_2O
   50ml stock buffer

5) Colour reagent 7.5g dimethylamino benzaldehyde
   11.25ml perchloric acid (60%)

   Hydroxyproline standards 1-10μg/ml in ddH_2O. This can be prepared in bulk and stored aliquots at -20°C.

Reagents 1, 2 and 3 were prepared in advance and stored at room temperature. Reagents 4 and 5 used to prepare prior to use and were stored at 4°C overnight.

3.1.3.2 Digestion of Micromass Dots

The micromass dots that were collected daily from the culture were hydrolysed in 400µl of concentrated HCl with the same volume of ddH_2O for 24 hours giving a final concentration of 6N HCl at 110°C. The samples were dried using a vacuum centrifuge at full speed overnight to remove all the HCl from the samples. Hydrolysed samples were resuspended in 400µl of ddH_2O (using the same volume of HCL that was used) and were used immediately for the hydroxyproline assay.

3.1.3.3 Measurement of Hydroxyproline Content

The standards and samples (30µl) were pipetted gently in triplicate into a 96- well plate and then 70µl of diluent were added into the wells and mixed gently three times. 50µl of oxidant was added into the wells and mixed gently. The 96 well plate was then mixed on a plate shaker at room temperature for 5-10 minutes. Colour reagent (125µl) was added into the wells and the plate shaken for 5 minutes. The plate was then incubated in a water bath at 70°C for 10-30 minutes until samples went a peach colour. The plate was read immediately using a plate reader (Labsystem Multiscan MS Spectrophotometer) at a wavelength of 540nm. Further dilution was required with some of the samples that gave readings outside the limits of the standard curve 1-10 µg/ml. These samples were diluted in ddH_2O. The 96-well plate was read immediately after the incubation at 70°C. All standards were made up using ddH_2O as the real samples

3.1.4 Analysis of Proteins Content in the Micromass Culture

The protein content of samples was measured using Bio-Rad protein assay. Samples were collected every 24 hours for 8 days. In this analysis the micromass dots were washed three times in PBS and removed carefully by scraping the dots from the surface of the dish culture. The micromass dots of different cell phenotypes were collected separately in eppendorf tubes. The samples were stored at -80°C until they were used for this assay after 8 days.

3.1.4.1 Preparation of Protein Assay Reagents

One part of the Bio-Rad protein assay dye was diluted with 4 parts of ddH_2O. The diluted dye was filtered through a Whatman (110mm) filter paper.

3.1.4.2 Dissolving of Micromass Dots

The micromass samples were thawed on the bench top for 10 minutes at room temperature. Fifty µl of the dissolving buffer (50 mM NaH_2PO_4, 300 mM NaCl and 0.5% Titron X-100, pH 8.0) were added to each sample. The samples were vortexed for one minute and incubated in a water bath at 37°C for 30-60 minutes. The samples were vortexed three times during the incubation until the content of each sample was clear and had become homogenous. One part of the concentrated sample was diluted with 4 parts of the dissolving buffer. The diluted samples were then used for the protein assay. A serial dilution was prepared using 50mM NaH_2PO_4, 300mM NaCl, and 0.5 % Triton X-100 stock, with BSA standards ranging from 0.05mg/ml to 7mg/ml. All standards were made up using ddH_2O.

3.1.4.3 Measurement of Protein Content in the Micromass Culture

The standard and sample solution (10µl) was pipetted separately into 96-well plate. The diluted dye reagent (200µl) was added to each well. The samples and reagent were mixed thoroughly by a multi-channel pipette. The plate was incubated at room temperature for 10 minutes and then the plate was read using a plate reader at a wavelength of 595nm.

3.1.5 Statistical Analysis

Data of three replicate samples from each type of tissues (caudal, middle and cephalic) for each group of treatment (cyclopamine treated and non-treated) were statistically analysed using paired tests in MINITAB. This computerised software allows test for normality to be carried out using the Anderson-Darling test as described by Fry, (1993). The parametric Paired t test was used to determine if the mean difference, treated and non-treated groups, is significantly different from zero (Fry, 1993).

3.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.2.1 Isolation of RNA from Micromass Culture

Micromass dots were digested in collagenase / trypsin for 1 hour. The cells were pipetted into 15ml tubes and were centrifuged at 1800xg for 5 minutes, the cell pellet was resuspended in 5ml of DMEM-10 % FCS medium and the tubes were centrifuged at the same speed for another 5 minutes. This process was then repeated three times. The three different types of chondrocytes were then counted with a haemocytometer slide. Using the manufacture's recommended protocol (Qiagen) the number of cells used for RNA isolation was (3-4x 10^6). The pellets were lysated in 350µl of RLT buffer and vortexed for 30 seconds. The lysate was pipetted directly onto a QIA shredder spin column placed in a 2ml collection tube and the columns were centrifuged for 2 minutes at maximum speed. One volume of 70 % (v/v) ethanol was added to the homogenized lysate and mixed well by pipetting. The lysate (700µl) was then applied to a RNeasy mini column placed in a 2 ml collection tube. All tubes were closed gently and were centrifuged in microcentrifuge for 15 seconds at 8000xg (≥10,000rpm) and the flow-through was discarded.

During this procedure, DNase digestion was performed by using the RNase-Free DNase kit (Qiagen). RW1 buffer (350µl) was pipetted into the RNeasy mini column which was centrifuged for 15 seconds at 10,000 rpm to wash, and then the flow-through was discarded. The columns were placed on the bench at room temperature and 10µl of DNase I stock solution (see RNase-Free DNase kit's hand book; Qiagen) were added to 70µl of RDD buffer and the mixture was mixed gently by inverting the tube. The DNase

I incubation mix (80μl) was directly applied on the RNeasy silica-gel membrane, and then the columns were placed on the bench top at room temperature (20-30°C) for 15 minutes. After the 350μl of RW1 buffer were pipetted into RNeasy mini columns, they were centrifuged for 15 seconds at 10,000 rpm. The flow-through and collection tubes were discarded and the columns were transferred into new 2ml collection tubes, and 500μl of RPE buffer were pipetted onto the RNeasy columns. The tubes were closed gently and were washed by centrifuging the tubes for 15 seconds at 10,000 rpm, and the flow-through was discarded. Another 500μl of buffer RPE were pipetted onto the columns, and then the tubes were centrifuged once again for another 2 minutes at the same speed. The columns were placed into new collection tubes and they were centrifuged for 1 minute, in order to eliminate any possibility of buffer RPE carryover. The tubes were transferred to 1.5ml RNase DNase free collection tubes and 30-50 μl of RNase-free water were applied directly onto the RNeasy silica-gel membrane. The tubes were closed gently and then were centrifuged for 1 minute at 10,000 rpm to elute the RNA. The concentration of the RNA samples was calculated using the spectrophotometer at the absorbance of 260nm and 280nm.

3.2.2 cDNA Synthesis by Reverse Transcription-PCR

The RNA was reversed transcribed to cDNA by mixing the listed reagents as follows:

Reagents	Amount
5x MMLV RT buffer	10μl
Random primers (Promega)	1μl
dNTPs 25mM (Promega)	1μl
RNase inhibitor (Promega)	0.5μl
MMLV reverse transcriptase (Promega)	0.5μl
RNA sample (1μg/μl)	2μl
DNase and RNase free water (Sigma)	34μl
Total	**50μl**

Table 3.1: Preparation of RT product (cDNA) for RT-PCR

The reaction mixture was incubated in the 9700 thermal cycler (Applied Biosystem) according to the following sequence:

25°C for 10 minutes → 45°C for 60 minutes → 95°C for 10 minutes.

The RT-Product was used immediately for PCR reactions or stored at -80°C until required.

3.2.3 Preparation of Reverse Transcription-PCR Reaction

The reagents were added in the following order:

Reagents	Amount
RT product	0.5µl
10xPCR buffer (Promega)	5µl
$MgCl_2$ 1.5mM (Promega)	3µl
Forward primer (50µg/ml)	1µl
Reverse primer (50µg/ml)	1µl
dNTPs 10mM (Promega)	1µl
Taq polymerase (Promega)	0.25µl
DNase and RNase free water (Sigma)	36.75 µl
Total	**50 µl**

Table 3.2: Standard preparation of RT-PCR reaction

The PCR reaction was cycled at:

95°C for 2 minute → [95°C for 30seconds, At (X)* °C for 1minute, 75°C for 30seconds] × 35cycles → 75°C; 5minutes, 4°C ∝

(* the appropriate annealing temperature, see Table 3.3)

The primer pairs used in the RT-PCR experiment were chick Ihh, chick SAP, chick type X collagen, chick patched, chick PTHrP and chick GAPDH. Sequences of the primers used are listed in Table 3.3.

Primers	Sequence 5′ to 3′		Annealing temperature	Product size of cDNA
Chick Ihh	Forward	^{5}CAT CAT CTT CAA GGA CGA GGA GAA C^{3}	58	224
	Reverse	^{5}TAC TTG TTG CGG TCC CTG TCT GAC^{3}		
Chick SAP	Forward	^{5}GCC TTG TTA TCG GAG ACC ACA G^{3}	55	360
	Reverse	^{5}TTT GGT TTC TGG GAC CGC AG^{3}		
Chick Type X collagen	Forward	^{5}AGC AGG AGC AAA TCA AGC^{3}	55	340
	Reverse	^{5}CAG AGG AAT AGA GAC CAT^{3}		
Patched	Forward	^{5} GTG GAA GTT GGT GGA CGA GT ^{3}	60	280
	Reverse	^{5} CAT GTA CTC TGC TGG CCT GA ^{3}		
GAPDH	Forward	^{5}AAG GCT GAG AAC GGG AAA CTT G^{3}	58	560
	Reverse	^{5}TCA ACA ACA GAG ACA TTG GGG G^{3}		
PTHrP	Forward	^{5}CCT AAG CCT GCT ACC AAC AC ^{3}	58	260
	Reverse	^{5} GCT CGC CTC TTT TTC TTC TC ^{3}		

Table 3.3: Forward and reverse sequences of the primers used in this project.

3.3 Results

3.3.1 Optimization of the Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The present study used RT-PCR to study the effect of cyclopamine on the expression of SAP, type X collagen, Ihh, PTHrP and Patched. GAPDH was also used as a house keeping gene throughout this project. A series of PCR cycles were primarily carried out using a set of the appropriate primers in order to determine the optimum number of PCR cycles required for the study. Figure 3.1 shows the intensity of the DNA products obtained by varying the number of PCR cycles in the experiment. The results showed that the intensity of the PCR products at cycle 30 is similar to that at cycle 40. Therefore, cycle 30 was used in all subsequent PCR reactions in order to ensure that the results obtained from different PCR reactions can be compared and that the reaction products still fall within the experimental phase of DNA amplification. Jemtland *et al* (2003) also reported that the optimum number of PCR cycles for use in their study of the expression pattern of various genes was 30 cycles. The present study also included additional test to determine the optimum amount of cDNA required for the subsequent experiments. Figure 3.2 shows results obtained from PCR analyses with various concentrations of DNA template. The test indicated that the appropriate concentration that still produced a well defined band of high density was 0.5μg/μl of cDNA. Therefore, this concentration of cDNA was used throughout the study.

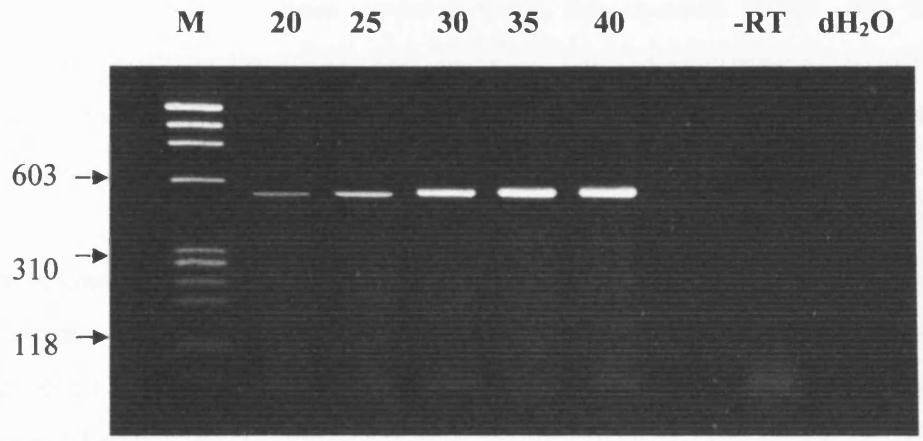


Figure 3.1: Agarose gel showing that there is a gradual increase in the amount of GAPDH PCR product from 20 cycles to 40 cycles with an increment of 5 cycles. M = X174 DNA/*Hae* III Marker.

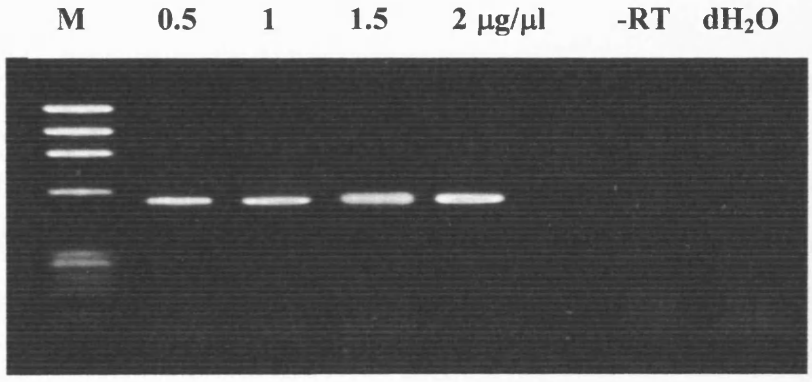


Figure 3.2: Agarose gel illustrating various amount of cDNA used in PCR reaction of GAPDH. 0.5 µl of the cDNA was used as an optimum amount of template into the PCR reaction. M = X174 DNA/*Hae* III Marker.

3.3.2 Expression of Type X collagen, Ihh, SAP, Patched and PTHrP in Cyclopamine Treated and Untreated Micromass Dot Cultures at Different Time Points

In this study the expression of different genes has been studied in the micromass culture of 17-day chick chondrocytes isolated from the sternum tissue. The effect of cyclopamine (a drug that block the hedgehog signalling pathway) on changes in expression pattern of six different genes was also examined. These genes included: GAPDH, type X collagen, Ihh, Patched, PTHrP and SAP.

The expression analysis of these genes was studied in three different phenotypes of chondrocytes: proliferative (caudal region), prehypertrophic (middle region) and hypertrophic (cephalic region). The cells ($2x10^5$ cells) were cultured using micromass technique. Cyclopamine treated and untreated cultures were established. The micromass dots were grown in small culture dishes, each contained 15 dots. One dish of each phenotype of chondrocytes was harvested daily from the cyclopamine treated and untreated cultures for RNA isolation. Examination by microscopy revealed that, some of the chondrocytes that had separated from the edges of the micromass dots had differentiated and became fibroblasts.

In order to prevent the samples from being contaminated with RNA from fibroblasts, the micromass dots cultures were carefully removed from the culture dishes using sterile forceps. Each isolated sample was transferred into sterile RNA/DNA free tubes, and then the RNA was isolated and reverse transcribed for the analysis of gene expression using the RT-PCR. The experiment that used the fibroblast free cultures was repeated three times The PCR results obtained from the first set of experiments are shown in Figures 3.3, 3.4 and 3.5 and those from the other two sets of experiments are shown in Appendix (Figures A.6, 7, 8, 9, 10 and 11). In addition, an experiment using micromass dots cultures that contain fibroblasts was also included in the study. This experiment was done only once to ascertain whether the inclusion of the fibroblasts affected the gene expression pattern and the results obtained from this experiment are shown in Figure 3.6. The results indicate that the patterns of expression of GAPDH, Type X, Ihh, SAP and PTHrP in the cultures without fibroblasts (Figures 3.3, 3.4 and

3.5) are similar to those in the cultures containing fibroblasts (Figure 3.6). However, there are variations in the pattern of Patched expression in these cultures. For example, in all cultures (caudal, middle and cephalic tissues) containing fibroblast, Patched was detected at all time points (Figure 3.6). With regard to those cultures containing no fibroblast, the pattern of Patched expression was variable depending on the types of cultures. The cultures of caudal region showed Patched expression from day 5 onward (Figure 3.3) whereas those of the middle region expressed Patched from day 0 to day 5 (Figure 3.4). In the cultures of cephalic region, Patched was only detected from day 0 to day 4 (Figure 3.5).

It is also noteworthy that the cultures containing no fibroblasts showed some variations in the expression pattern of Ihh, Patched and PTHrP, noticeably those treated with cyclopamine (see Appendix, Figures A.6, 7, 8, 9, 10 and 11). For example, in one set of the experiments using treated caudal region of the tissue (Figure A.6), Ihh showed no expression until day 4 whereas in the other (Figure A.9) it was detected at day 3. Furthermore, the expression of PTHrP in these tissues was found to be less pronounced from day 3 in the former set of the experiments (Figure A.6) whereas in the latter (Figure A.9), there was no reduction in the level of PTHrP expression until day 5. There were also changes in the level of PTHrP expression in the middle region of the tissue (Figures A.7 and A.10). In one experiment (Figure A10), PTHrP was no longer detected at day 2 but in the other (Figure. A.7), its expression was still found up to day 4. Changes in the level of Patched expression pattern were obvious in both the treated and untreated cephalic region (Figure A.8 and A.11). One set of the experiments using the untreated samples (Figure A8), Patched was found up to day 2 whereas its expression was not detected until day 5 (Figure A11).

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3.3.3 RT-PCR Analysis of Micromass Dot Cultures of Chondrocytes without Fibroblasts

3.3.3.1 Chondrocytes Isolated From Caudal Region of Sternum

Figure 3.3A represents one of the three sets of experiments being carried out. This representative figure shows the expression patterns of GAPDH, Type X collagen, Ihh, SAP, Patched and PTHrP in the untreated cultures of caudal chondrocytes without fibroblasts from 0 to 7 days. The intensity of GAPDH expression appears somewhat similar at all different time points (0, 1, 2, 3, 4, 5, 6 and 7 days). Type X collagen and Ihh were not expressed throughout the period of cultures. SAP showed the same levels of expression from time 0 up to day 4 and more intense expression from day 5 up to day 7. However, the expression of Patched and PTHrP was somewhat variable, depending on the time points. Most noticeable was the difference in the expression intensity of Patched; the chondrocytes did not express Patched until their culture reached day 5. PTHrP showed similar expression levels from time 0 up to day 3 but its expression gradually became less pronounced from day 4 to day 7. The expression of GAPDH, Type X collagen, Ihh, SAP, Patched and PTHrP showed a similar pattern in other two sets of experiments (see Table 3.4 and Appendix).

Figure 3.3B illustrates the typical results obtained from the analysis of cyclopamine treated cultures of chondrocytes. Cyclopamine appeared to have no effect on GAPDH as the treated cultures showed similar levels of expression at all time points. However, there were striking differences in the expression levels of other genes. Type X collagen showed no expression in the cultures until day 4 when a faint band appeared and the expression became gradually more intense from day 5 up to day 7. The chondrocytes started to show Ihh and Patched expression at day 3 and the intensity increased thereafter. SAP expression was obtained at time 0 but there was no SAP expression at other time points. And similarly PTHrP was expressed from time 0 but its expression started to decrease at day 2 and no bands appeared after day 4. The other two sets of experiments showed similar results (see Table 3.4 and Appendix, Figures A.6 and A.9).

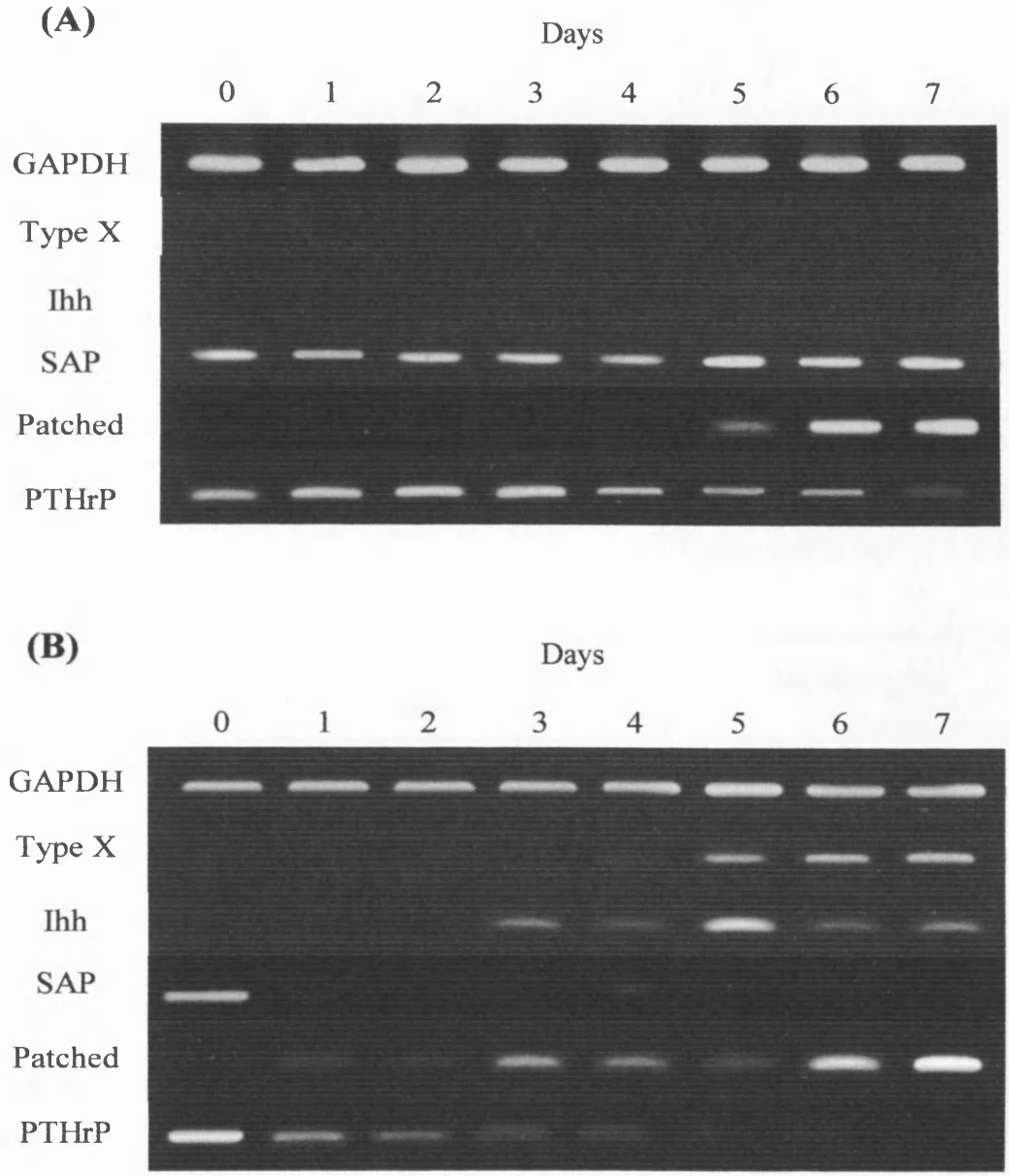


Figure 3.3: Agarose gel of PCR products showing the typical observation out of 3 sets of experiments. The expression of different genes in the untreated and treated micromass cultures of the caudal region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

Gene	Untreated	Treated
GAPDH	No change in expression	No change in expression
Type X collagen	No expression	Expression at day 5, 6 and 7
Ihh	No expression	Expression at day 4, 5, 6 and 7
SAP	Expression from the time 0 to day 7	No expression
Patched	Expression at day 5, 6 and 7	Expression from day 3 to day 7
PTHrP	High expression from the time 0 and gradually decreased to day 7	High expression at the time 0 and then gradually decreased to day 4

Table 3.4: Summary of the expression of different genes obtained by the RT-PCR in the caudal region of the treated and cyclopamine treated samples

3.3.3.2 Chondrocytes Isolated from Middle Region of Sternum

As shown in Figure 3.4A GAPDH and type X collagen show similar expression levels at all different time points (0, 1, 2, 3, 4, 5, 6 and 7). However, there were noticeable variations in the intensity of expression of the other genes. Indian hedgehog showed low level of expression from time 0 to day 3 but the expression became more intense at day 4 and thereafter. In contrast, SAP expression was intense from time 0 to day 3 and its expression became less intense thereafter. The Patched expression was pronounced from time 0 to day 4 and decreased thereafter. On the other hand, the expression of PTHrP appeared strong from time 0 to day 3 and the bands started to lose their intensity at day 4 but the levels of expression remained similar at day 5 up to day 7.

As shown in Figure 3.4B the levels of GAPDH expression in the cyclopamine treated cultures were similar at all time points. Other genes showed remarkable variations in the expression intensity. Noticeably, SAP showed only a strong band of expression at time 0 and no expression at the other time points. PTHrP also showed a strong band at time 0 but the expression became much less at day 2 and no band was observed from day 3. Patched showed strong bands from time 0 until day 4 and the bands became less intense at other time points.

The above results are representative and summarised in Table 3.5. Those from other two sets of experiments are shown in Appendix, Figures A.7 and A.10.

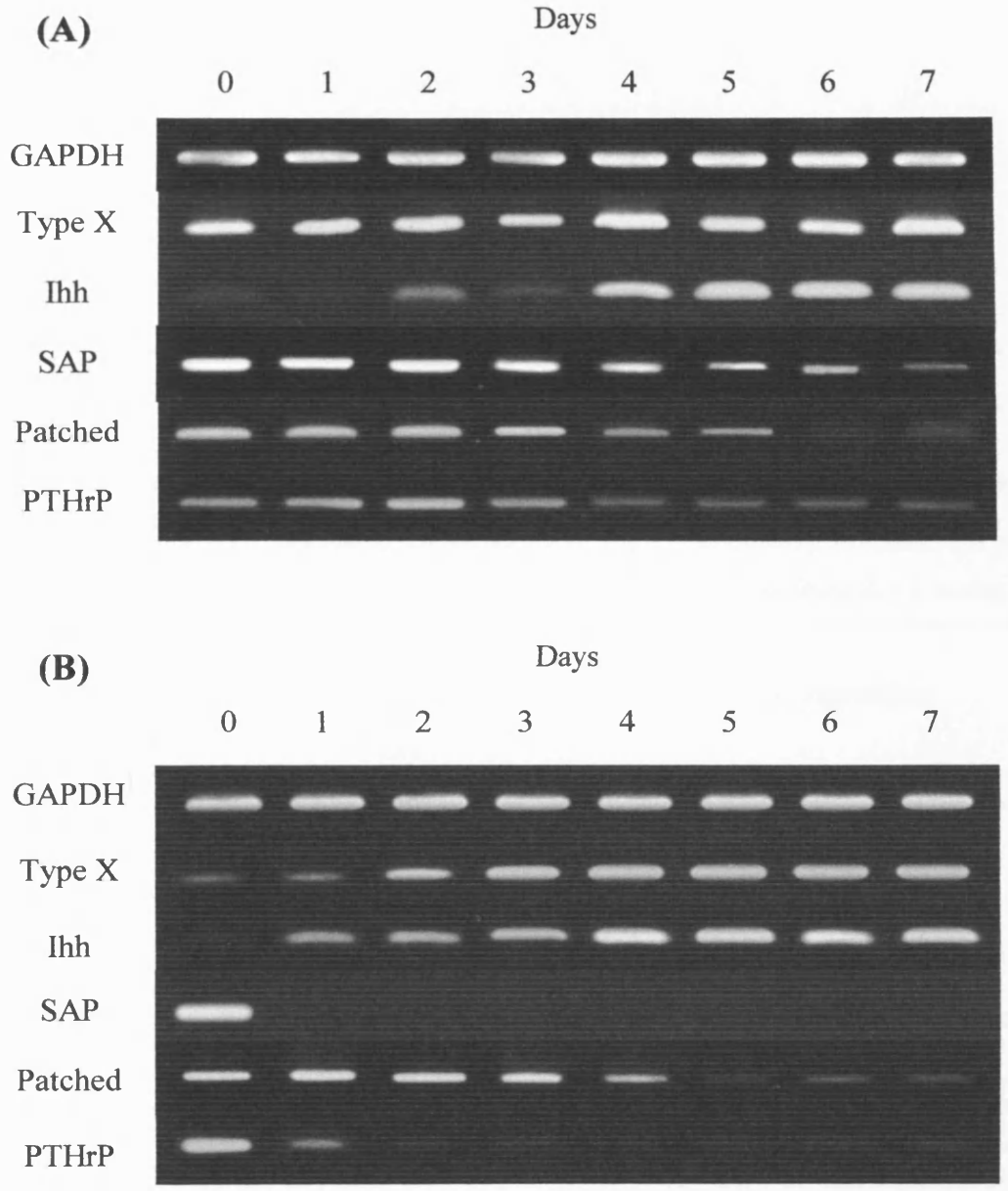


Figure 3.4: Agarose gel of PCR products showing the typical observation out of 3 sets of experiments. The expression of different genes in the untreated and treated micromass cultures of the middle region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

Gene	Untreated	Treated
GAPDH	No change in expression	No change in expression
Type X collagen	Expression at all time points	Expression at all time points
Ihh	Gradually increased in expression from day 3 to day 7	Gradually increased in expression from day 1 to day 7
SAP	High expression from the time 0 and then gradually decreased to day 7	No expression
Patched	High expression from the time 0 and then gradually decreased to day 7	High expression from the time 0 and then gradually decreased to day 7
PTHrP	High expression from the time 0 and then gradually decreased to day 7	High expression at time 0,1 and suddenly decreased from day 2

Table 3. 5: Summary of the expression of different genes obtained by the RT-PCR in the Middle region of the treated and cyclopamine treated samples

3.3.3.3 Chondrocytes Isolated from Cephalic Sternum

Reverse transcription -PCR analysis of the cephalic region of both, the cyclopamine treated and untreated sternum, shows that all 3 genes (GAPDH, Type X collagen and Ihh) were expressed and the levels of their expression were somewhat similar throughout the time points (Figure 3.5A). The remaining genes (SAP, Patched and PTHrP) exhibited significant variations in the intensity of expression, depending on the time points. In the untreated samples, SAP was expressed at time 0 but the bands of expression started to loose their intensity from day 1 and then no further expression was recorded as from day 6. The pattern of decreasing levels of expression was also observed in Patched and PTHrP. The Patched expression was intense from day 1 and the intensity of its expression was gradually reduced from day 4. In the treated samples, SAP and PTHrP expression was recorded at time 0 but showed no further bands from day 1 to day 7. In contrast, Patched did not show up until day 2 and its expression started to increase gradually from day 5 right through to day 7 (Figure 3.5 B).

The above results represent one set of the experiments and summarised in Table 3.6. The other two sets of experiments also showed similar results that are presented in Appendix, Figure A.8 and A.12.

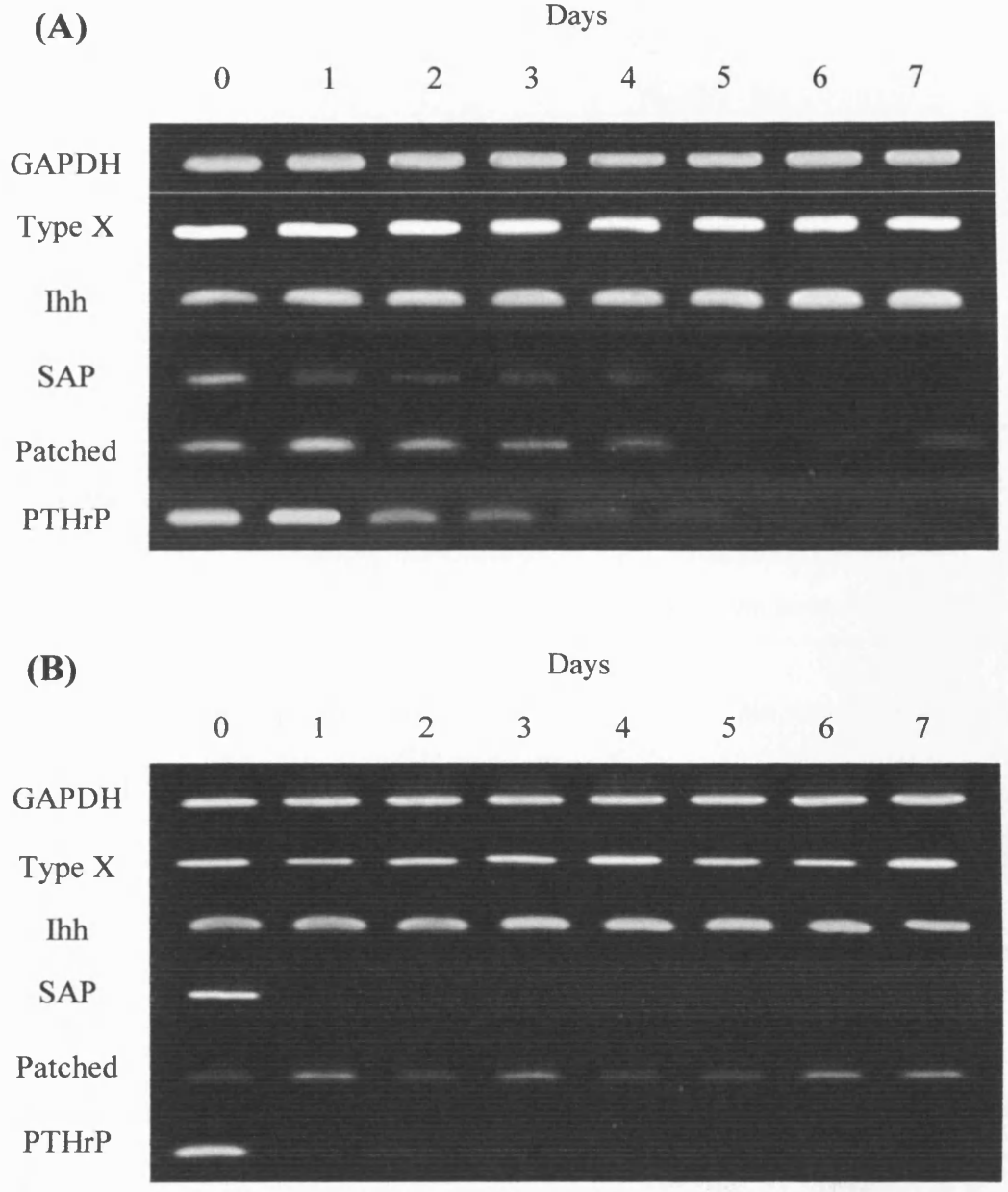


Figure 3.5: Agarose gel of PCR products showing the typical observation out of 3 sets of experiments. The expression of different genes in the untreated and treated micromass cultures of the cephalic region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

Gene	Untreated	Treated
GAPDH	No change in expression	No change in expression
Type X collagen	Expression at all time points	Expression at all time points
Ihh	Gradually increased in expression from day 3 to day 7	Gradually increased in expression from day 1 to day 7
SAP	High expression from the time 0 and then gradually decreased to day 5	No expression
Patched	High expression from the time 0 and then gradually decreased to day 7	High expression from the time 0 and then gradually decreased to day 7
PTHrP	High expression from the time 0 and then gradually decreased to day 7	High expression at time 0 and suddenly non expression from day 1

Table 3.6: Summary of the expression of different genes obtained by the RT-PCR in the cephalic region of the treated and cyclopamine treated samples

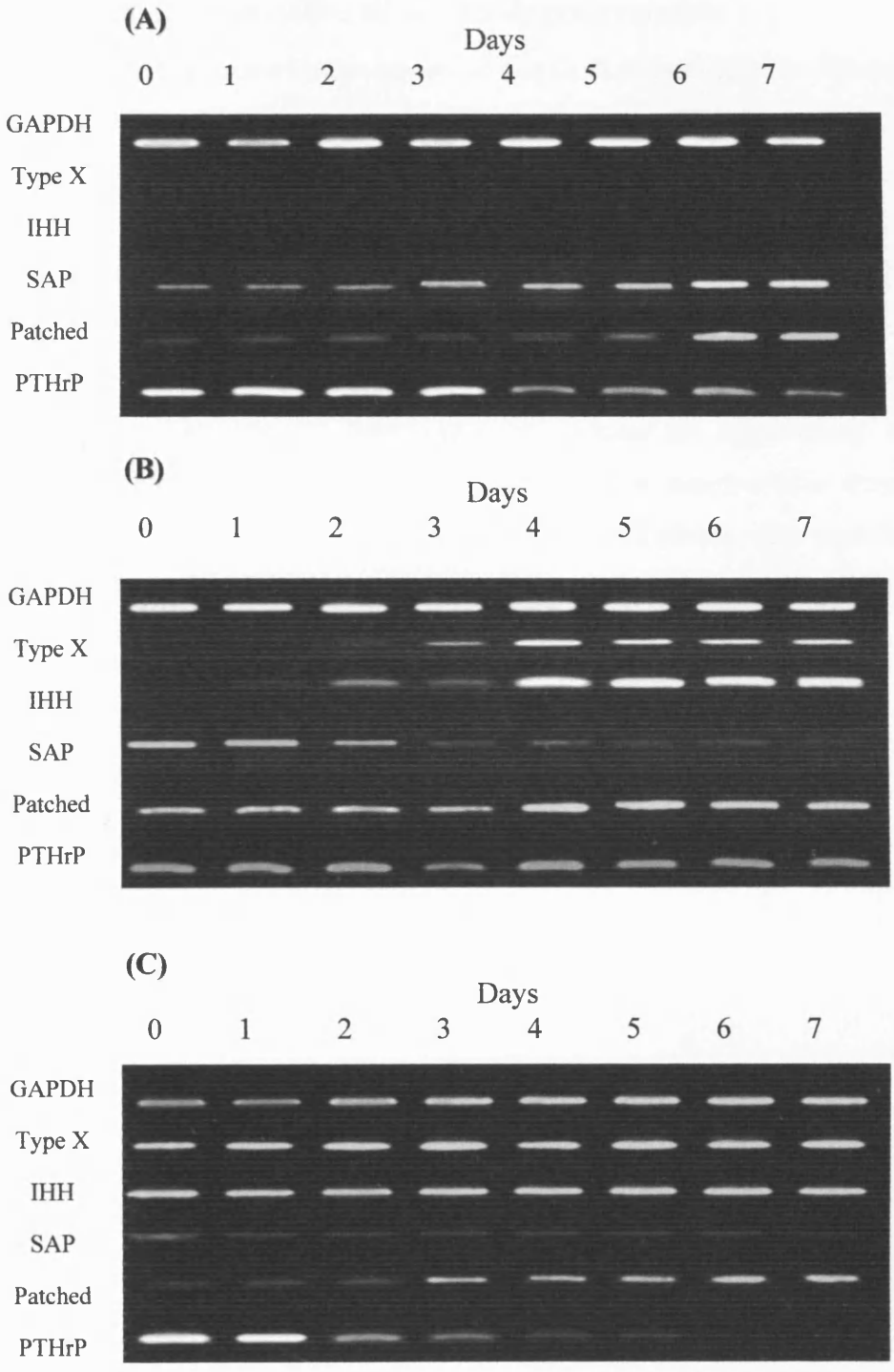


Figure 3.6: PCR reaction products of different regulatory genes in the untreated micromass culture of 17 day chick embryo. The RNA used in these reactions was isolated from the whole micromass culture containing the dedifferentiated fibroblasts. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Caudal region **(B)** Middle region **(C)** Cephalic region

3.3.4 Glycosaminoglycans (GAGs) and Collagen synthesis

3.3.4.1 Effect of Cyclopamine on the Levels of GAGs Released into the Micromass Medium during 8 Days Culture

The experimental results and the statistical analysis are shown in Table 3.7

The controls taken from caudal, middle and cephalic regions showed a gradual increase in the levels of GAG release into the medium throughout the period of culture from day 2 to day 8. The same pattern of GAG release was also observed in the cyclopamine treated samples. Furthermore, the levels of GAG release are significantly higher ($P<0.05$) in the cyclopamine treated than those in the control samples taken from both the caudal and middle regions. However, the levels of GAG release were significantly ($P<0.05$) less in the cyclopamine treated than those in the control samples taken from the cephalic region.

Caudal Samples

Day	Untreated (μg/ml) mean	SE	Treated (μg/ml) mean	SE	P Value
2	0.38	± 0.006	14.33	± 0.012	0.02
4	12.26	± 0.015	31.69	± 0.002	0.01
6	45.64	± 0.002	52.51	± 0.007	0.03
8	63.42	± 0.004	60.28	± 0.003	0.031

Middle samples

Day	Untreated (μg/ml) Mean	SE	Treated (μg/ml) mean	SE	P Value
2	10.87	± 0.001	21.86	± 0.005	0.010
4	23.24	± 0.003	28.42	± 0.001	0.020
6	26.52	± 0.002	29.88	± 0.002	0.047
8	30.49	± 0.001	43.47	± 0.006	0.006

Cephalic samples

Day	Untreated (μg/ml) Mean	SE	Treated (μg/ml) mean	SE	P Value
2	7.43	± 0.001	2.93	±0.005	0.040
4	12.19	± 0.003	9.49	± 0.006	0.044
6	21.22	± 0.012	11.71	± 0.001	0.049
8	31.90	± 0.005	18.15	± 0.017	0.048

Table 3.7: The release of GAGs by three different regions of the 17 day chick embryonic sternum into the medium of the treated and untreated micromass cultures. SE = Standard error, replicate number (n) = 3

3.3.4.2 Effect of Cyclopamine on the Levels of GAGs Production in the Micromass Mass during 8 Days Culture

The results and the statistical analysis are summarised in Table 3.8

In the untreated (control) caudal sample, the level of GAG production constantly increased throughout the period of culture (8 days). The production of GAG in this sample increased from 27.26 μg/mg of protein on day 2 to 100.17 μg/mg of protein on day 8 (Table 3.8). In the cyclopamine treated sample, the production of GAG also increased constantly throughout the same period of culture from 34.30 μg/mg of protein on day 2 to 103.15 μg/mg on day 8. Increases in the level of GAG production have also been observed throughout the period of cultures in the cyclopamine treated and untreated middle region. The level of GAG production in the untreated middle region reached 6.67 μg/mg on day 2 and gradually increased to 70.65 μg/mg of protein on day 8 (Table 3.8). The production of GAG in the cyclopamine treated middle region showed a similar pattern; it reached 19.07 μg/mg of protein on day 2 and increased to 93.93μg/mg on day 8 (Table 3.8). A similar pattern of increasing levels of GAG production was also found in the cephalic tissue, either treated or untreated. The level of GAG production in the untreated sample of the cephalic region was at 9.38 μg/mg of protein on day 2 and peaked at 55.47 μg/mg of protein on day 8 (Table 3.8). In the treated cephalic region, the level of GAG production was found to be 3.20 μg/mg on day 2 and gradually increased to 14.39 μg/mg on day 8 (Table 3.8).

Statistical analysis of the data shows that the levels of GAG production in the cyclopamine treated sample were significantly ($P<0.05$) higher than those in the untreated taken from both caudal and middle regions (Table 3.8). However, the cephalic region showed a reverse trend, that is the levels of GAG production in the cyclopamine treated sample were significantly less ($P<0.05$) as compared to those in the control samples (Table 3.8). Furthermore, it is interesting to note that the levels of GAGs produced by the cephalic region were less than those in the middle and caudal regions regardless of whether the samples were treated or untreated by cyclopamine.

Caudal Samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	27.26	± 0.003	34.30	± 0.001	0.038
4	49.24	± 0.003	73.63	± 0.003	0.009
6	86.64	± 0.011	108.56	± 0.006	0.020
8	100.17	± 0.009	103.15	± 0.006	0.032

Middle samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	6.67	± 0.003	6.67	± 0.003	0.035
4	32.35	± 0.004	32.35	± 0.003	0.004
6	61.73	± 0.003	61.73	± 0.001	0.008
8	70.65	± 0.003	70.65	± 0.002	0.010

Cephalic samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	9.38	± 0.002	3.20	± 0.001	0.036
4	17.82	± 0.003	7.42	± 0.003	0.035
6	41.58	± 0.003	11.24	± 0.003	0.005
8	55.47	± 0.003	14.39	± 0.007	0.008

Table 3.8: Shows the amount of GAGs production in the mass of the treated and untreated micromass cultures during one week culture and the P value between the three different regions of the 17 day chick sternal chondrocytes. SE = Standard error, replicate number (n) = 3

3.3.4.3 Effect of Cyclopamine on the Levels of GAGs Production in the Micromass Mass after Two Weeks in Culture

In the results shown in Table 3.9 the micromass cultures were grown for two weeks. This was to examine and investigate the phenotype of the chondrocytes in the micromass culture. In the cartilaginous tissue the undifferentiated and mature chondrocytes are responsible for ECM synthesis which can be examined by measuring the production of GAGs and hydroxyproline. From the result obtained from the 8 days culture period it was necessary to examine if the chondrocytes can maintain their chondrocytic phenotype beyond the initial period of culture ie 8, Therefore the culture period was extended to 14 days, and the micromass mass were analyses at three different time points (12, 13 and 14 days) during the second week of culture.

In the experimental results are shown in Table 3.9. In the absence of cyclopamine, all three regions of the sternum (caudal, middle and cephalic) showed a gradual increase in the levels of GAG production throughout the period of cultures from day 12 to day 14. In the presence of cyclopamine, the levels of GAG production by all treated cultures gradually decrease during the same period. The amount of GAG found in the treated caudal and middle regions was significantly ($P<0.05$) higher than that in the untreated samples. In contrast, the levels of GAG in the treated cephalic region were found to be significantly ($P<0.05$) lower than those in the untreated samples.

Caudal Samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	113.42	± 0.011	77.78	± 0.010	0.005
13	118.14	± 0.003	90.81	± 0.003	0.001
14	123.97	± 0.007	101.19	± 0.009	0.001

Middle samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	102.31	± 0.013	81.88	± 0.007	0.073
13	108.69	± 0.006	87.95	± 0.006	0.046
14	112.31	± 0.002	90.46	± 0.004	0.040

Cephalic samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	48.13	± 0.003	34.14	± 0.007	0.013
13	70.56	± 0.003	33.30	± 0.006	0.004
14	83.54	± 0.001	43.97	± 0.003	0.002

Table 3.9: Shows the amount of GAGs production in the mass of the treated and untreated micromass cultures during two weeks culture and the P value between the three different regions of the 17 day chick sternal chondrocytes. SE = Standard error, replicate number (n) = 3

3.3.4.4 Effect of Cyclopamine on the Release of Collagen in the Micromass Mass during 8 Days Culture Period

The results and the statistical analysis are summarised in Table 3.10

Collagen synthesis was measured by analysis of hydroxyproline. In all untreated samples, the levels of hydroxyproline production constantly increased throughout the period of culture (8 days). The production of hydroxyproline in the caudal region increased from 0.12 μg/mg of total protein on day 2 to 13.73 μg/mg of total protein on day 8. The level of hydroxyproline production in the untreated middle region reached 2.55 μg/mg of total protein on day 2 and gradually increased to 12.44 μg/mg of total protein on day 8 (Table 3.10). A similar increasing pattern of hydroxyproline production was also found in the untreated cephalic tissue. The hydroxyproline production in the untreated sample of the cephalic region was at 0.91 μg/mg of total protein on day 2 and peaked at 4.13 μg/mg of total protein on day 8.

In the cyclopamine treated caudal and middle regions, the production of hydroxyproline also increased constantly throughout the same period of culture. The level of hydroxyproline found in the treated caudal started from 2.50 μg/mg of total protein on day 2 increased to 20.08 μg/mg of total protein on day 8. The level of hydroxyproline production in the cyclopamine treated middle region reached 0.31 μg/mg of total protein on day 2 and increased to 8.81 μg/mg of total protein on day 8. However, a similar increasing pattern of hydroxyproline production was also found in the treated cephalic tissue but only up to day 6 after which this level dropped to 1.85 μg/mg of total protein on day 8 (Table 3.10).

Caudal Samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	0.12	± 0.001	2.50	± 0.003	0.039
4	5.41	± 0.002	9.70	± 0.001	0.004
6	7.77	± 0.003	12.99	± 0.002	0.009
8	13.73	± 0.003	20.08	± 0.001	0.026

Middle samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	2.55	± 0.001	0.31	± 0.001	0.001
4	3.44	± 0.001	4.01	± 0.001	0.004
6	5.83	± 0.001	8.26	± 0.008	0.030
8	8.81	± 0.002	12.44	± 0.008	0.009

Cephalic samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
2	0.91	± 0.004	0.11	± 0.002	0.012
4	2.99	± 0.001	1.47	± 0.009	0.047
6	3.05	± 0.001	2.39	± 0.002	0.006
8	4.13	± 0.001	1.85	± 0.001	0.019

Table 3.10: Shows the amount of hydroxyproline released into the mass of the treated and untreated micromass cultures during one week culture and the P value between the three different regions of the 17 day chick sternal chondrocytes. SE = Standard error, replicate number (n) = 3

3.3.4.5 Effect of Cyclopamine on the Release of Collagen in the Micromass Mass after Two Weeks of Culture

The experimental results and the statistical analysis are summarised in Table 3.11. In the absence of cyclopamine, all three regions of the sternum (caudal, middle and cephalic) showed a gradual increase in the levels of hydroxyproline production throughout the period of cultures from day 12 to day 14. In the presence of cyclopamine, the levels of hydroxyproline production by all treated cultures gradually increase throughout the same period of cultures. The amount of hydroxyproline found in the treated caudal and middle regions was significantly ($P<0.05$) higher than that in the untreated samples (Table 3.11). In contrast, the levels of hydroxyproline in the treated cephalic region were found to be significantly ($P<0.05$) lower than those in the untreated samples (Table 3.11).

Caudal Samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	17.50	± 0.009	28.03	± 0.001	0.047
13	23.80	± 0.001	29.08	± 0.003	0.024
14	26.62	± 0.003	34.55	± 0.002	0.024

Middle samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	11.68	± 0.003	18.95	± 0.001	0.039
13	14.37	± 0.001	21.95	± 0.001	0.014
14	18.30	± 0.003	27.28	± 0.002	0.025

Cephalic samples

Day	Untreated (μg/mg) mean	SE	Treated (μg/mg) mean	SE	P Value
12	4.03	± 0.001	3.03	± 0.005	0.048
13	7.02	± 0.002	4.28	± 0.001	0.009
14	7.93	± 0.001	4.75	± 0.001	0.011

Table 3.11: Shows the amount of hydroxyproline released into the mass of the treated and untreated micromass cultures during two weeks culture and the P value between the three different regions of the 17 day chick sternal chondrocytes. SE = Standard error, replicate number (n) = 3

Summary of the Biochemistry Analysis Results

- The culture medium of the treated caudal and middle micromass dots contains high level of GAGs as compared to that of the untreated samples.
- The level of GAGs in the culture medium of the treated cephalic micromass dots is less than that in the untreated samples.
- The level of GAGs was high in the caudal and middle micromass dots grown in the cyclopamine containing medium for 8 days as compared to that in the untreated samples.
- The level of GAGs was low in the cephalic micromass dots grown for 8 days in the medium containing no cyclopamine as compared to that in the untreated samples.
- The level of GAGs in the two weeks cultures of the treated caudal, middle and cephalic regions was less than that in the untreated samples.
- The level of hydroxyproline production was generally higher in the one week cultures of the treated caudal and middle regions except that at day 2.
- The level of hydroxyproline in the one week culture of the treated cephalic region was less than that in the untreated samples.
- The level of hydroxyproline was high in the two weeks culture of the treated caudal and middle regions as compared to that in the untreated samples.
- The level of hydroxyproline in the two weeks culture of the treated cephalic region was less than that in the untreated samples.

3.4 Discussion

In the previous experiments (see Chapter 2), histological and *in situ* hybridisation methods demonstrated that most of the chondrocytes in the caudal region of the sternum showed a phenotype similar to the cells of the proliferative zone of the tibia whereas those in the middle region appeared to be similar to the prehypertrophic chondrocytes, and those in the cephalic region are similar to the hypertrophic chondrocytes in the tibia (Gibson, 1997; 1998). Furthermore, the results obtained from these experiments have shown that small acidic protein (SAP) was predominantly localised in the proliferative chondrocytes although low intensity of expression was also observed in the prehypertrophic and hypertrophic cells. While type X collagen was only detected in the hypertrophic chondrocytes, Ihh was found in both the prehypertrophic and hypertrophic cells. Studies made in earlier years have reported that the prehypertrophic and hypertrophic phenotypes also showed the presence of PTHrP and patched (van der Eerden *et al.*, 2000).

Since cyclopamine, a plant steroidal alkaloid, is known to have the ability to inhibit cell receptor signalling, its inhibitory effect on the expression of SAP, Ihh, PTHrP, Patched and type X collagen was examined in the present study using 17 day old chick sternum. Much attention was focused on whether the non expression of SAP affects the expression pattern of the other genes of interest. The results obtained from this study could be used, not only to confirm the above results but also to ascertain whether SAP interacts with Ihh, PTHrP, Patched and type X collagen, all of which are known to be involved in the chondrocyte differentiation. Three different phenotypes of chondrocytes were isolated from their respective (caudal, middle and cephalic) regions of the sternum and grown in an appropriate culture system.

It has been well established that cyclopamine inhibits cellular response to hedgehog (Hh) signal by blocking its signalling pathway (Kindblom *et al.*, 2002). In a number of tissues, deregulation of the Hh signalling pathway can inhibit cell differentiation and promote tumorigenesis (Altaba *et al.*, 2004). However, the effect of deregulating Hh signalling on tumour growth can be reversed by cyclopamine treatment (Altaba *et al.*, 2004). According to Sanchez *et al* (2004), blocking the pathway of Hh with

cyclopamine inhibits cell proliferation and differentiation. Furthermore, Qualtrough *et al* (2004) have demonstrated that cyclopamine treatment resulted in decreased cell yield and induction of apoptosis in vivo and that no evidence of necrotic cell death was observed in treatment of cell lines, which suggested that cyclopamine is not toxic.

In order to determine the appropriate culture system for use throughout this study, different types of cultures were initially tested: monolayer, organ and micromass dots. The test showed that in the monolayer culture system, the cells were differentiated into fibroblasts after a few days in culture. Moreover, attempts to isolate RNA from the organ culture proved very difficult if not impossible and often resulted in RNA degradation. The latter culture system in which the cells were grown in micromass dots was found to have several advantages over the former two: (1) it was easy to extract a high quality RNA from the cells; and (2) the cells were surrounded by their extracellular matrix, thus maintaining their in vivo environment. Furthermore, in this system the cells were found to maintain their chondrocytic phenotype and since they were densely packed together in dots, this facilitated the interaction and signalling pathway between these cells and the ECM (Swalla *et al.,* 1986; Archer *et al.,* 1992).

This culture system was initially developed to study chondrogenesis in the limb bud (Ahrens *et al.,* 1977; Daniels *et al.,* 1996). This system allowed the formation of a three dimensional, multilayered organisation of closely associated cells with pericellular and extracellular matrix (ECM) entrapped to potentiate differentiation. The sequence of chondrogenic events within the micromass culture recapitulates the sequence observed in vivo. According to Daniels *et al* (1996), the micromass faithfully reproduces three basic concepts in developmental biology: pattern formation, morphogenesis, and differentiation in vitro. Since the micromass dot culture system was found to offer several advantages over that of monolayer and organ system, it was used throughout the present study.

This study used reverse transcription polymerase chain reaction (RT-PCR) method to examine the effect of cyclopamine on the expression of type X collagen, Ihh, SAP, Patched and PTHrP in all three regions of the sternum: caudal, middle and cephalic. The

results obtained from the RT-PCR study indicate that cyclopamine has an effect on all five genes: type X collagen, Ihh, SAP, Patched and PTHrP (Figures 3.3, 3.4 and 3.5). However, the response of these genes to cyclopamine varied depending on the duration of the treatment and cell phenotypes (proliferative, prehypertrophic and hypertrophic). In the absence of cyclopamine, the proliferative chondrocytes showed no expression of type X collagen and Ihh (Figure 3.3 A). However, these cells expressed type X collagen and Ihh following exposure to cyclopamine (Figures 3.3 B, 3.4 B and 3.5 B). Furthermore, all untreated proliferative chondrocytes showed high levels of SAP expression whereas there was no SAP expression in any treated cells throughout the time points of the cultures. These findings suggest that cyclopamine has a strong inhibitory effect on SAP expression in the three different phenotypes of chondrocytes (Figures 3.3B, 3.4B and 3.5B).

The present study also revealed that the proliferative cells and those at early stage of differentiation exhibited high levels of PTHrP expression in all cyclopamine-free cultures whereas those exposed to cyclopamine showed a significant reduction in the levels of PTHrP expression. This inhibitory effect was only observed after a prolonged exposure to cyclopamine after 3 days of culture. Furthermore, the PCR analysis of prehypertrophic chondrocytes in the middle region of the sternum revealed that while cyclopamine had no significant inhibitory effect on the expression of type X collagen and Ihh, it completely knocked out that of SAP and PTHrP (Figures 3.3 B & 3.4 B and 3.5 B). In the untreated and treated cultures, no significant variation in the levels of expression of Patched was observed. The results obtained from the PCR analysis of the hypertrophic chondrocytes in the cephalic region indicate that cyclopamine did not inhibit the expression of type X collagen, Ihh and Patched whereas it showed a strong inhibitory effect on SAP and PTHrP (Figures 3.3 B & 3.4 B and 3.5 B). The detection of PTHrP expression in the untreated caudal cells seems to confirm an earlier report that PTHrP was immunostained in proliferating chondrocytes (Moseley *et al.,* 1991).

Earlier studies using tibia indicated that these genes coordinate the rate of chondrocyte proliferation and determine the time when the cells should leave the proliferative stage to begin differentiation (Kobayashi *et al.,* 2002; Maclean *et al.,* 2005). As suggested by

Vortkamp *et al* (1996) Ihh and PTHrP are the major regulators of chondrocyte proliferation and differentiation, and they work together in a negative feedback loop. Moreover, earlier studies using growth plate have shown that cyclopamine inhibited PTHrP expression by blocking Ihh signalling which directly affects the expression of PTHrP in the chondrocytes (Kim *et al.,* 1998; Chen *et al.,* 2002). It has been further suggested that the non expression of PTHrP could cause the chondrocyte to become hypertrophic (Nakase *et al.,* 2001). Under normal conditions, the signal of Ihh activates the release of PTHrP and this signalling process happens through the Ihh receptors such as Smoothened (Smo) and Patched. In the presence of cyclopamine, the Ihh response is inhibited and also affected the balance between active and inactive forms of Smo (Taipale *et al.,* 2000). Therefore examining the expression of Smo will also be important in supporting the current conclusion.

In the present study, high levels of SAP expression were found in all untreated caudal cultures in which type X collagen and Ihh were absent. In contrast, in the cyclopamine treated caudal samples, SAP was knocked out completely as early as day 1 whereas type X collagen and Ihh were highly expressed after a longer exposure to cyclopamine. SAP expression was inhibited by cyclopamine, not only in the caudal region where the cells were proliferative but, also in the middle and cephalic regions in which the cells underwent differentiation. These findings indicate that the blocking of HH signalling may either affect SAP mRNA stability or inhibit SAP gene expression. PTHrP also showed similar sensitivity to cyclopamine in all cell phenotypes. The fact that SAP expression was of very low intensity in all the untreated cephalic cultures seems to confirm that SAP was downregulated as soon as the chondrocytes became hypertrophic. This observation confirms the results of the *in situ* hybridisation studies presented in chapter 2, which showed that SAP was expressed in the prehypertrophic rather than hypertrophic cells of the sternum (Chapter 2).

According to Webster's finding (1999), SAP may be a transcription factor since its secondary structure is a basic-Helix-loop-Helix. This bHLH structure is known to be one of the other transcription factors structures. This observation suggests that SAP would play an important role as a transcription regulator which either binds to other

transcription factors or upregulates the transcription of other gene(s) (Figure 3.7). The present study shows that under normal culture conditions (no cyclopamine treatment), SAP and PTHrP were highly expressed in the caudal (proliferative) and middle (prehypertrophic) regions of the sternum. However, the results also show that following cyclopamine treatment, the expression of SAP was completely inhibited in these regions and that of PTHrP was gradually downregulated. It was also noted that there was increasing Ihh expression soon after PTHrP expression was switched off; the middle region in particular showed high levels of Ihh expression. These observations suggest that cyclopamine may induce a chain of events in these regions where the inhibition of SAP was found to be followed by the downregulation of PTHrP expression. If this is the case, SAP may be involved in the transcription of PTHrP and indirectly affects the Ihh-PTHrP feed back loop. This could be investigated using a PTHrP promoter- reporter gene construct in transfected cells treated with various amount of SAP. The results obtained from the present study seem to confirm the observations in earlier studies that cyclopamine has the ability to inhibit cell response and signalling pathways (Incardona *et al* 1999; Chen *et al* 2002; Qualtrough *et al* 2004).

The PCR results presented in this study have revealed that SAP, Ihh and PTHrP are all expressed in the same region (prehypertrophic) of the embryonic sternum and that the non expression of either SAP or PTHrP or both results in the up regulation of Ihh in this region. This observation suggests that SAP may function in the same way as PTHrP in that it interacts with Ihh in a negative feedback loop. The fact that the up regulation of Ihh expression was followed by the down regulation of that of PTHrP seems to indicate for the very first time that the Ihh/PTHrP negative feedback loop has taken place in the embryonic sternum. Since this feedback loop has only been reported in the growth plate of mouse tibia, the results obtained from both the *in situ* hybridisation and PCR analyses confirm that SAP was specifically localised and highly expressed in the proliferative and prehypertrophic regions of the embryonic sternum. SAP was also shown to interact in a negative feedback fashion with Ihh, one of the endochondral ossification regulators. These findings seem to support the hypothetical view that SAP may play a part in endochondral ossification process.

As far as Patched is concerned, the present study showed no major variations in its expression between the untreated and treated cultures (Figures 3.3, 4, 5, 6, 7 and 8). PCR analysis of the hypertrophic chondrocytes in the cephalic region indicates that cyclopamine did not inhibit the expression of Patched. However, cyclopamine has previously been reported to have the ability to disrupt the action of Patched, one of the Ihh receptors. Patched has been shown to be one of the downstream targets of Ihh signalling (Kamenetsky *et al.,* 2002) and both the present study and earlier reports showed that Patched was expressed in proliferative as well as in mature (prehypertrophic and hypertrophic) chondrocytes. This interesting observation required further examination to study the possible mechanism and function of SAP, Patched and Smo in endochondral ossification.

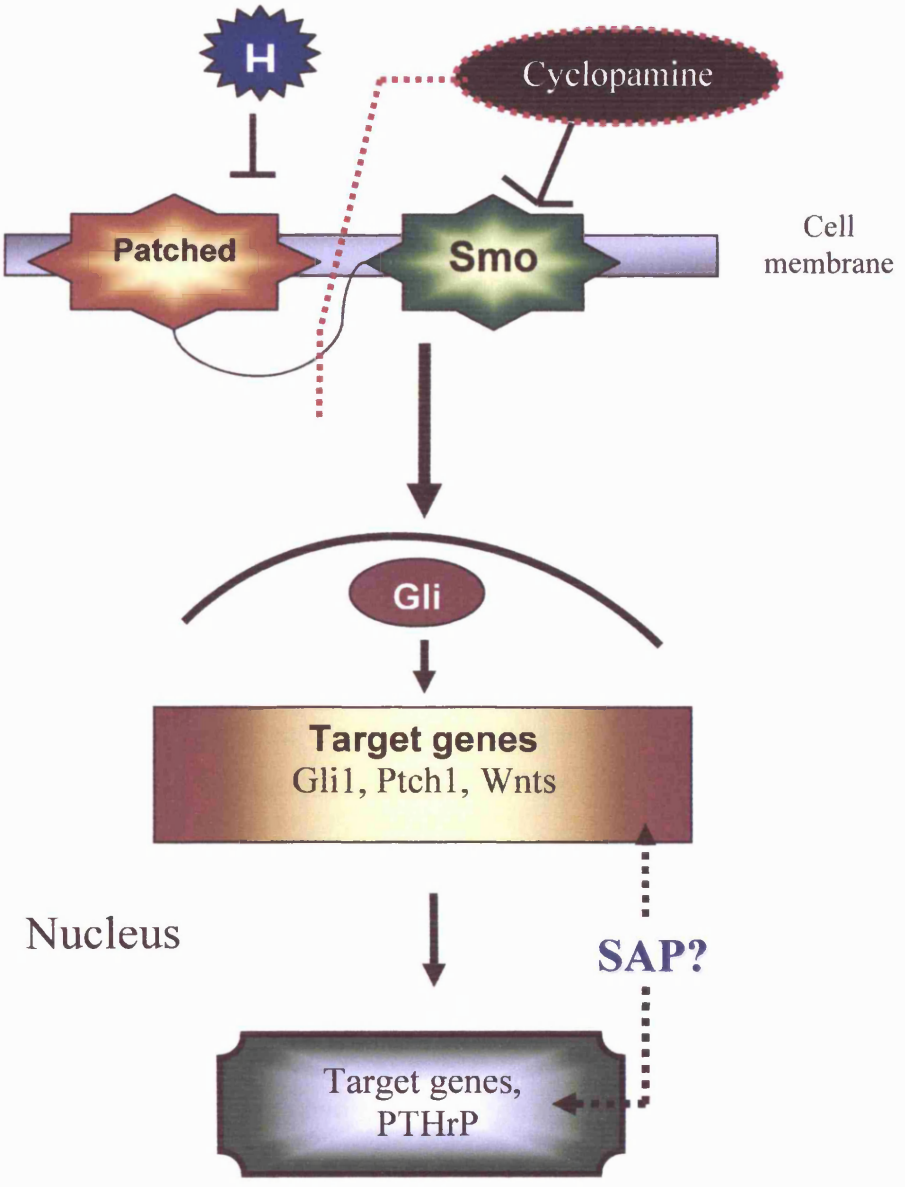


Figure 3.7: Diagram summarising the expected involvement of SAP within the Ihh signalling pathway. In the presence of Cyclopamine, the Ihh signalling pathway is inhibited, and as a result its receptors such as Patched (Patch) and Smoothened (Smo) are deactivated. The deactivation of Smo signal inhibits the expression of Gli in the nucleus and consequently the Ihh target genes are also affected, mainly PTHrP. SAP has been predicted to be a transcription factor (Webster, 1999). Cyclopamine is shown in the present study to inhabit the expression of SAP and PTHrP. These observations may suggest that SAP could be a transcription factor for either Gli target genes or the PTHrP gene.

The present study also examined the effect of cyclopamine on the production of glycosaminoglycans (GAGs) and collagen by various types of chondrocytes using the DMMB staining and hydroxyproline assay methods as described in the 'Materials and Methods'. Changes in the level of GAGs and hydroxyproline productions may be used as an indicator for cell maturation since it is well known that proliferative chondrocytes produce more ECM and collagens as compared to the hypertrophic cells.

In the absence of cyclopamine, the production of GAGs and collagen in the caudal, middle and cephalic regions was found to increase constantly throughout the 14 days period of cultures. The caudal produced more GAGs and hydroxyproline than the middle and cephalic regions. Comparing the middle and cephalic regions shows that the former produced more GAGs and hydroxyproline than the latter. The caudal region has been demonstrated by the *in situ* hybridisation (Chapter 2) to contain proliferative chondrocytes. The fact that the levels of production of GAGs and hydroxyproline in this region were much higher than those in the other two regions indicates that the proliferative chondrocytes underwent active mitotic division as well as actively synthesised the structural components of the extracellular matrix (ECM), namely GAGs and collagens. A slowdown in the production of GAGs and hydroxyproline in the middle region suggests that early cell differentiation took place in this region. The cephalic region where the cells reached full differentiation stage to become hypertrophic, showed a further decline in the production of GAGs and hydroxyproline, resulting in decreased ECM yield.

In the presence of cyclopamine, the levels of GAGs and hydroxyproline in the caudal and the middle increased throughout the same period of cultures. However, differences were noted in the levels of GAGs and hydroxyproline production between the cyclopamine treated and untreated cultures and these variations depended on the regions from which the chondrocytes were isolated. The analysis of the 8 days cultures shows that the levels of GAGs and hydroxyproline produced in the treated caudal and middle were significantly ($P<0.05$) higher than those in the untreated culture. Except that in day 8 the GAGs production was significantly ($P<0.05$) less in the treated caudal region. By contrast, the levels of GAGs and hydroxyproline produced in the cyclopamine treated

cephalic region were significantly ($P<0.05$) lower than those in the untreated samples and these significant differences were observed throughout the 8 days and 14 days period (Tables 3.7, 3.8, 3.9. 3.10 and 3.11). A decrease in the production of GAGs and hydroxyproline in the treated cephalic samples could suggest that cyclopamine affects the production of the extra cellular matrix and the synthesis of type X collagen which is known to be the major collagen synthesised by the hypertrophic chondrocytes.

GAGs and collagen are found highly abundant in vertebrate embryos. These structural components of the extracellular matrix interact with a wide variety of proteins in performing major developmental functions. The present study shows that under the influence of cyclopamine, the chondrocytes at the early stage of proliferation and prehypertrophy (caudal and middle samples) increased the production of GAGs (Figure3.8) whereas those at the later stage of the processes (two weeks cultures) showed a decrease in GAGs production (Figure3.9). This decline in GAGs production in the later days of culture may be due to the fact that the proliferative and prehypertrophic cells differentiated into hypertrophic chondrocytes and therefore they produced less GAGs. The present study has also suggested that cell maturation may be regulated by the Ihh/PTHrP loop and that it can be affected by cyclopamine treatment. In the caudal region in particular, the cells do not usually express type X collagen as this collagen is specific to hypertrophic chondrocytes. The fact that this type X gene was found by the PCR analysis to be expressed in the cyclopamine treated caudal region seems to suggest that in the presence of cyclopamine, the proliferative cells accelerate the process of differentiation; thus the cells became hypertrophic or matured earlier than normally expected. In addition, the results obtained from the RT-PCR analysis showed that the expression of PTHrP which has been reported to be expressed in the proliferative and the prehypertrophic chondrocytes (Nakase *et al.,* 2001) is gradually decreased in the caudal and middle regions in the present of cyclopamine. This fact further supports the notion that chondrocytes from these two regions become hypertrophic more rapidly. This finding is confirmed by the reduced production of GAGs in these two regions during two weeks of culture when the cells were exposed to longer treatment of cyclopamine.

With regard to the cephalic region where the cells already reached a fully differentiated stage, cyclopamine was found to reduce the production of GAGs and hydroxyproline: The levels of GAGs production were significantly ($P<0.05$) less than those observed in the controls. It is apparent that the production of GAGs and hydroxyproline in the hypertrophic region was affected by the cyclopamine treatment. However, the levels of GAGs and hydroxyproline produced appeared to increase gradually with time in both cyclopamine treated and untreated cephalic cultures. This shows that cyclopamine was not toxic to the cells. There was no morphological evidence of cell death in these cyclopamine treated culture. The decline in the production of GAGs and hydroxyproline in the cephalic region indicates a continuing slowdown in the synthesis of GAGs and collagens, resulting in less production of extracellular matrix. These observations seem to support the findings reported by Qualtrough *et al* (2004) that treatment of cell lines by cyclopamine induced decreased cell yield and apoptosis in tumour cells and that it did not cause cell death by necrosis.

Regardless of whether the cells were treated or not with cyclopamine, the levels of GAGs and hydroxyproline in the caudal and middle regions were always found to be much higher than those in the cephalic region; this observation suggests that the proliferative and prehypertrophic chondrocytes were more actively involved in the production of the extracellular matrix components than the hypertrophic cells. In another word, the synthesis of GAGs and hydroxyproline was down regulated as soon as the chondrocytes became fully differentiated or matured cells

CHAPTER 4

Over expression of Small Acidic Protein (SAP) *in vitro* using an avian RCAS vector

4 Introduction

In this project it has been mentioned that the biological functions of the novel Small Acidic Protein (SAP) is not yet known. The main aim of the work presented in this chapter is therefore to investigate the biological functions of SAP especially focused on its involvement in the process of chondrocyte maturation in chondrocyte culture and embryonic limb development. This study was carried out using the avian Replication Competent ALV LTR with a Splice acceptor (RCAS).

The RCAS vector has been developed by Hughes *et al.,* (1987) and used successfully by a rapidly expanding number of groups to assess the biological function of gene directly in *ovo.* This proviral vector is derived from the Rous Sarcoma Virus (RSV) which has a unique *ClaI* restriction sites in place of the region normally encoding the src onco genes, into which foreign DNA fragments of up to approximately 2.4 kb can be inserted. This proviral vector can be efficiently transfected into cultured chick embryo fibroblast (CEFs) using standard techniques, and supernatant containing infectious viruses easily collected and concentrated to yield high-titer viral stocks, that can then be used to infect chick embryos of susceptible strains. Alternatively, transfected CEF cells can be grafted directly into host embryos. The viral surface glycoprotein encoded by the *env* gene primarily determines the host range specificity of the virus. Avian specific vectors are currently available that contain env genes derived from subgroups A, B and D (Figure 4.1) (Hughes *et al.,* 1987). More recently, modified RCAS vectors containing env gene derived from murine leukaemia viral glycoprotein have also been generated that can infect (but not replicate in) mammalian cells efficiently thus making it possible to also use the RCAS system for gene transfer in mice (Barsov *et al.,* 1999). Strains of chicken exist that differ in their susceptibility to infection with viruses of different RCAS subgroups. C, A and B cells are resistant to infection with A and B subgroup viruses and there are cells which are not resistant to any of the five subgroups. It is possible to graft infected cell pellets derived from a sensitive strain into the limb bud of an insensitive strain. This provides a convenient way to generate a local source of transgene expression while avoiding subsequent spread of the virus following application of the graft. The replication-competent avian vectors are able to carry out all the functions of the virus life cycle in addition to encoding an additional transcript of

the inserted transgene. It is also therefore possible for the virus to spread from cell to cell after the initial infection following injection into the embryo. This can result in a widespread infection in the host embryo and therefore broad missexpression of the transgene following the initial injection.

The gene of interest (SAP) was copied using the RT-PCR and cloned into the RCAS vector and over-expressed *in vitro* using 17-day embryonic chick chondrocytes.

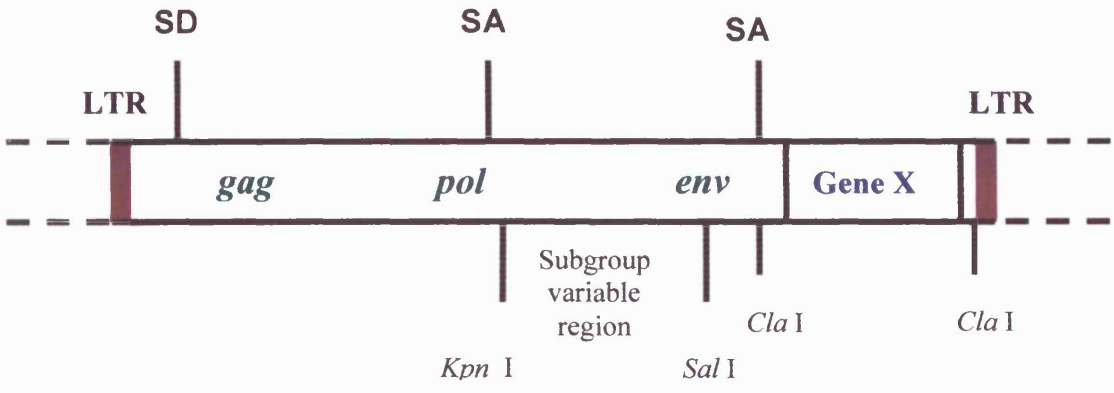


Figure 4.1: RCAS retroviral vector. Avian leukaemia virus long terminal repeat (LTR) sequences (red shading) flank the retroviral gag, pol and env genes and the site used to insert the transgene (gene X). The *Kpn*I- *Sal*I sites were used to insert envelops genes of different subgroups (A, B and E). The *Cla*I sites used to insert the transgene. SD, splice donor, SA splice acceptor.

4.1 Materials & Methods

4.1.1 Preparation of RCAS Vector

The RCAS vector used in this project was cloned into competent XL-1 *E.Coli* as follows:

The XL-1 blue competent cells were thawed on ice for 15 minutes and then 150µl of cell suspension mixed gently with 1µl of RCAS vector (50ng) by flicking the tube for 4-5 times. The tube was placed on ice for 20 minutes, followed by a heat-shock incubation in a pre-warmed water bath without shaking for exactly 45 seconds at 50°C. The tubes were returned immediately to ice and left for 10 minutes. SOC medium (200µl) was added into the tube containing the cells. The tube was incubated at room temperature for 25 minutes. The content of the tube was then spread on two *Luria broth* (LB) Ampicillin agar plates, incubated at room temperature for 30 minutes, and then overnight at 37°C.

On the following day colonies were picked up and inoculated separately into 5 ml of freshly prepared sterile LB medium. The tubes were incubated with continuous shacking (250 rpm) at 37°C over night.

4.1.2 Isolation of RCAS Plasmids from the Competent Cells XL-1

This procedure was done using the plasmid mini prep (Qiagen), all buffers used are included in the kit.

After overnight incubation 1.5 ml of each culture was transferred into a sterile DNase/RNase free eppendorf microcentrifuge tube. The remaining cultures of the tubes were frozen in liquid nitrogen and then stored at -80°C for future use. All 1.5ml tubes containing the cells were centrifuged in a microcentrifuge for 5 minutes at 10,000 rpm. The resulting supernatant in each tube was discarded and the bacterial pellet resuspended in 250µl of P1 buffer. P2 buffer (250µl) was added gently into the tubes which were then inverted 4-5 times. Buffer N3 (350µl) was added into the solution, and the tubes were inverted immediately 4-6 times and centrifuged for 10 minutes at 13,000rpm.

The supernatants were pipetted onto QIAprep spin columns and centrifuged for 60 seconds. The flow-through was discarded. The QIAprep spin columns were washed by adding 0.5ml of PB buffer and centrifuged for 60 seconds. The resulting flow-through was again discarded and the QIAprep spin columns were washed by adding 0.75ml of PE buffer and centrifuged at full speed (13,000 rpm) for 60 seconds. The same procedure was repeated again at the same speed to remove residual wash buffer. The QIAprep spin columns were placed into fresh DNase/RNase free 1.5ml microcentrifuge tubes, and then the DNA was eluted by adding 40µl of EB buffer (10nM Tris-Cl, pH 8.5) to the centre of each QIAprep spin columns. The columns were left on the bench surface at room temperature for 1-2 minutes. They were then centrifuged at maximum speed 13,000 rpm for 1 minute. The DNA concentration of RCAS plasmid was measured by using a DNA/RNA spectrophotometer.

4.1.3 Cutting RCAS Plasmid with *ClaI* Restriction Enzyme

The chicken SAP open reading frame was inserted into the RCAS plasmid using the *Cla*I restriction sites. Briefly, 50ng of the plasmid was restricted with 2ml of *Cla*I according to the following Table:

Reagents	Amount
Buffer C (Promega)	5 µl
BSA (Promega)	0.2 µl
RCAS plasmid (50ng)	2 µl
*Cla*I restriction enzyme	2 µl
dH_2O	10.8 µl
Total	**20 µl**

Table 4.1: Cutting RCAS plasmid

The reaction was incubated overnight at 37°C.

In order to examine that the restriction enzymes were cut the plasmid, the cut plasmid and uncut plasmids were run on 1% agarose gel, see (Figure 4.3)

4.1.4 Dephosphorylation of the Cut RCAS Plasmid

Dephosphorylation of linearised plasmids is a method employed to prevent the majority of plasmids simply recircularising during the ligation step. It is especially useful when both protruding termini of the linearised plasmid have been cut with the same enzyme (sticky ends) but is also used in blunt-ended ligations. The cut RCAS plasmid was dephosphorylated using alkaline phosphates as follows:

Reagents	Amount
Alkaline phosphates	4 µl
Buffer supplied	4 µl
Cut RCAS plasmid	3 µl
dH_2O	9 µl
Total	**20 µl**

Table 4.2: Dephosphorylation of the cut RCAS plasmid

The reaction was incubated at 37°C for 1 hour.

4.1.5 Isolation of RNA from the Sternal Chondrocytes

The 17 day chick sterna were dissected from the chick embryo bodies and rinsed twice with DMEM serum free medium to remove all blood contamination. The sterna were chopped into small pieces of tissue and digested in collagenase/trypsin for 2 hour at 37°C. The resulting suspension was then filtered through a falcon cell strainer. The suspension was centrifuged at 1800 rpm for 5 minutes at room temperature. The cell pellet was resuspended in 5ml of DMEM-10% FCS and the tubes were centrifuged at the same speed for another 5 minutes. This process was repeated three times. The pellets were lysed in 350µl of RLT buffer and vortexed for 30 seconds. The lysate was pipetted directly onto a QIAshredder spin column placed in a 2ml collection tube and the columns were centrifuged for 2 minutes at maximum speed. One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The lysate mixture (700µl) was then applied to a RNeasy mini column placed in a 2ml-collection tube. All tubes were closed gently and were centrifuged in a microcentrifuge for 15 seconds at 10,000rpm and the flow-through was discarded.

During this procedure, DNase digestion was performed by using the RNase-Free DNase kit (Qiagen). Aliquots (350µl) of RW1 buffer were pipetted into the RNeasy mini column and they were centrifuged for 15 seconds at 10,000 rpm to wash and then the flow-through was discarded. The columns were placed on the bench at room temperature and 10µl of DNase I stock solution (see RNase-Free DNase kit's hand book; Qiagen) were added to 70µl of RDD buffer and the mixture was mixed gently by inverting the tube. The DNase I incubation mix (80µl) was directly applied on the RNeasy silica-gel membrane, and then the columns were allowed to stand at room temperature (20-30°C) for 15 minutes. After the 350µl of RW1 buffer were pipetted into RNeasy mini columns, they were centrifuged for 15 seconds at 10,000 rpm. The flow-through and collection tubes were discarded and the columns were transferred into new 2ml collection tubes and 500µl of RPE buffer were pipetted onto the RNeasy columns. The tubes were closed gently and were washed by centrifugation for 15 seconds at 10,000 rpm, and the flow-through was discarded. The mini columns were further washed with another 500µl of RPE buffer and centrifuged for another 2 minutes at the same speed. The columns were placed into new collection tubes and they were centrifuged for 1 minute to eliminate any possibility of buffer RPE carryover. The tubes were transferred to 1.5ml RNase DNase free collection tubes and 30-50 µl of RNase-free water were applied directly onto the RNeasy silica-gel membrane. The tubes were closed gently and then centrifuged for 1 minute at 10,000 rpm. The RNA isolated from the 17 day chick embryo was used for preparing cDNA using the RT-PCR as described in chapter 3 (Table 3.1).

4.1.6 RT-PCR Reaction of the Open Reading Frame of SAP Fragment

In order to be able to clone the open reading frame of SAP gene into RCAS viral vector we designed a pair of primers that amplify the open reading frame of SAP sequence. These primers were designed to have *Cla*I restriction sites (ATCG) attached to the end of each (forward and reverse) primer. These primers amplify a fragment of 557 bp of the open reading frame SAP gene. Also, a 6xHis-tag sequence was added to the C-terminal of the reverse primer. The 6xHis-tag would be useful in subsequent immunolocalisation of the recombinant SAP in transfected chondrocytes using the 6xHis-tag antibody.

SAP forward primer with *ClaI* restriction site:

ClaI Site SAP forward

$^{5'}$ TTA A ATCG A TGA GCT CGG CCC GGG AGT G $^{'3}$

SAP reverse primer with ClaI restriction site and 6xHis-tag sequence:

ClaI Site 6xHis-Taq SAP reverse

$^{5'}$ TATA ATCG ATTA GTG GTG GTG GTG ATG ATG TGA ACC ACT GCT TTA AC ‘

The PCR reaction was cycled at:

95°C for 2 minute → [95°C for 30seconds, At* 55 °C for 1minute, 75°C for 30seconds] × 30cycles → 75°C; 5minutes, 4°C ∝

This PCR reaction was repeated for 30 cycles.

* The optimum annealing temperature of open reading frame SAP primers in the PCR reaction.

4.1.7 Ligation of the Open Reading Frame SAP Fragment into RCAS Vector

The ligation of SAP open reading frame cDNA, using the RCAS viral vectors was prepared as follows:

The tube containing the RCAS vector was briefly centrifuged and placed on ice. The ligation reaction mixtures were set up as shown in Table 4.3. To give a maximum number of transformants, the tubes were incubated at room temperature for 24 hours.

Reagents	Amount
2 X ligation buffer	2 µl
Cut RCAS vector	2 µl
SAP cDNA	2 µl
T4 ligase	1 µl
dH_2O	3 µl
Total	**10 µl**

Table 4.3: standard protocol for RCAS vector ligation reaction

4.1.8 Transformation of RCAS-SAP Vectors into XL-1 Blue Cells

High-efficiency competent cells (XL-1 blue *E.Coli* strain) were used for the transformation of RCAS vector containing SAP open reading frame fragment. The XL-1 competent cells were thawed on ice for 15 minutes. The tube containing the ligation reaction was centrifuged to collect the contents at the bottom of the tube. The competent cells were mixed gently by flicking the tube for 4-5 times, and 100µl of these cells was added into ligation tube and mixed gently. The tube was placed on ice for 20 minutes, followed by a heat-shock incubation in a pre-warmed water bath without shaking for exactly 45 seconds at 50°C. The tube was returned immediately to ice and left for 10 minutes. SOC medium (200µl) was added into the tube containing the cells which was transformed with ligation reaction; the tube was incubated at room temperature for 25

minutes. The content of tube was spread on the top of two *Luria broth* (LB) agar plates and incubated at room temperature for 30 minutes, and then over night at 37°C.

4.1.9 Isolation of RCAS-SAP Plasmids from the XL-1 Competent Cells

After over night incubation, 20 colonies were selected (10 colonies from each plate) for the isolation of RCAS plasmids containing SAP open reading frame fragment with *Cla*I sites. All these selected colonies were inoculated separately in fresh 2ml of LB-broth containing Ampicillin (100µg/ml). All tubes containing cultures were grown overnight at 37°C with continuous shaking (300 rpm). After the incubation period (16 hours); 1.5 ml of each culture was transferred into a sterile DNase/RNase free tube. The remaining cultures were kept at 4°C for future use. All prepared tubes containing the cells were centrifuged in a microcentrifuge for 5 minutes at 10,000 rpm. The resulting supernatant in each tube was discarded.

For isolating the RCAS plasmids from the Xl-1; Wizard plus SV Mininprep DNA purification system kit (Promega) was used. The bacterial pellets were resuspended thoroughly in 250µl of cell resuspension solution. The cell lysis solution buffer (250µl) was added gently into each sample and the tubes were inverted 4-5 times. Alkaline protease solution (10µl) was added into each tube and the samples were inverted 4 times to mix. All samples were placed onto 1.5ml rack and incubated at room temperature for 5 minutes. Neutralization solution (350µl) was added into the samples and the tubes were inverted again 4 times. All samples were centrifuged at top speed (13,000 rpm) for 10 minutes at room temperature. During the centrifuging process the spin columns provided with the kit were inserted into collection tubes. The resulting cleared lysate were decanted into the spin columns. The tubes were then centrifuged at full speed for 1 minute at room temperature and the flowtrough was discarded. The columns were reinserted into the collection tubes followed by adding 750µl of wash solution (ethanol added) into each column. The columns were centrifuged at full speed for 2 minutes at room temperature. All columns were transferred to sterile 1.5ml microcentrifuge tubes. 50µl of nuclease-free water were added to the spin columns and centrifuged at full speed for 1 minute at room temperature. The columns were discarded and the RCAS-SAP plasmids (DNA) were stored at -20°C.

4.1.10 Cutting RCAS-SAP Plasmids with *ClaI* Restriction Enzyme

In order to check that the RCAS plasmids isolated from 20 different colonies contained the open reading frame SAP fragment. All 20 samples were cut with *Cla*I restriction enzyme. This cutting procedure should release SAP fragment (557 bp) from the RCAS vector. This cutting producer was prepared as illustrated in Table (4.5)

Reagents	Amount
10 x buffer (Promega)	2µl
Spermidine (100mM/µl)	1µl
ClaI enzyme 10u/µl (Promega)	1µl
dH_2O (Sigma)	6µl
DNA samples (RCAS)	10µl
Total	**20µl**

Table 4.5: cutting RCAS plasmid with *ClaI* restriction enzyme

The reaction mix was prepared for 20 samples. All samples were centrifuged briefly for 30 seconds and incubated for 2 hours at 37°C. The reaction was stopped by incubating the samples at 65 °C for 5 minutes. Aliquots 10µl of each sample were mixed with 3µl of loading dye and run on 1% agarose gel. Samples number 9 and 17 were selected for the PCR reaction with SAP open reading frame primer. These two samples were the only samples that gave bands of 557 bp on the agarose gel. The sizes of these two bands were the same size as that of the SAP fragment inserted into the RCAS vector. These two samples (plasmids 9 and 17) were then used as template for the PCR reaction with SAP full length primers to make sure that they give the exact size of SAP open reading frame fragment (Figure 4.3).

4.1.11 PCR Reactions of the RCAS-SAP Plasmids with SAP Open Reading Frame Primers

The two selected samples (9 and 17) were used as a template for the PCR reactions. The primers used in this reaction were the SAP open reading frame primers (Table 4.6) these primers were originally used to amplify the SAP open reading frame fragment.

Primers	Sequence
Forward	5' TTA A ATCG A TGA GCT CGG CCC GGG AGT G '3
Reverse	5' TATA ATCG ATTA TGA ACC ACT GCT TTA AC '3

Table 4.6: SAP primers sequence

The reaction was prepared as follow:

Reagents	Amount
10x PCR buffer	5µl
$Mgcl_2$ (25mM/µl)	3µl
dNTPs (10mM/µl)	1µl
Forward SAP primer (50mM/µl)	1µl
Reverse SAP primer (50mM/ml)	1µl
cDNA (RCAS plasmid;1:20 dilution)	1µl
Taq polymerase	0.25µl
dH_2O (Sigma)	37.75µl
Total	**50µl**

Table 4.7: PCR reaction of SAP open reading frame fragment

The reaction was run for 30 cycles and the annealing temperature used was 55°C, and 15µl of the PCR products of each sample (SAP 9 and SAP 17) were mixed with 3µl of lading dye and run on 1.5% agarose gel. The 174 DNA/*Hae* III DNA marker (10µl) was run beside the two samples (Figure 4.4).

4.1.12 Termination of the Correct Orientation of SAP Open Reading Frame Fragment Cloned into RCAS Vector by using RT-PCR

After SAP full length fragment was generated and cloned successfully into the RCAS vector. It was necessary to determine the correct orientation of the cloned sequence. This determination was done by designing two forward and reverse primers for RCAS vector just before the cloning site of the vector.

The two samples (9 and 17) which contain the correct size of SAP were used again as templates for the PCR reactions. In this PCR reaction, two different pairs of primers were used (SAP primers) which is shown in Table 4.6 and (RCAS primers) illustrated in Table (4.9)

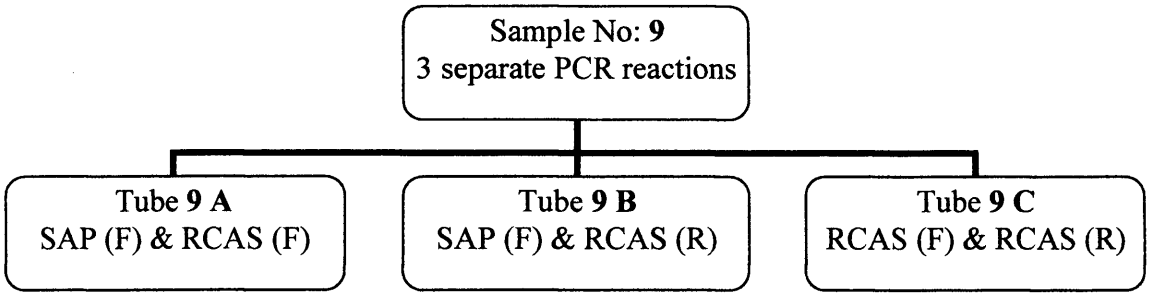
Primers	Sequence
Forward	5' ACG CTT TTG TCT GTG TGC TGC '3
Reverse	5' ATC TCT GCA ATG CGG AAT TCA GTG '3

Table 4.9: Primers of RCAS plasmid

4.1.13 PCR Reaction of SAP and RCAS Primers

The protocol of the PCR reaction used in this experiment was exactly as shown in Table 4.7. However, in this reaction two pairs of primers (SAP and RCAS) were used into this PCR reaction. These primers were used with the two DNA samples (9 and 17). The use of these primers into the PCR reaction was as shown in diagram (4.2). The PCR reaction was run for 30 cycles and the optimum annealing temperature of both SAP and RCAS primers was 58°C.

(A)



(B)

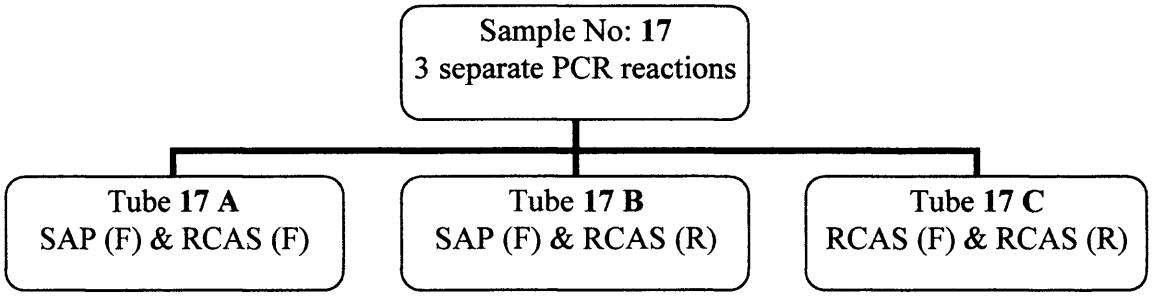


Figure 4.2: A diagram shows the experimental design of the PCR reactions using the SAP and RCAS primers.

(A) Shows the forward and reverse primers used into three different PCR reactions of samples 9.

(B) Shows the forward and reverse primers used into three different PCR reactions of samples 17.

4.1.14 Sequencing RCAS Plasmid Containing SAP Open Reading Frame Sequence

The PCR results obtained from the experiment in section 4.1.13 has led to determine the RCAS plasmid that has to be sequenced. RCAS plasmid No: 9 was selected to be the correct sample for sequencing. ABI Prism Bigdye terminator cycle sequencing ready reaction kit was used for sequencing the RCAS plasmid. The reaction was prepared as follow:

Reagent	Quantity
Terminator ready reaction mix	4.0µl
Template (RCAS plasmid) 500 ng	5 µl
Primer (3.2 pmol)	1µl
dH_2O	3.6 µl
Total	**10µl**

Table 4.10: Preparation of the sequencing mixture of the PCR reaction

The primers that were used for sequencing included SAP and RCAS forward and reverse primers are listed in Table 4.11

Primers		Sequence
SAP	Forward	5' TTA A ATCG A TGA GCT CGG CCC GGG AGT G 3'
	Reverse	5' TATA ATCG ATTA TGA ACC ACT GCT TTA AC 3'
RCAS	Forward	5' ACG CTT TTG TCT GTG TGC TGC 3'
	Reveres	5' ATC TCT GCA ATG CGG AAT TCA GTG 3'

Table: 4.11: Primers used for sequences the RCAS plasmid containing the SAP fragment

The master mix was made for three sequencing reaction. The contents of each tube were mixed well and spin briefly.

4.1.15 Cycle sequencing of RCAS Plasmid on the Gene Amp PCR System 9700

The tubes were placed in the PCR thermal cycler and the following PCR cycle was repeated for 25 cycles.

Rapid thermal ramp to 96°C; (96 °C for 10 seconds), Rapid thermal ramp to 55°C; (55°C for 5 seconds), Rapid thermal ramp to 60°C; (60°C for 4 minutes).

Rapid thermal ramp at 4°C and was hold until purification process.

4.1.16 Precipitation of the PCR Sequencing Reaction of RCAS Plasmid Containing the Open Reading Frame of SAP Fragment

The contents of all three different PCR reactions were centrifuged briefly for 10 seconds. Double dH_2O (40µl) was added separately into each tube. The reaction mix of each tube was transferred individually into fresh 1.5 ml DNA/RNA free tubes. NaAc pH 5.2 (5µl) was added into each reaction followed by adding 125 µl of 95 % ethanol. The content of each tube was mixed and vortex briefly and then incubated at room temperature for 20 minutes. The reactions were centrifuged at full speed (13,000 rpm) for 15 minutes. The supernatants were removed carefully and 175µl of 70% ethanol were added gently into each reaction. The tubes were centrifuged at full speed for 2 minutes. The 70% ethanol was removed slowly and the tubes were air dry at room temperature for 20-30 minutes.

4.2 Preparation of primary cell line

One dozen of eggs were incubated at 37°C until day 10 (approximately stage 36). The embryos were removed from the eggs and laid in a 10-cm Petri dish. The limbs and heads were removed using a sterile razor blade and eviscerated with forceps. The remained embryo trunks were collected in a fresh 10-cm Petri dish and minced thoroughly with a razor blade (this step was important as it greatly increase the efficiency at which the trypsin solution dissociates the cells). The chopped embryos were collected into Erlenmeyer flask containing 10ml of 0.25% trypsin, 1mM EDTA solution (Gibco). The contents was shaken gently for 60 minutes on a rotator, also the flask was shaken by hand every few minutes. When the small chunks of tissue were disappeared the flask was taken off the rotator and left on the bench surface for a few

minutes until all big chunks of tissue were settled. The supernatant was transferred to a 50ml centrifuge tube (Falcon sterile plastic tube). Another 50ml of fetal calf serum (FCS) were added into the suspension and mixed gently for a few seconds. The mixture was left to stand for 5 minutes in the tissue culture hood to allow any remaining tissue chunks to settle. The suspension mixture was decanted into a fresh 50ml tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in 10ml of 100% FCS, the mixture was spun again, and resuspended in DMEM supplemented with 10% FCS and 2% chicken serum (Sigma).

The number of cells in the suspension was counted using a conventional haemocytometer. The entire preparation was plated onto the appropriate number of 10cm tissue culture dishes at three different concentrations: 10^7cell/dish, 10^6cells/dish and 3×10^5 cells/dish. The cultures were monitored daily until they began to recover and started to divided very rapidly. As soon as the healthiest dishes became close to confluent, it was taken and harvested. The cells were harvested by adding 0.10% trypsin for 15 minutes. The enzyme was removed and the cells were rinsed with two washes of PBS, another 1ml of 0.10% trypsin was added for a few minutes. As soon as the cells began to lift off the dish and separated, 3ml of DMEM supplemented with 10% FCS and 12% DMSO were added to the solution and mixed gently until the cells clumps were disappeared. An aliquot amount of 0.5ml was placed into 1.5ml sterilized tubes and all tubes were put on dry ice and were stored at -80°C.

4.3 Transfection of Viral Supernatant Production

Two tubes of the frozen chicken fibroblast cell line stock were thawed on ice. The cells were plated onto 10cm dishes and cultured overnight in 10ml DMEM (supplemented with 10%FCS and 2% CS and Penicillin-streptomycin solution, Gibco-BRL). After overnight incubation the cells reached approximately 70-80% confluence, 10ml of fresh medium were added to the culture 4 hours before the transfection. RCAS-transgene plasmid DNA (10μl) were diluted with distilled water to a final volume of 450μl, followed by adding 50μl of 2.5 M $CaCl_2$. The solution and was mixed well and incubated at room temperature for 20-30 minutes. After this incubation period, the DNA

(RCAS plasmid) solution was added drop wise onto the dish of the cells while slowly swirling the medium in the plate. The culture was incubated overnight at 37°C.

The following day, the cells were confluent and split to two 15cm dishes. The cultures were incubated overnight again. Next day a small aliquot of the 0.1 % trypsin was added into each culture and the cultures were incubated for 30 minutes. The cells were rinsed and washed twice with completed DMEM medium and the cells were plated on a 24-well cell culture plate. In order to confirm that the transfection procedure was successful, after a day of culture these cells were stained with the 3C2 monoclonal antibody (mAb), which recognizes the viral MA antigene.

4.4 Results and Discussion

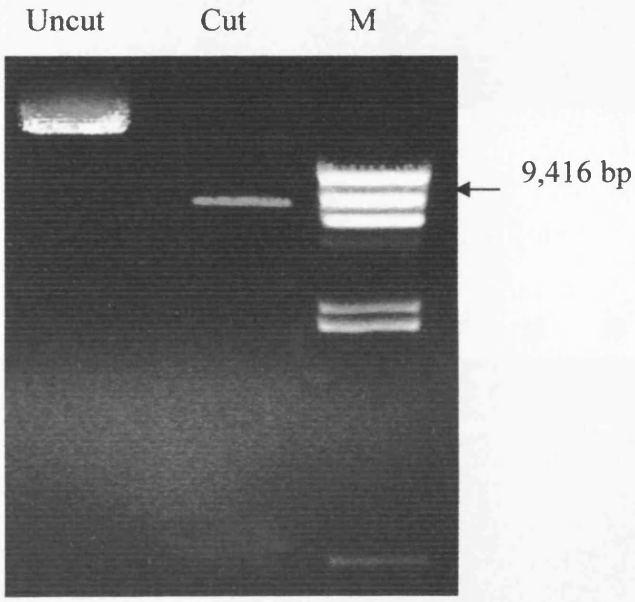
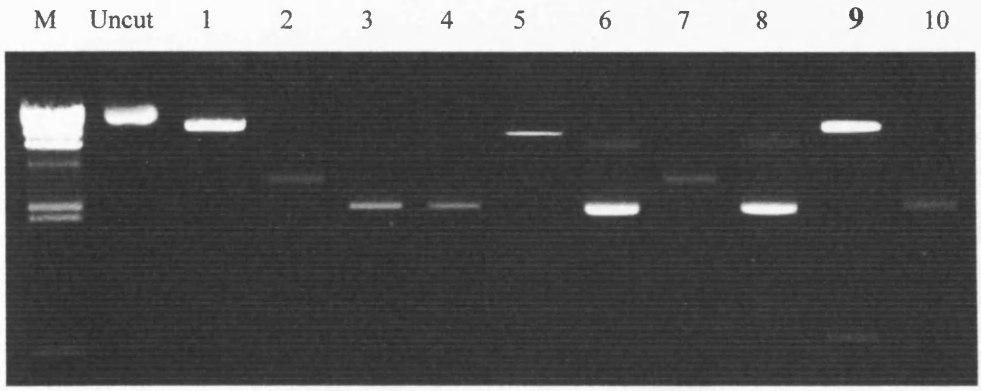


Figure 4.3: Gel electrophoresis photos showing the uncut (lane 1) and the RCAS plasmid cut with *Cla*I restriction enzyme (lane 2) plasmids. M = lambda *Hind* III marker.

(A)



(B)

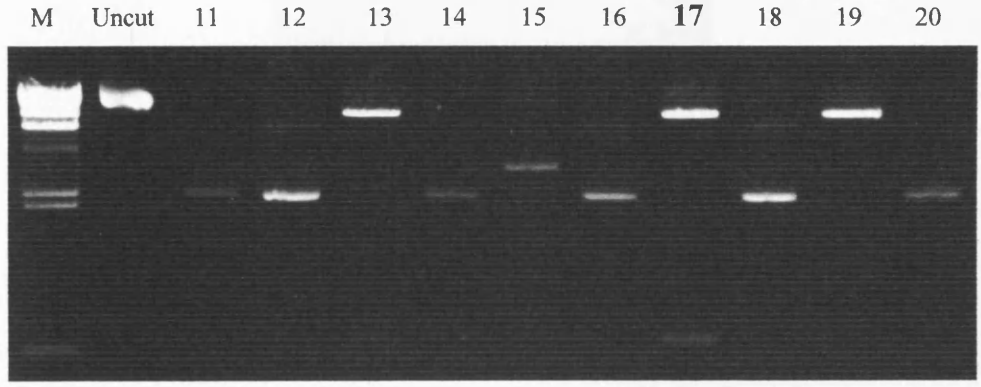


Figure 4.4: Agarose gel showing the uncut plasmid (RCAS) and the 20 samples of cut RCAS plasmid containing the open reading frame SAP sequence (557bp). All samples were cut with *Cla*I restriction enzyme to release the inserted open reading frame sequence of SAP. Lanes 1-20 are RCAS plasmids isolated from different colonies. Only two samples lanes 9 and 17 showed the corrected size of the inserted gene SAP whereas the other samples showed different sizes of inserts. These sizes are not equal to the SAP open reading frame sequence therefore they were not containing the correct insert (SAP).

(A) Showing the uncut and cut RCAS plasmids. Sample 9 is showing the correct size of SAP fragment.
(B) Showing the uncut and cut RCAS plasmids. Sample 17 is showing the correct size of SAP fragment.

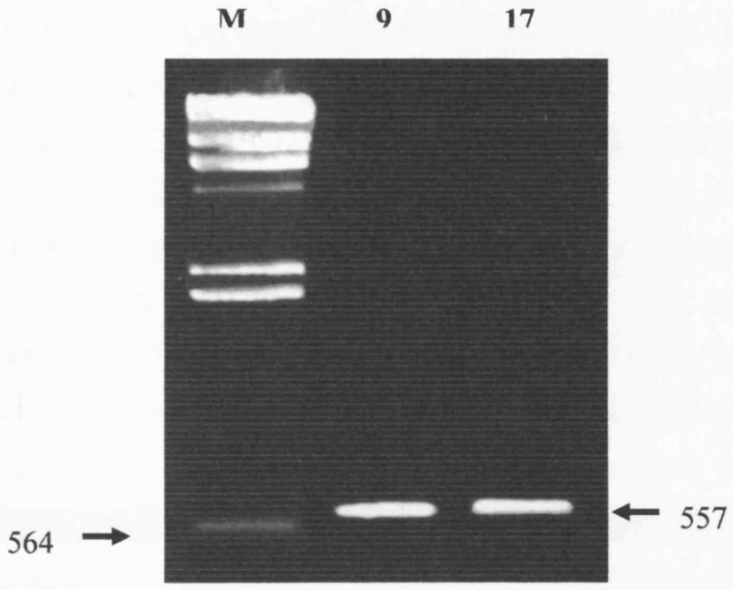


Figure 4.5: Gel electrophoresis showing RT-PCR products of SAP open reading frame fragment. The cDNA used in this PCR reaction were samples 9 and 17 of the RCAS plasmids containing SAP open reading frame sequence. Two bands of samples 9 and 17 showing the correct size of the insert.

CGG AGC TGA GCT GAC TCT GCT GGT GGC CTC GCT CTA CCA CTG
TGG C ATCG ATG AGC TCG GCC CGG GAG TCG CAG GCT CGC CAC GGC
CTC AAG CGA GCG GCC TCT CCC GAC GGC TCC GGC AGC TGG CAG
GCG GCG GAC CTC GGC AAC GAG GAG AGG AAG CAG AAG TTC CTG
AGG CTG ATG GGC GCG GGG AAG AAA GAA CAT ACT GGC CGC CTT
GTT ATC GGA GAC CAC AGA TCA ACC TCT CAC TTC AGG ACA GGG
GAA GAA GAC AAG AAA ATG AAT GAA GAA CTG GAG TCT CAG TAC
CAG CAG AGC ATG GAC AGC ACG ATG TCT GGA CGA AAC CGG CGC
CAT TGT GGA CTG GGT TTC AGT GAG TTT CAG GAA GGT GAA GAG
GAG GCA GCT GGG CAT TCC TCT GAC CAT GAG AGT TCA GAG GAC
TCC GAA AGT GGC TCT GAT TCA GAG CAA GAT GAA TCT GCA GAG
GAA CTG CAA GCT GCT GAA AAA CAC GAT GAA GCT GCG GTC CCA
GAA AAC AAA AAG GAA GCA AAA AGC AAT TAT AAA ATG ATG TTT
GTT AAA GCC AGT GGT TCA CAT CAT CAC CAC CAC CAC **TAA** T CGAT
G CGA TGT ACG GGC CAG ATA TAC GCG TAT CTG AGG GGA CTA GGG
TGT GTT TAG GCG AAA AGC GGG GCT TCG GTT GTA CGC GGT TAG
GAG TCC CCT CAG GAT ATA GTA GTT TCG CTT TTG CAT AGG GAG

Figure 4.6: The sequence of RCAS plasmid containing the open reading frame of SAP fragment. Assession number: CR353822.

*Cla*I restriction sites

SAP forward and reverse primers

6xHis-Tag sequence

ATG Start code

TAA Stop code

The present study aims to investigate the effect of over expression of SAP in vitro in embryonic chick using an avian viral vector (RCAS A). All preparative procedures used throughout this project are described in detail in the section 'Materials and Methods'. A brief account of the main preparative steps is also described in this section as required.

First of all, a small amount of RCAS vector (donated by Dr. I. Khan, Cardiff School of Biosciences) was cloned into XL-1 *E.Coli* competent cells which were then allowed to grow for 24 hours. Some of the colonies were collected under sterile conditions following the 24 hours culture, and incubated overnight in LB medium. The RCAS plasmids were isolated from these cells. 1µl and 5µl of the plasmids were run on 1% agarose gel along side with 5µl of lambda *Hind* III marker. This RCAS plasmid was then cut with *Cla*I restriction enzyme to create a *Cla*I restriction site in which the open reading frame of SAP fragment should fit. The cut and uncut RCAS plasmids were run side by side on agarose gel with the uncut one used as a control. As shown in Figure. 4.3, the cut RCAS plasmid appears as a single band and is about 9.4 kb.

Following dephosphorylation of the cut RCAS with Alkaline phosphatase, the ligation of SAP open reading frame cDNA was performed using the avian viral vector (RCAS A). The RCAS vector containing the SAP open reading frame fragment was then transformed with XL-1 competent cells and spread on the top of two *Luria broth* (LB) agar plates. After an overnight incubation, 20 colonies were selected (10 colonies from each plate) from the isolation of RCAS plasmids containing the SAP open reading frame fragment with ClaI sites. All these selected colonies were inoculated separately in fresh 2ml of LB-broth. In order to check that the RCAS plasmids that were isolated from 20 different colonies contained the open reading frame SAP fragment, all 20 samples were cut with *Cla*I restriction enzyme. This cutting procedure should release SAP fragment (557 bp) from the RCAS vector. As shown in Figure. 4.4, two samples (lane 9 and lane 17) show a band at 557 bp, the same size as that of SAP fragment inserted into the RCAS vector. These samples (SAP 9 and SAP 17) were used as template for the PCR reaction with SAP primers. The PCR was run for 30 cycles and the reaction products were mixed with loading dye and run on agarose gel along side with 174 DNA/*Hae* III DNA marker. As shown in Figure. 4.5, the two bands show the correct size of the insert.

After the SAP open reading frame fragment was generated and cloned successfully into the RCAS vector, it was necessary to determine the correct orientation of the cloned sequence. This was done by designing two forward and reverse primers for the RCAS vector. The two samples (9 and 17) which contained the correct size of SAP were used again as templates for the PCR reactions. In this case, two different pairs of primers were used (SAP primers) and (RCAS primers) with their respective DNA. The PCR reaction was run for 30 cycles. The resulting sequence reveals the open reading frame of cloned SAP fragment and confirms that the gene was cloned into the RCAS vector at the correct orientation as shown in Figure. 4.6. This open reading frame of cloned SAP fragment was used to transfect the sternal chondrocytes.

The chondrocyte cell lines were prepared as described in detail in the 'Materials and Methods'. In brief, the embryos were removed from chicken eggs (12 in total) after 10 day incubation. With the limbs and heads removed, the remaining embryo trunks minced, chopped and trypsinised. Following a succession of resuspension in FCS followed by DMEM and centrifugation steps, the number of cells in the suspension was counted using a conventional haemocytometer and plated onto culture dishes at three different concentrations (10^7cell/dish, 10^6cells/dish and 3 $\times 10^5$ cells/dish).. The confluent cells were harvested, trypsined, washed in PBS, mixed with supplemented DMEM. As required, the cells were plated, cultured overnight in 10ml supplemented DMEM and used for transfection. After the cells reached approximately 70-80% confluence, diluted RCAS-transgene plasmid DNA solution was added. After an overnight incubation, the cells were trypsinised, washed with DMEM medium and plated on a 24-well cell culture plate. After 24 hours culture, these cells were stained with the 3C2 monoclonal antibody (mAb). This antibody binds to the transfected chondrocytes and gives a blue colour which can be used as an indication for a successful transfection. However, the results had shown no blue colour. This indicates that the chondrocytes were not successfully transfected with RCAS/SAP vector .

A number of factors may contribute to this unsuccessful transfection. It is generally known that cells become infected by retroviruses as a result of the interaction that takes place on the surface of the cells between the viral envelope glycoprotein and a related receptor. This interaction ultimately causes the viral membrane to fuse with the membrane of the target cell. As a result of this fusion, the virion core enters the cytoplasm of the target cell. Different retroviral envelopes recognise different receptors on the surface of the host cells. For example, the avian sarcoma-leukosis virus (ASLV) family that included RCAS, ALV and RSV vectors have a variety of envelop subtypes such as A-J and A-E. According to Bates *et al* (1993), the cellular receptor for the A envelope is tva, a protein that is related to an LDL receptor repeat. Amongst these vectors, that of RCAS was found to replicate efficiently in avian cells. Since most chicken strains contain endogenous pro-viruses that are closely related to the family of avian sarcoma leukosis virus (ASLV), these pro-viruses are also related to the RCAS vectors that originate from ASLV. Consequently, this can lead to recombination between the RCAS vector and these endogenous viruses. This problem has been previously reported by Hughes (2004).

Furthermore, it has been generally acknowledged that there are potential problems when designing inserts for RCAS vectors. Some of these problems are as follows: (1) whereas some sequences contain direct repeats or duplicate other regions of the vector genome, the others can interfere with the expression of the full length viral genome; (2) there are also sequences that are too large for the vector as well as those that are toxic for the host cells when expressed; (3) it is difficult to use the RCAS vectors to express genes that are toxic and/or interfere with cell growth; (4) cells that are made to express proteins that are toxic or interfere with cell growth, will be overgrown by cells that don't express these proteins; and (5) attempts to use the RCAS vectors to express toxic proteins often result in the loss of the inserted gene.

Another potential problem is that viral passage can lead to the loss of inserted sequences. When the cells are not fully infected by an RCAS vector, proviral DNA also becomes unstable.

The RCAS (Replication Competent ALV LTR with a Splice acceptor) vector was used in the present study because it has been reported to offer the following advantages over the replication-defective vectors: (1) there is no problem with recombination between the vector and the inserted sequences; (2) there is no need to provide a selectable marker or select infected cells; (3) The RCAS contains a short sequence that can be recognise by an antibody (4) because a replication-competent vector spreads rapidly, it will infect all the cells in a culture dish in a short time. However, it is apparent in the present case that the cells have not been successfully transfected with the use of RCAS vectors. One of the possible causes is that the inserted gene may be toxic to the cells that are supposed to express SAP, but instead encourages the overgrowth of those that do not express this protein. This consideration suggests using a different cell line. As a rule of thumb, the host cell must be in a healthy state if viral replication is to be efficient. Furthermore, retroviral vectors can efficiently transfer the genes they carry into the genome of the host cells, only if retroviral integration is efficient.

The RCAS-SAP vector can be used to infect chondrocyte culture and therefore over expression SAP in these cells. The high level of SAP expression can be monitored using an anti 6xHisTag antibody. This will show whether the newly expressed recombinant SAP is localised mainly within the nucleus. Thus confirming SAP is a nuclear protein, the expected results from such investigation would be a reduction in the maturation rate of infected chondrocytes. The expected changes between the normal and infected cultures are summarised as follows:

	Infected	Normal	
Alkaline phosphatase	↓	↑	with time
Type X Collagen	↓	↑	with time
Mineral deposition (Alizarin red staining)	↓	↑	
MMP 13	↓	↑	
GAGs and Hydroxyproline	↑	↓	
	Slow down cell Maturation	Mature faster	

CHAPTER 5

General discussion

5 General Discussion

The skeleton develops through two mechanisms, intramembranous and endochondral ossification. The former, during which mesenchymal cells develop into osteoblasts, is involved in the formation of flat bones as well as the appositional growth of most long bones. The latter is responsible for the development of most other bones. Endochondral ossification involves a two stage mechanism, whereby cells (chondrocytes) form a matrix template for example the growth plate, in which cellular events eventually lead to the replacement of the cartilage template by newly formed bone. This process requires careful coordination of proliferation, cell differentiation and the deposition of bone. Many regulatory genes have been shown to take part in the coordination of chondrocyte proliferation and differentiation (Vortkamp 1999). Ihh and PTHrP are implicated in this coordination. Earlier studies using murine tibial growth plate indicated that these genes coordinate the rate of chondrocyte proliferation and determine the time when the cells should leave the proliferative stage to begin differentiation (Kobayashi *et al.,* 2002; Maclean *et al.,* 2005). As suggested by Vortkamp *et al* (1996), Ihh and PTHrP are the major regulators of chondrocyte proliferation and differentiation, and they work together in a negative feedback loop.

The detection of high Ihh expression in the prehypertrophic cells by the present investigation adds further evidence to support the findings of several previous studies that Ihh is exclusively produced by prehypertrophic chondrocytes (Bitgood *et al.,* 1995; Koyama *et al.,* 1996; Lanske *et al.,* 1996; St-Jacques *et al.,* 1999 Seth et *al.,* 2000; Colnot *et al.,* 2000). The localisation of Ihh in the embryonic chick sternum and tibial sections obtained from the present investigation together with those of the earlier studies support the observation that Ihh is involved in the early stages of chondrocyte differentiation and that Ihh plays two important roles in chondrocyte proliferation, a direct stimulator and a regulator of this process. First of all, Ihh stimulates perichondrial cells to produce PTHrP through its receptor (Patched) and then co-ordinates with the PTHrP to regulate the rate of chondrocyte proliferation as well as the timing for cell differentiation (Long *et al.,* 2001; Karp *et al.,* 2000; St-Jacques *et al.,* 1999; Lanske *et al.,* 1998; Vortkamp *et al.,* 1996). The Ihh/PTHrP interaction prevents the cells from reaching maturation. Interestingly, there are recent reports that the Ihh/PTHrP

interaction takes place in a negative feedback fashion. (Yoshida *et al.,* 2001; Kindblom *et al.,* 2002 Karp *et al.,* 2000, Vortkamp, 1999, St-Jacques *et al.,* 1999 Bitgood *et al.,* 1995). One typical example of the Ihh/PTHrP negative feedback interaction is that in fetal bone, Ihh stimulates the periarticular cartilage to express PTHrP, which then acts on the prehypertrophic chondrocytes to inhibit cell differentiation (Kindblom *et al.,* 2002; Vortkamp, 1999). Vortkamp (1996) remarked that in the postembryonic growth plate, postproliferative chondrocytes secreted Ihh which increased secretion of PTHrP to slowdown hypertrophy and that consequently; the slowing down of hypertrophy will reduce the production of cells that secreted Ihh.

A novel gene product recently identified as a Small Acidic Protein (SAP), has also been suggested to play a part in endochondral ossification. Embryonic cartilaginous tissue or growth plate of tibia has been extensively used in most of the earlier studies on endochondral ossification and in particular the development of the cartilage growth plate. Another type of cartilaginous tissue, the embryonic chick sternum has also been used to study bone development in recent years but to a lesser extent. While in most cases, both the sternum and tibia have been used to localise type X collagen, a well known marker of chondrocyte hypertrophy, the latter has been an important model system in the elucidation of the Ihh/PTHrP negative feedback loop in the regulation of endochondral ossification. SAP has so far not been extensively studied especially its involvement in chondrocyte maturation. This Small Acidic Protein was first reported in quail (Gong *et al.,* 1997) and then later in chick (Webster, 1999). SAP has also been reported in cells from mammalian tissues including the human heart, brain, liver and placenta. As reported by Webster (1999), the SAP gene is short in quail, chick and human. The chick mRNA is 758 nucleotides in length including the poly- A tail and the predicted peptide is 173 amino acids long. The mRNA and peptide sequences are highly conserved amongst different species. Sequence comparisons revealed that it shares 92% and 60% nucleotide homology and 95% and 74% amino acid identity with the quail and human SAP sequence respectively. Comparison of the human and chick genomic sequences indicated remarkable conservation of the gene structure. Both human and chick SAPs are coded for by 5 exons with highly conserved exon/intron boundaries. This high level of evolutionary conservation suggests that SAP has an important biological function. Previous studies by Webster (1999) have suggested that SAP may

reside within the nucleus and together with the predicted basic-helix-loop-helix secondary structure. This structure is one of the four forms transcription factors (see Chapter 3) SAP may function as a transcription regulator either by binding other nuclear proteins or directly to DNA. Although the molecular structure of SAP has been partially characterized and differential display PCR screen has shown that SAP gene expression is down regulated in hypertrophic chondrocytes *in vitro*, the localisation of SAP mRNA in the growth plate of the tibia and sternum has not yet been studied. Moreover, there has been little insight into what role SAP plays in the process of endochondral ossification.

The sternum has not been widely used in the studies of the bone development of cartilage growth plate. This observation has prompted the inclusion of both tissues in the present work. My project aims to examine the biological roles of SAP in endochondral ossification in particular the regulation of its expression. The experimental approaches employed in my project include *in situ* hybridisation, RT-PCR and biochemical analysis of the embryonic chick sternum and tibia in vivo. The experimental findings are summarised as follows:

- ISH showed that SAP was down regulated in the cephalic region of the embryonic sternum of the sternum.
- ISH showed that SAP was expressed in the proliferative and prehypertrophic zones f the tibia.
- ISH showed that SAP was expressed in the caudal and middle regions of the sternum.
- ISH showed that SAP was down regulated in the hypertrophic zone in the tibia.
- ISH showed that Ihh was expressed in the middle and cephalic region of sternum.
- ISH showed that Ihh was expressed in the prehypertrophic zone of the tibia.

- ❖ RT-PCR showed that type X expressed in the middle and cephalic regions of the untreated micromass culture.
- ❖ RT-PCR showed that type X collagen was expressed in the cyclopamine treated caudal chondrocytes micromass cultures.
- ❖ RT-PCR showed no differences in Ihh expression between the treated and untreated cultures.
- ❖ RT-PCR showed that SAP was expressed predominantly in the caudal and middle micromass cultures.
- ❖ RT-PCR showed that SAP expression was strongly suppressed in the cyclopamine treated micromass cultures.
- ❖ RT-PCR showed that PTHrP expression was inhibited in the treated micromass culture.
- ❖ RT-PCR showed that there was no difference in the expression pattern of Patched within the treated and untreated cultures.
- ❖ GAGs production was reduced in the treated micromass culture from all three different regions.
- ❖ Collagen biosynthesis was reduced in the treated micromass culture from all three different regions.

From a cellular/developmental perspective, tibia and sternum show a close similarity as confirmed by the present histological studies. The embryonic chick sternum is composed of three characteristic zones (caudal, middle and cephalic) distinguishable by their respective chondrocyte phenotypes: proliferative, prehypertrophic, and hypertrophic.

The present *in situ* hybridisation studies have shown for the very first time that SAP is predominantly localised in the proliferative chondrocytes although low intensity of expression is also observed in the prehypertrophic and hypertrophic cells. This pattern

of SAP expression is observed in both, sternum and tibia. Ihh is highly expressed in the prehypertrophic chondrocytes in both tissues and the expression continues throughout this transitional zone where the majority of cells are stained strongly, but the reactivity becomes less pronounced throughout the hypertrophic zone. Unlike SAP and Ihh, type X collagen is found to accumulate exclusively in the hypertrophic chondrocytes zone in these tissues.

In this project, *in situ* hybridisation was used initially to examine the expression pattern of SAP *in vivo*. Indian hedgehog and type X collagen mRNA localisations were used to define the boundaries of the prehypertrophic and hypertrophic zones respectively, since these are well known markers for cell maturation in cartilage. Also their expression domains have been clearly defined by other investigators. In the present study it was interesting to note that SAP is expressed in the prehypertrophic chondrocytes where Ihh is expressed suggesting that a possible functional link between SAP and Ihh. Therefore subsequent investigations were designed to examine any possible interactions between SAP and the Ihh/PTHrP feedback loop.

The expression of SAP was then investigated using RT-PCR. The PCR studies revealed that under normal culture conditions, the proliferative cells show high level of SAP, PTHrP and Patched expression but do not express Ihh and type X collagen. However, when treated with cyclopamine, these cells exhibited the reverse trend. In other words, the proliferative chondrocytes no longer express SAP and PTHrP but started to express Ihh and type X collagen. It is interesting to note that the response of the proliferative chondrocytes to cyclopamine with respect to the expression of SAP, Ihh and PTHrP varies depending on the duration of the treatment and cell phenotypes. Cyclopamine has a strong inhibitory effect on SAP and PTHrP expression not only in the proliferative but also in the prehypertrophic as well as hypertrophic chondrocytes. This inhibitory effect is observed throughout the duration of the experiments. By contrast, cyclopamine did not inhibit the expression of Ihh and type X collagen in the prehypertrophic chondrocytes and hypertrophic chondrocytes; instead, it up regulated the expression of these genes in the proliferative chondrocytes in which they are not normally expected to be expressed. The present study is consistent with the earlier observations that cyclopamine has the ability to inhibit cell response to Ihh receptor signalling (Incardona

et al., 1999; Chen *et al.,* 2002; Qualtrough *et al.,* 2004). Moreover, earlier studies using murine growth plates have shown that cyclopamine inhibited PTHrP expression by blocking Ihh signalling which directly affects the expression of PTHrP in the chondrocytes (Kim *et al.,* 1998; Chen *et al.,* 2002). In addition, it has been reported that the missexpression of PTHrP in mouse limbs could cause the chondrocytes to become prematurely hypertrophic (Nakase *et al.,* 2001). Taken together, it is concluded that there is a strong possibility that SAP is an integral part of the Ihh/PTHrP regulation in endochondral ossification.

Cyclopamine is shown to have no significant inhibitory effect on Patched expression. This is an unexpected finding since Patched is also a downstream target gene of the Hh signalling pathway. This observation may also suggest that the cyclopamine used in these experiments was not effective in blocking Ihh signalling. However, the effect of cyclopamine on PTHrP suggests that the Ihh signalling pathway was indeed inactivated. Further experiments examining the expression of other Hh signalling downstream targets such as the Gli proteins (Gli 1, 2 and 3) and Smo would be important to ascertain the effect of cyclopamine on the Ihh pathway in this system. Although a slight difference in the intensities of the Patched PCR products was observed with or without cyclopamine treatment, it was not possible to determine whether such changes are significant. Quantitative PCR would be a more suitable approach to examine any quantitative changes in the expression of the hedgehog target genes. Due to time constraint and problems with the primers and probes at the time, this approach was considered but not implemented. On the other hand, cyclopamine has previously been reported to have the ability to disrupt the Hh signalling (Kamenetsky *et al.,* 2002). Therefore the premise that cyclopamine has inhibited the Hh-signalling pathway in the present cell culture system was adopted.

It is evident from the PCR studies that SAP and PTHrP are expressed by the proliferative chondrocytes and that their expression is down regulated when the cells start to differentiate. In this project a concomitant up regulation of Ihh with the down regulation of SAP and PTHrP expression has been observed. These findings confirmed that Ihh expression was up regulated when chondrocytes leave the proliferative phase. Another interesting observation is that SAP, Ihh and PTHrP are shown to be

simultaneously expressed in the middle (prehypertrophic) region of the embryonic sternum and that the reduced expression of either SAP or PTHrP or both resulted in the up regulation of Ihh in this region. This observation tentatively suggests that SAP may function in the same way as PTHrP in that it inhibits cell maturation. The findings seem to support the hypothetical view that SAP may play a part in the endochondral ossification process, either involving in the transcription of PTHrP directly or indirectly affecting the Ihh/PTHrP feedback loop. This could be further investigated using a PTHrP promoter-reporter construct to examine whether there is a transcriptional control of PTHrP gene expression by SAP. It can also be studied in a SAP missexpression system that the over expression of SAP may lead to the up-regulation of PTHrP.

In the presence of cyclopamine, the levels of GAGs and hydroxyproline increased throughout the same period of cultures. However, differences were noted in the levels of GAGs and hydroxyproline production between the cyclopamine treated and untreated cultures and these variations depended on the regions from which the chondrocytes were isolated. The analysis of the micromass cultures shows that the levels of GAGs and hydroxyproline produced in the treated caudal and middle were significantly higher than those in the untreated cultures. By contrast, the levels of GAGs and hydroxyproline produced in the cyclopamine treated cephalic region were significantly lower than those in the untreated samples. This decrease of hydroxyproline in the cephalic samples could suggest that cyclopamine affect the synthesis of type X collagen which is know to be the major collagen synthesised by the hypertrophic chondrocytes. The levels of GAGs and hydroxyproline in all cyclopamine treated cultures were significantly less than those in the untreated samples with an exception that the day 12 of the middle region showed no significant difference in the levels of GAGs produced between the treated and untreated cultures

GAGs and hydroxyproline are found highly abundant in vertebrate embryos. These structural components of the extracellular matrix closely associate with a wide variety of proteins in performing major developmental functions. The present study shows that under the influence of cyclopamine, only the chondrocytes at the early stage of proliferation or differentiation (8 days cultures) increased the production of GAGs whereas those at the later stage of the processes (14 days cultures) showed a decline in

their production. Since the cell proliferation process was shown to be regulated by the Ihh/PTHrP loop (Vortkamp 1996; 1999), the present study has also suggested that this process may be regulated by the Ihh/PTHrP loop and that it can be affected by cyclopamine treatment. In the caudal region in particular, the cells do not usually express type X collagen as this collagen is specific to hypertrophic chondrocytes. The fact that this type X gene was found by the PCR analysis to be expressed in the cyclopamine treated caudal region seems to suggest that in the presence of cyclopamine, the proliferative cells accelerated the process of differentiation; thus the cells became hypertrophic or matured earlier than normally expected. In addition, the results obtained from the RT-PCR analysis showed that the expression of PTHrP which has been reported to be expressed in the proliferative and the prehypertrophic chondrocytes (Nakase *et al.,* 2001) is gradually decreased in the caudal and middle regions in the present of cyclopamine. This fact further supports the notion that chondrocytes from these two regions become hypertrophic more rapidly. This finding is confirmed by the reduced production of GAGs in these two regions during two weeks of culture when the cells were exposed to longer treatment of cyclopamine.

With regard to the cephalic region where the cells already reached a fully differentiated stage, cyclopamine was found to reduce the production of GAGs and hydroxyproline: the levels of GAGs production were significantly ($P<0.05$) less than those observed in the controls. It is apparent that the production of GAGs and hydroxyproline in the hypertrophic region was affected by the cyclopamine treatment. However, the levels of GAGs and hydroxyproline produced appeared to increase gradually with time in both cyclopamine treated and untreated cephalic cultures. This shows that cyclopamine was not toxic to the cells. There was no morphological evidence of cell death in these cyclopamine treated cultures. The decline in the production of GAGs and hydroxyproline in the cephalic region indicates a continuing slowdown in the synthesis of GAGs and collagens, resulting in reduced extracellular matrix production.

Regardless of whether the cells were treated or not with cyclopamine, the levels of GAGs and hydroxyproline in the caudal and middle regions were always found to be much higher than those in the cephalic region; this observation suggests that the

synthesis of GAGs and hydroxyproline was down regulated as soon as the chondrocytes became fully differentiated.

From this study, it is apparent that several important areas require further investigation. In addition to the future experiments mentioned earlier, one of the urgent issues is to examine whether cyclopamine affects SAP gene transcription or SAP mRNA stability which resulted in the low levels of SAP PCR product. Transcriptional control of SAP by cyclopamine could be ascertained by performing nuclear run on assays. Also in order to confirm that SAP is a nuclear protein, an antibody could be raised to localized SAP in the nucleus. As an adequate amount of purified SAP is not available, it is planed to raise an antibody to a syntactic peptide in the future. Alternatively, the SAP 6x-HisTag fusion protein can be localised in the chondrocytes transfected with RCAS. More recently, other investigators in the laboratory have shown that over expression of human SAP in chick chondrocytes inhibited mineralisation *in vitro* in monolayer cultures. These observations further support the hypothesis that SAP regulates cell maturation by inhibiting this process and that SAP is an integral part of the Ihh/PTHrP loop. In addition, the expression of SAP could be directly modulated using siRNA of SAP. This would enable a variable gene knockdown to be achieved and the effect on mineralisation to be monitored.

Throughout this project, I have adopted the assumption that the Ihh/PTHrP signalling loop also operates in the embryonic chick sternum since the developmental sequence of chondrocytes in this tissue is almost identical to that of the tibial growth plate. The results from my RT-PCR investigation showed fluctuations of the mRNA levels of key components of the Ihh/PTHrP loop i.e: Ihh, PTHrP. Also when PTHrP expression was inhibited by the treatment of cyclopamine, type X collagen expression was up regulated. These observations suggest that the Ihh/PTHrP loop also operate in the developing chick sternum. This negative feedback loop has only been reported previously in the tibia. However, this is evidence circumstantial and definitive demonstration of the existence of the Ihh/PTHrP loop in the embryonic chick sternum requires a series of in situ hybridisation studies using serial sections from the different anatomical regions of the sternum.

CHAPTER 6

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Appendices

Appendix 1. Molecular Weight Markers

1.1 X174 DNA/BsuRI (*Hae*III) Marker

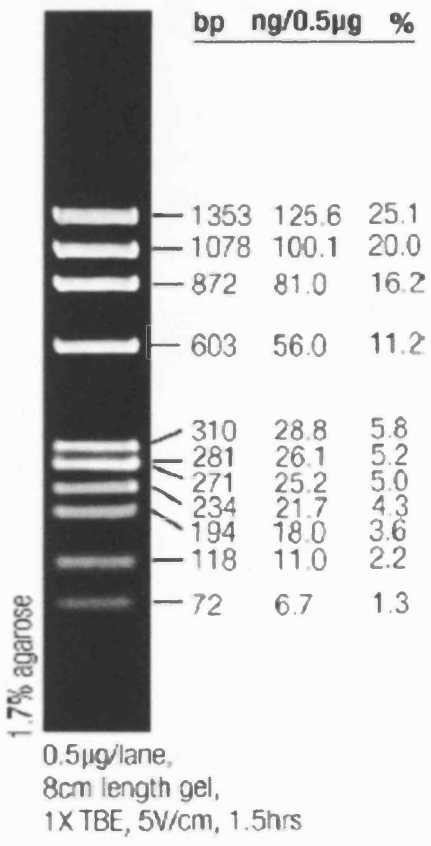


Figure A.1: X174 DNA/Hae III marker

1.2 Lambda DNA/*Hind*III Marker

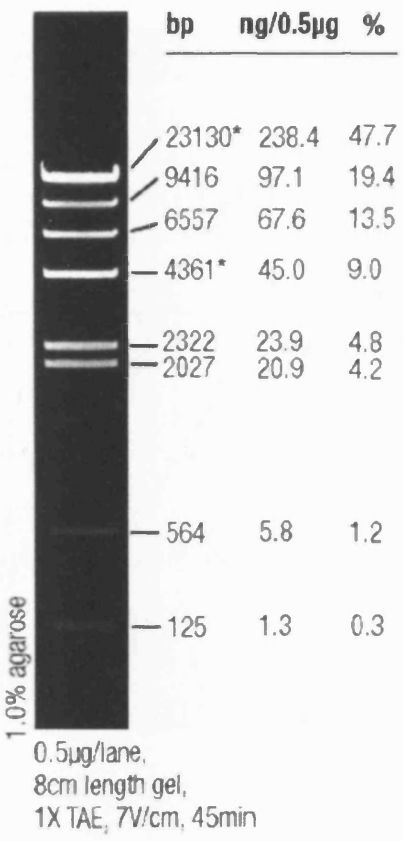
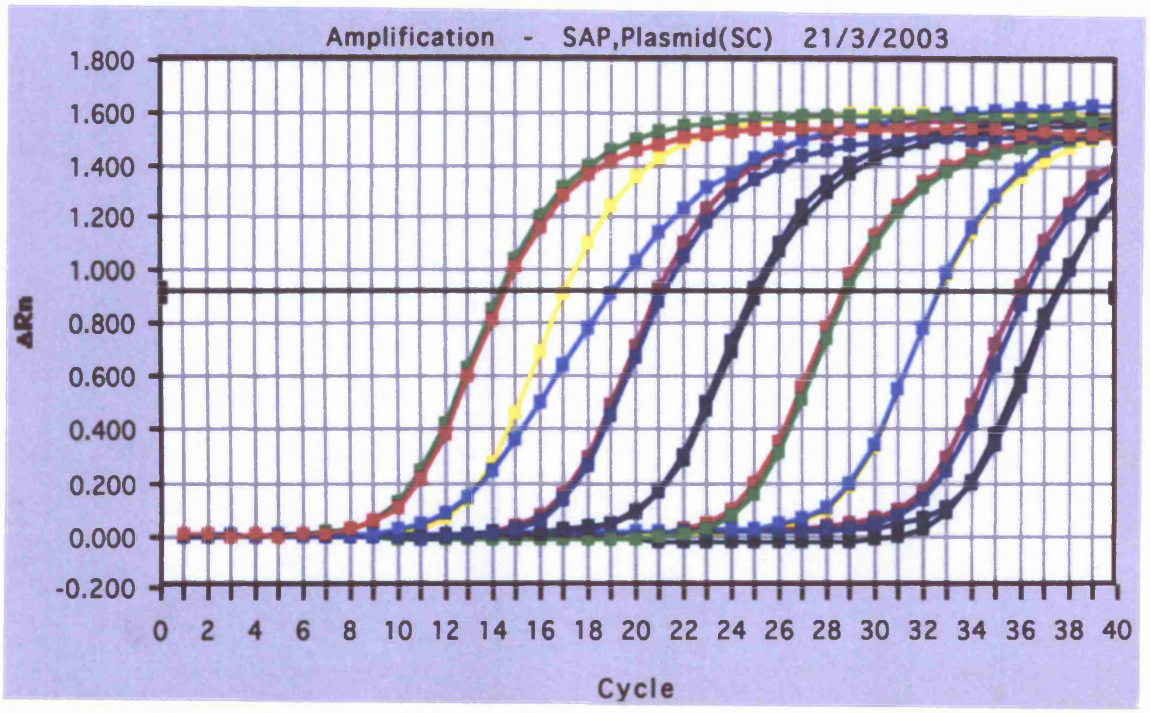


Figure A.2: Lambda DNA/*Hind* III Marker

1.3 Quantitative -PCR Result



FigureA.3: the standard curve of p-GEMT-SAP plasmid using the Q-PCR.

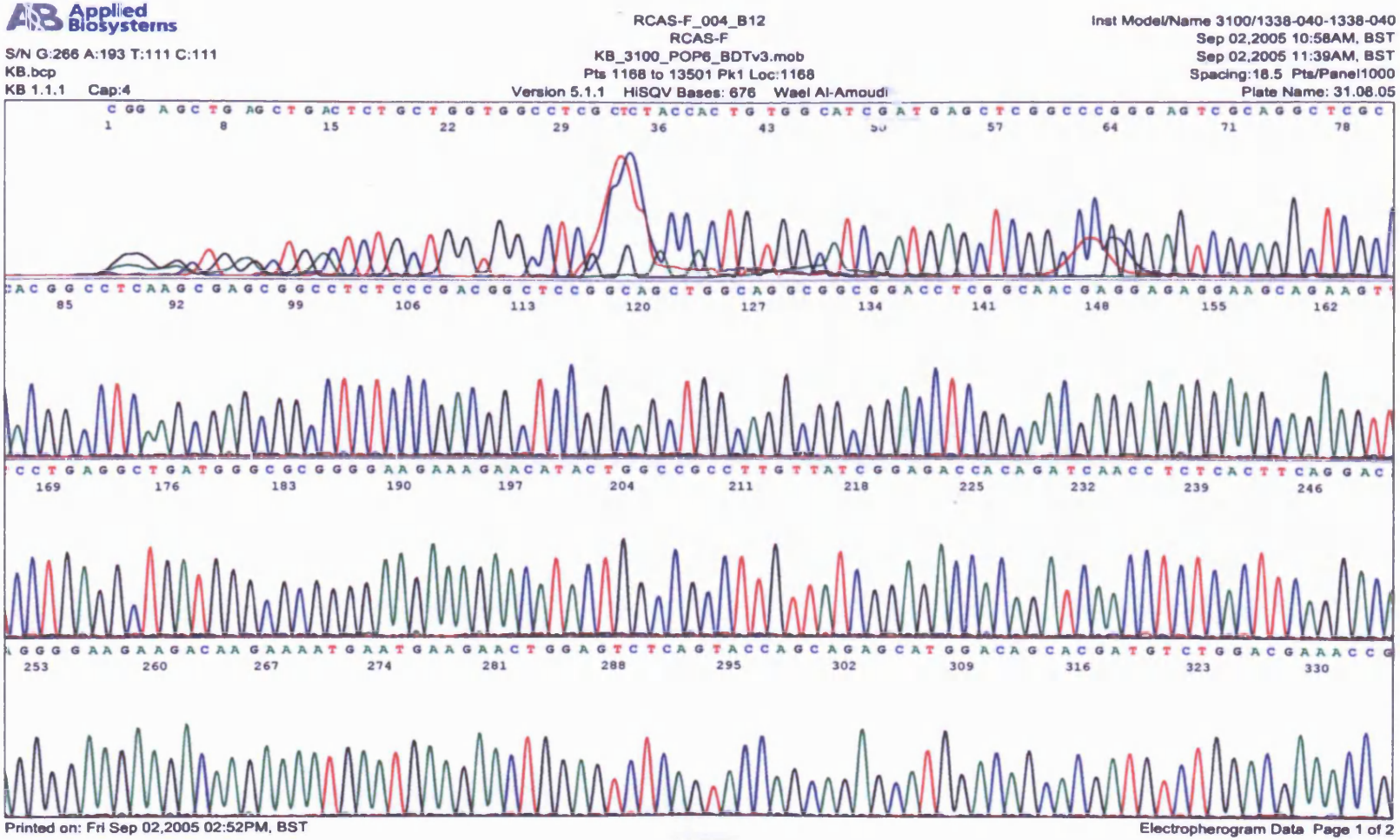


Figure A.4: The sequence of the open reading frame of SAP inserted into the RCAS vector (Sheet 1)

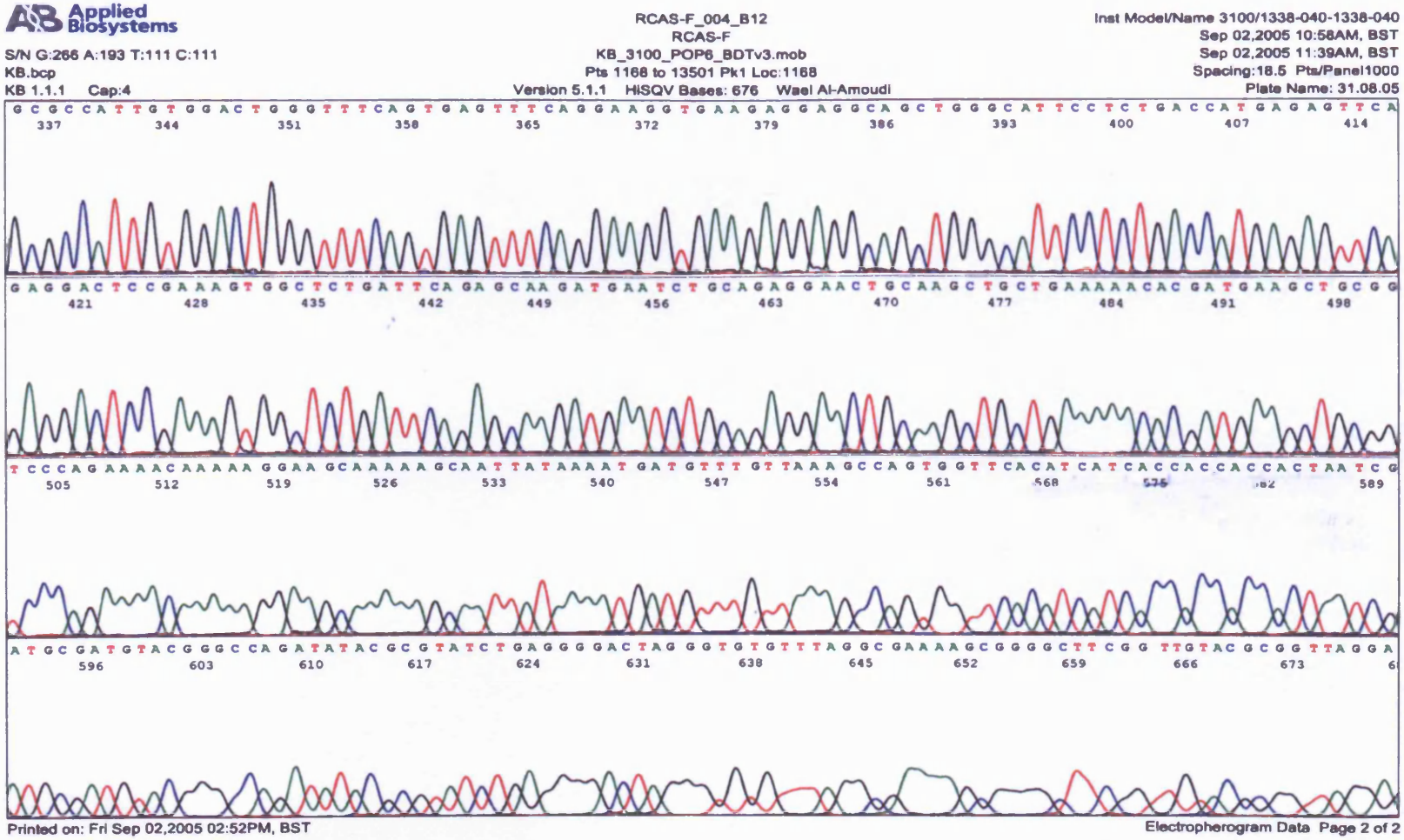


Figure A.5: The sequence of the open reading frame of SAP inserted into the RCAS vector (Sheet 2)

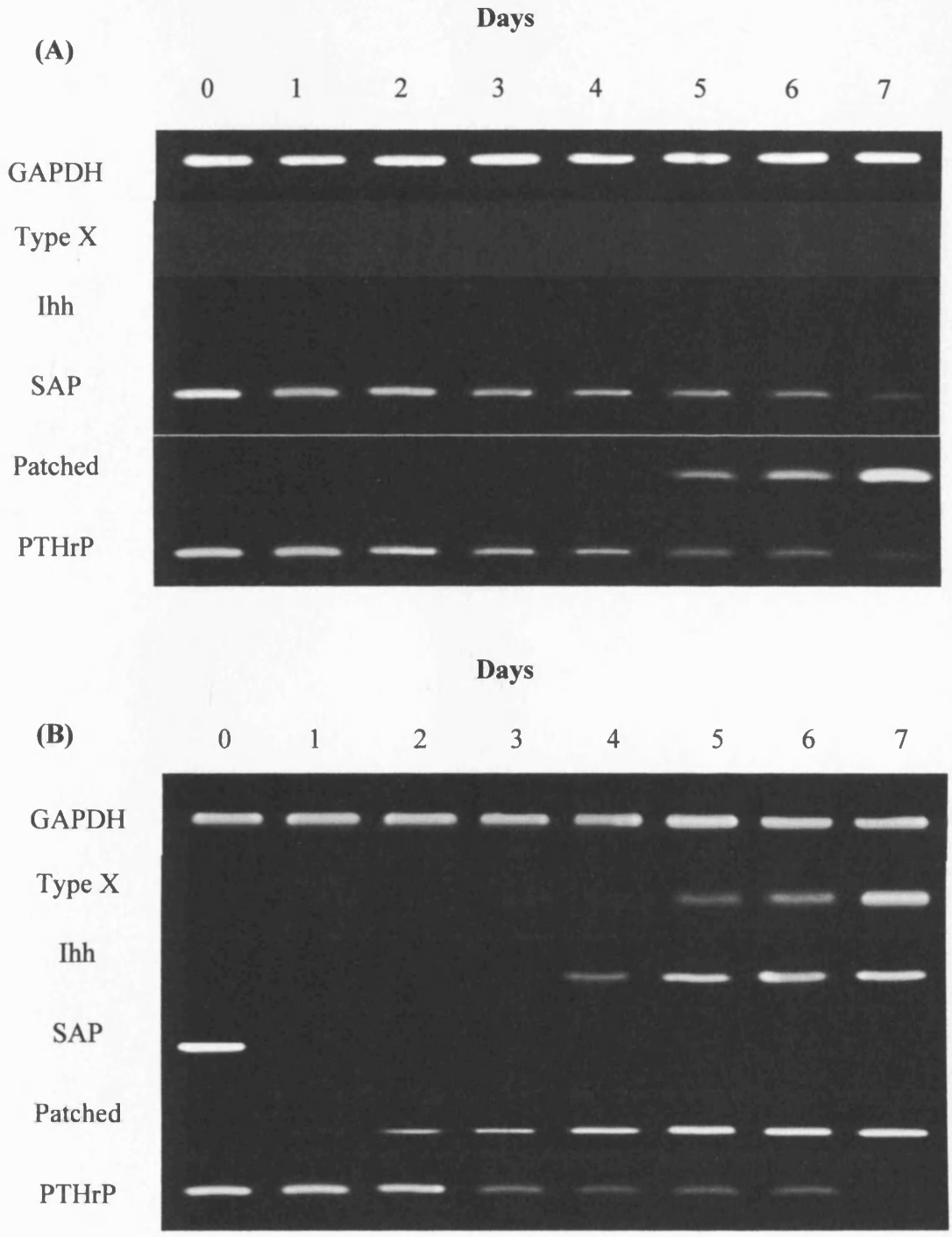


Figure A.6: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 2 of the caudal region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

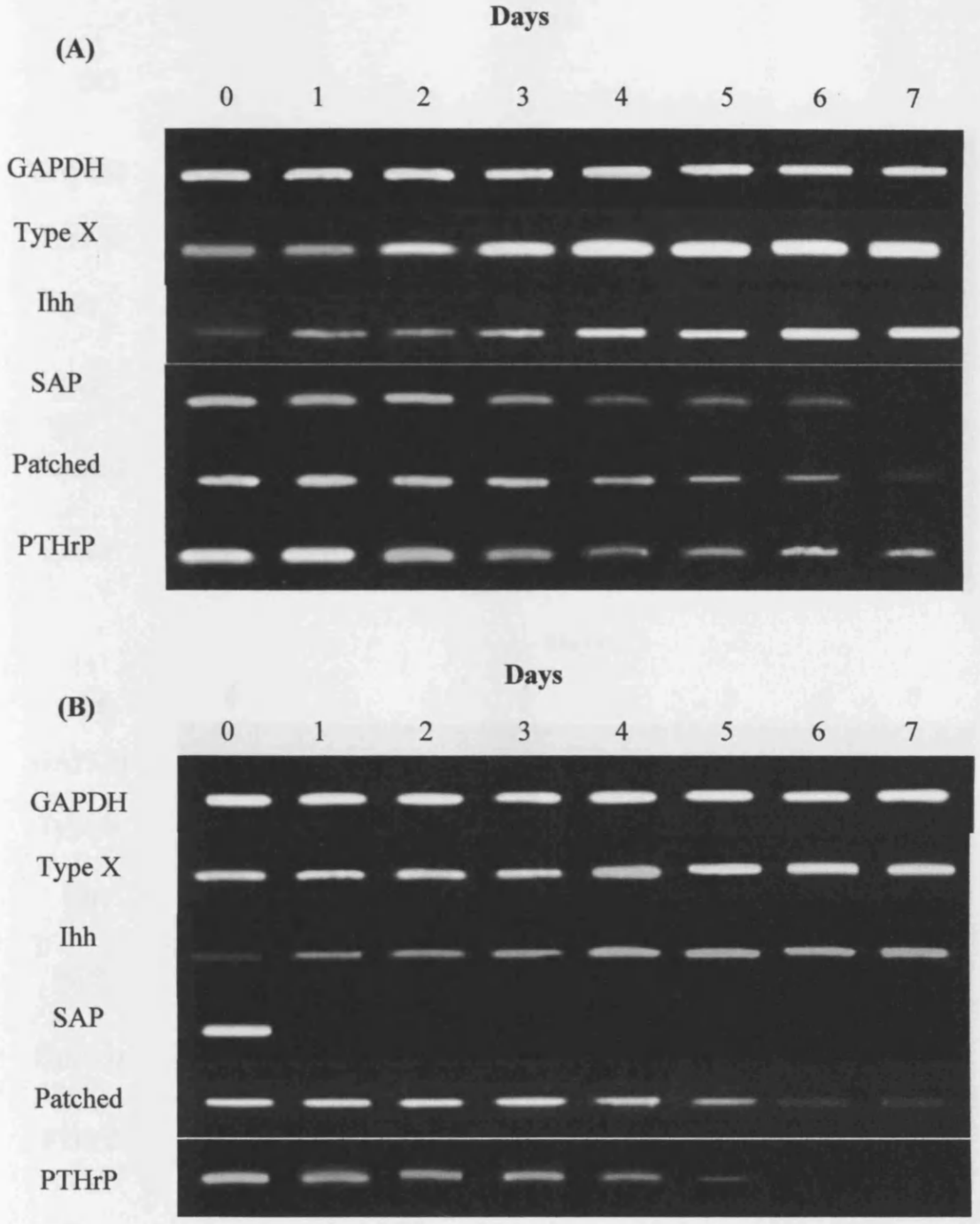


Figure A.7: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 2 of the middle region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

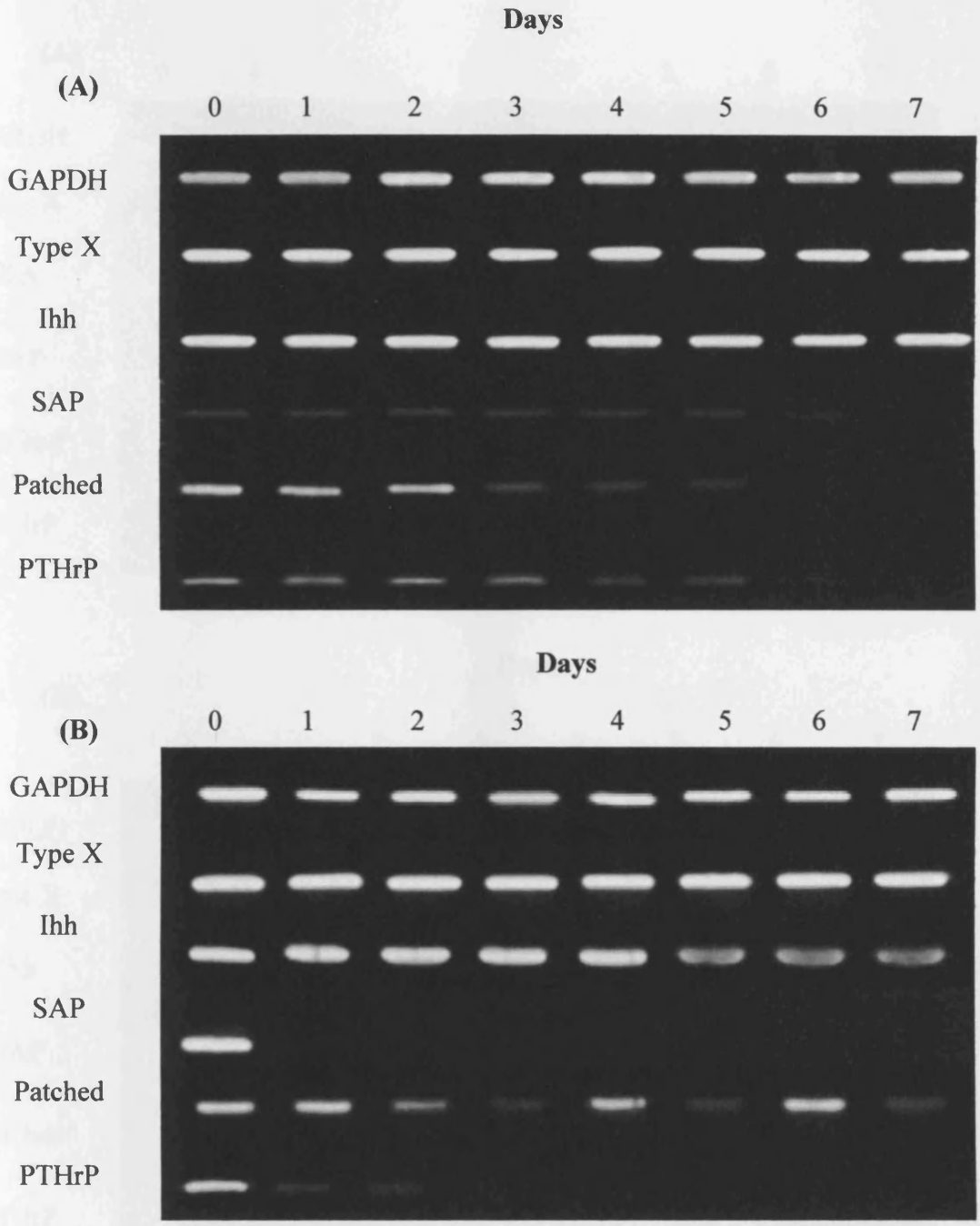


Figure A.8: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 2 of the cephalic region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

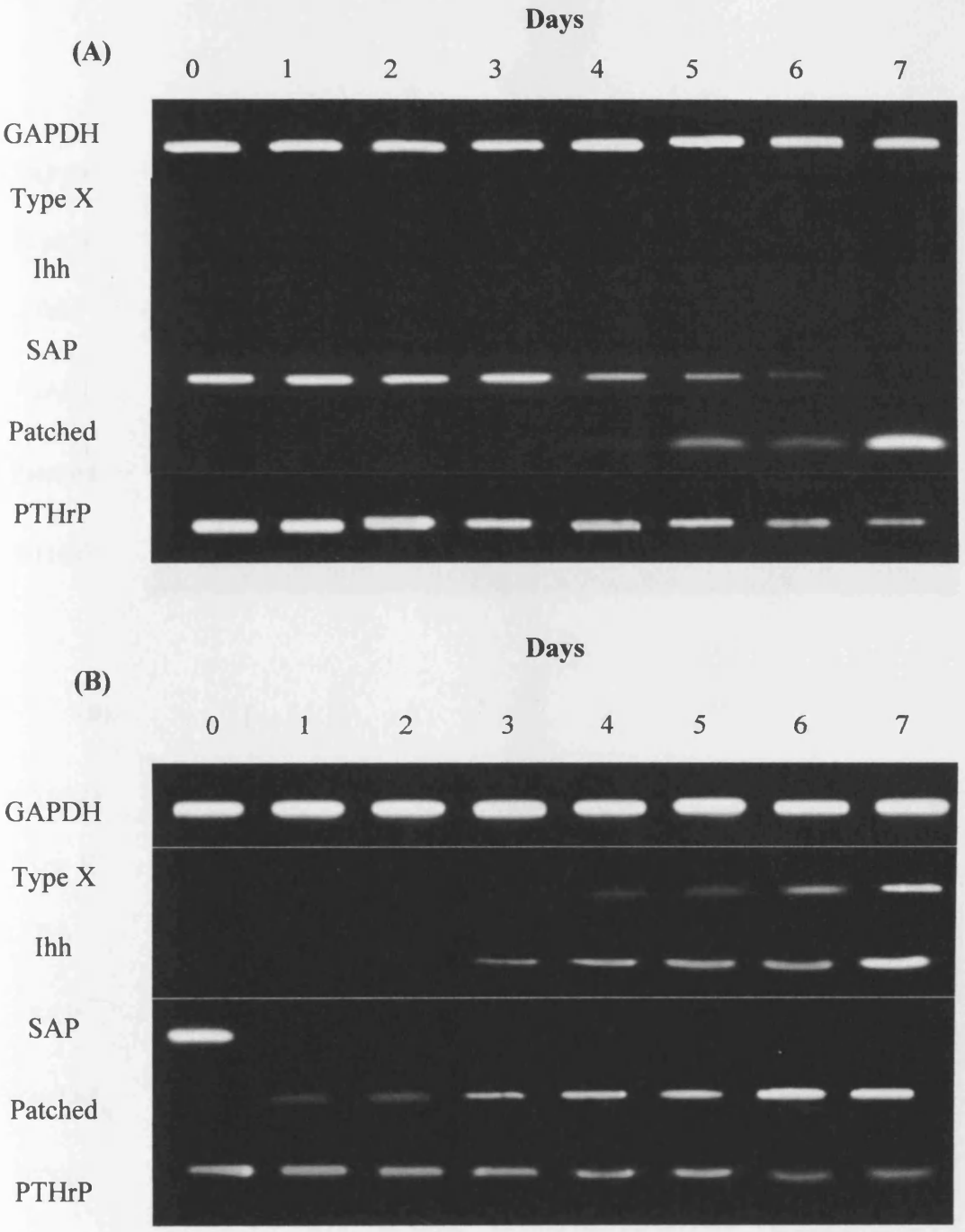


Figure A.9: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 3 of the caudal region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

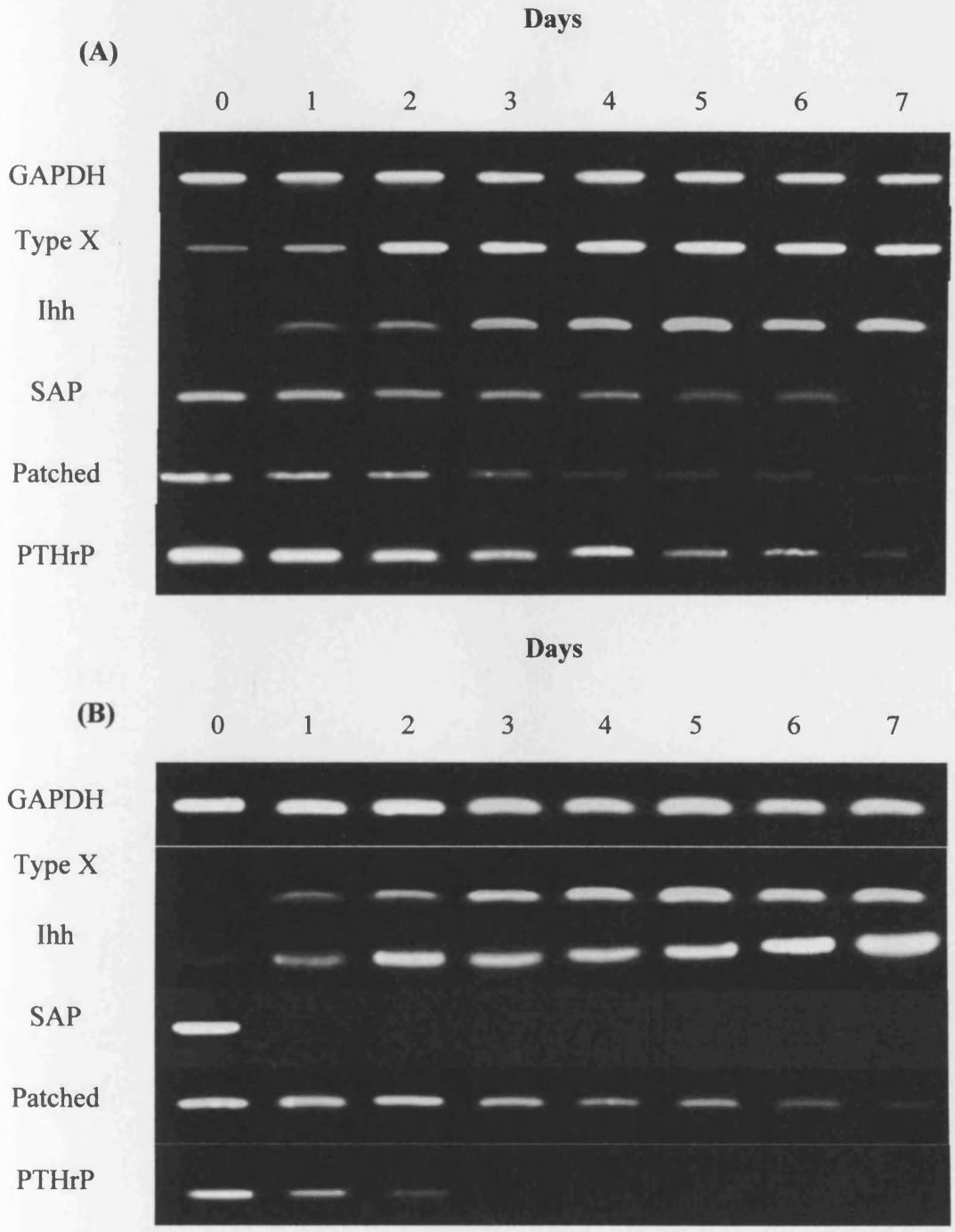


Figure A.10: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 3 of the middle region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.

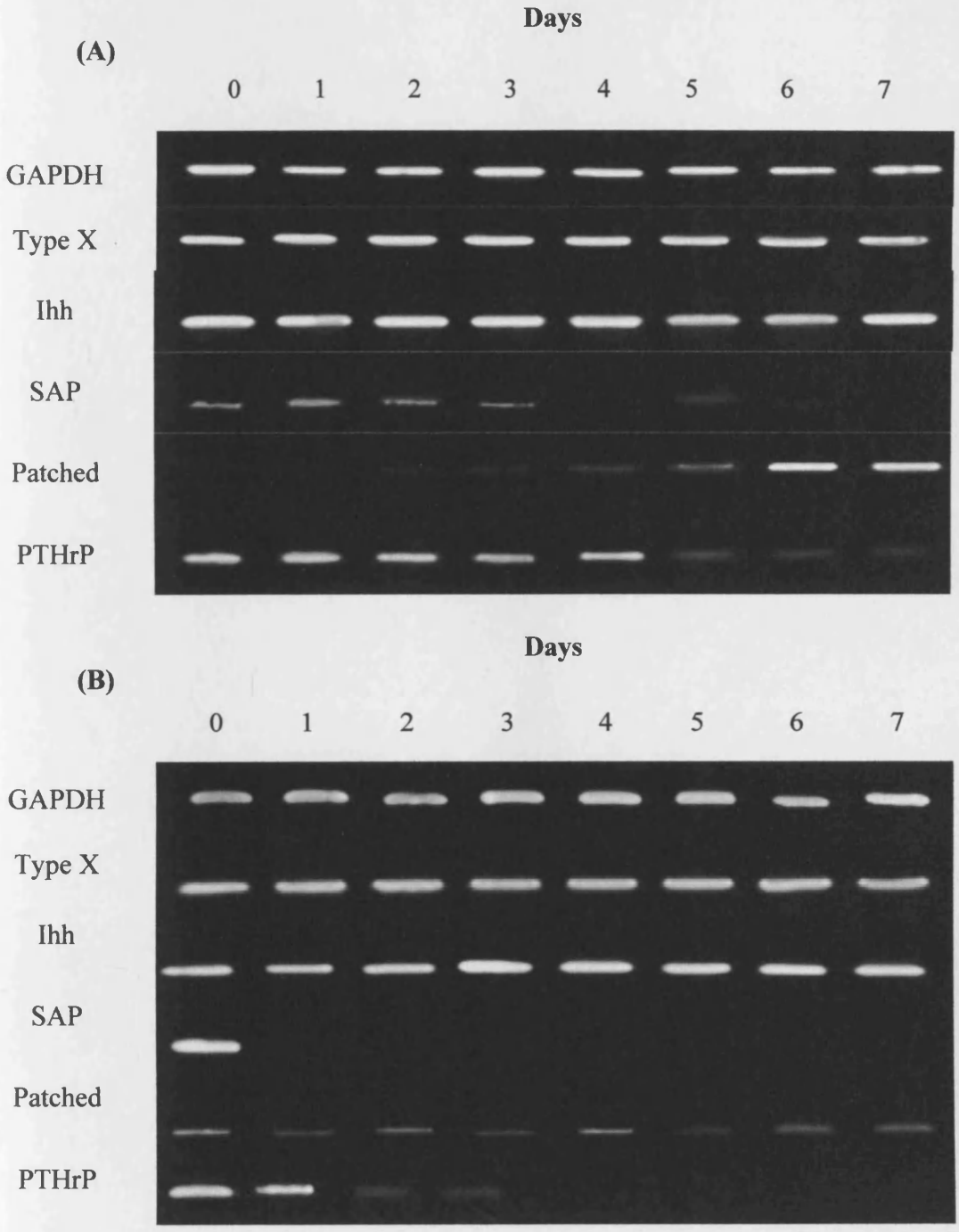


Figure A.11: Agarose gel of PCR products showing the expression of different genes in the untreated and treated micromass cultures set 3 of the cephalic region of the sternum of 17 day chick embryos. RNAs were isolated form individually collected chondrocyte micromass with out any surrounding cells. Every 24 hours the RNA was isolated from a 7 day culture period and was used for the RT-PCR analysis. Lane 0 = 0 time point. Lanes 1 - 7 = RNA isolated at days 1, 2, 3, 4, 5, 6 and 7 respectively.
(A) Untreated.
(B) Treated with cyclopamine.
(0) RNA was extracted from freshly isolated chondrocytes.