TRANSGENIC MODELS FOR THE STUDY OF NEUROENDOCRINE FUNCTION

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ABSTRACT

The mammalian suprachiasmatic nucleus (SCN) contains a biological clock that drives circadian rhythms that regulate physiological and behaviorial activities in relation to environmental cycles and provide an internal temporal framework. Transgenic rat models were used to investigate organisation of a mammalian circadian system, and the mechanisms by which the expression of the the circadian clock-regulated genes, vasopressin (VP) and *Period 1* (*Per1*), and the early growth response gene-1 (*egr-1*), is confined to, and physiologically regulated within, specific groups of neurons in the SCN (and pituitary gland). Initial studies of JP-17 and JP-59 (rat VP promoter / human growth hormone reporter gene) transgenic lines failed to detect expression of the reporter gene in the SCN. Therefore, no further analysis of circadian clock-regulated VP expression could be conducted in these lines. Four *mPer1* promoter / destabilised enhanced green fluorescent protein (d2EGFP) (Y) transgenic lines were generated, but failed to express the EGFP reporter gene. Subsequent studies in the *egr-1*/d4EGFP (57C) transgenic line detected tissue-specific constitutive and inducible expression in the brain. Region-specific and physiologically-regulated expression in the pituitary gland was also detected, as determined by northern blot and dual *in situ* hybridisation / immunohistochemical analyses. Nevertheless, direct GFP fluorescence remained undetectable in this line. Seven *egr-1*/d2EGFP (Z) transgenic lines were then generated, using a novel construct lacking the *egr-1* intron present in the 57C construct, each demonstrating tissue-specific constitutive and inducible expression in the brain, and region-specific expression in the pituitary gland, at all levels of detection: transcript, protein and direct fluorescence. These findings indicate that the *egr-1* intron is not required for directing tissue-specific and inducible expression in the rat. Analysis of GFP expression in the SCN and other brain nuclei indicates that the Z line rats will provide insights into the cellular progression of clock- and light- regulated responses in the brain.

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I would like to dedicate this thesis in loving memory of my grandmother, Tsang Lan Mui, whose genes have actively contributed to this thesis.

PUBLICATIONS

1. **Man, P.-S.**, Wells, T., Carter, D.A. (2004)
   Transcriptional analysis of *egr-1/zif268* in suprachiasmatic nucleus, retina and pineal gland of transgenic rats.
   34th Annual Meeting of the Society for Neuroscience, San Diego, 2004. EE-10 765.2. Abstract.

2. **Man, P.-S.**, Carter, D.A. (2003)
   Oestrogenic regulation of an egr-1 transgene in rat anterior pituitary.
   Journal of Molecular Endocrinology 30, 187-196.

3. Davies, J.S., **Man, P.-S.**, Carter, D.A., Wells, T. (2003)
   Photic stimulation inhibits growth hormone secretion in male rats: a hypothalamic mechanism for transient entrainment.
   33rd Annual Meeting of the Society for Neuroscience, New Orleans, 2003. Q-8 511.5. Abstract.

4. Slade, J.P., **Man, P.-S.**, Wells, T., Carter, D.A. (2002)
   Stimulus-specific induction of an Egr-1 transgene in the rat brain.
   Neuroreport 13 (5) 671-674.

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   31st Annual Meeting of the Society for Neuroscience, San Diego, 2001. E-59. Abstract.

6. Slade, J.P., **Man, P.-S.**, Wells, T., Carter, D.A. (2001)
   Characterisation of a novel *egr-1/NGFI-A* destabilised EGFP transgenic model.
   16th National Meeting of the British Neuroscience Association, Harrogate, 2001. 11.04. Abstract.

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ABBREVIATIONS

3V	third ventricle
AA-NAT	arylalkamine N-acetyltransferase
ACSF	artificial cerebral spinal fluid
ACTH	adrenocorticotrophic hormone
AP	anterior pituitary gland
AP-1	activator protein-1
APS	ammonium persulphate
Arc	arcuate nucleus
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BNST	bed nuclei stria terminalis
bp	base pairs
BSA	bovine serum albumin
CA	cornu ammonis
cAMP	cyclic adenosine monophosphate
CAR	calretinin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
cIEG	cellular immediate early gene
CKIε	casein kinase Iε
CNS	central nervous system
CON	control
COR	cortex
cpm	counts per minute
CRE	cAMP responsive element
CREB	cAMP responsive element-binding protein
CRH	corticotropin-releasing hormone
CRY	cryptochrome
C-terminus	carboxy-terminus
CTP	cytosine triphosphate
DAPI	4',6' diamidino 2-phenylindole dihydrochloride
DBP	D-element binding protein
DEC	differentially-expressed in chrondrocytes
dEGFP	destabilised enhanced green fluorescent protein
DEPC	diethylpyrocarbonate
DL	dorso-lateral
DM	dorso-medial
DMH	dorso-medial hypothalamus
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothrietol
EBFP	enhanced blue fluorescent protein
ECFP	enhanced cyan fluorescent protein
E.coli	*Escherichia coli*
EDTA	ethylene-diamine-tetra-acetic acid
EGFP	enhanced green fluorescent protein
egr-1	early growth response-1

ES cells	embryonic stem cells
et al	*et alter* (and others)
F1	first generation
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin-releasing hormone
GRP	gastrin-releasing peptide
GTC	guanidium isothiocyanate
GTP	guanosine triphosphate
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
hGH	human growth hormone
IEG	immediate early gene
Ig	immunoglobulin
IGL	intergeniculate nucleus
IGR	intergenic region
IHC	immunohistochemistry
i.p.	intraperitoneal
IRES	internal ribosomal entry site
ISH	*in situ* hybridisation
KCl	potassium chloride
kDa	kilo Daltons
lacZ	β-galactosidase
LB	Luria-Bertani
LGN	lateral geniculate nucleus
LH	luteinising hormone
LHRH	luteinising hormone-releasing hormone
LHβ	luteinising hormone beta chain
LS	lateral septal nucleus
luc	luciferase
M	mole/litre
MA	medial amygdaloid nucleus
MAE	MOPS-acetate-EDTA
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MET	metrazole
$MgCl_2$	magnesium chloride
MITE	micro-injection TE
mRNA	messenger RNA
Na_2HPO_4	sodium phosphate, dibasic
NaAc	sodium acetate
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen phosphate
NaOH	sodium hyroxide
NaPi	sodium phosphate

NGF	nerve growth factor
NGFI-A	nerve growth factor induced gene-A
NGS	normal goat serum
NIH	National Institute of Health
NMDA	N-methyl-D-aspartate
NT	neurotensin
NTG	non-transgenic
OC	optic chiasm
OD_{260}	optical density at 260nm
OLB	oligo-labelling buffer
ORF	open reading frame
OT	oxytocin
OVLT	organum vasculosum lamina terminalis
OVT	oviduct transfer
PAC	P1-derived artificial chromosome
PAF	paraformaldehyde
PB	phosphate buffer
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Triton X
P/C/IAA	phenol/chloroform/isoamyl alcohol
PCR	polymerase chain reaction
PeN	periventricular nucleus
PER	period
pH	negative logarithm of the hydrogen ion (H^+) concentration
PHI	peptide histidine isoleucine
PIT	pituitary gland
PK	prokineticin
POA	preoptic area
poly (A)	polyadenylation
PP	posterior pituitary
PRL	prolactin
PSCN	pre-suprachiasmatic area
PT	paratenial nucleus
PVDF	polyvinylidene fluoride
PVH	paraventricular hypothalamus
PVN	paraventricular nucleus
PVT	paraventricular nucleus thalamus
rev/min	revolutions per minute
RHT	retino-hypothalamic tract
RNA	ribonucleic acid
RNase	ribonuclease
(ROR)E	(retinoid-related orphan receptor) element
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SCN	suprachiasmatic nucleus
SD	Sprague-Dawley
SDS	sodium dodecyl sulphate
SER	serine
SON	supraoptic nucleus

SP	substance P
SRE	serum response element
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
SST	somatostatin
STE	SDS-Tris-EDTA
subPVA	sub-paraventricular area
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween
TEMED	tetra-methyl-ethylene-diamine
TE	Tris-EDTA
TG	transgenic
TGFα	transforming growth factor-alpha
TNB	Tris-HCl NaCl blocking buffer
TNT	Tris-HCl NaCl-Triton-X
TRH	tyrosine-releasing hormone
TSA	tyramide signalling amplification
TSH	thyroid stimulating hormone
TTP	thymidine triphosphate
UTP	uridine triphosphate
UV	ultraviolet
V	volts
VIP	vasoactive intestinal polypeptide
VL	ventro-lateral
VMN	ventromedial nucleus
VP	vasopressin
VPAC2	vasoactive intestinal polypeptide receptor subtype 2
VTU	ventral tuberal area
v/v	volume by volume
w/v	weight by volume
YAC	yeast artificial chromosome
ZI	zona interna

CHAPTER 1

GENERAL INTRODUCTION

1.1 OVERVIEW AND AIMS OF THESIS

Since the late 1980's, transgenesis has become a key technique in neuroendocrinology. Germline transgenic animal models have allowed neuroendocrinologists to study the role and regulation of genes within the context of the whole animal, sometimes without any prior knowledge of the gene's regulation or function. The various studies described in this thesis involve 4 different promoter / reporter transgenic rat models, as follows:

i) rat vasopressin (rVP) / human growth hormone (hGH), designated the JP-17 and JP-59 lines;

ii) mouse *Period 1* (*mPer1*) / destabilised enhanced green fluorescent protein (d2EGFP), designated the Y6, Y7 and Y16 lines;

iii) early growth response-1 gene (*egr-1*) / destabilised enhanced green fluorescent protein (d4EGFP), designated the 57C line;

iv) *egr-1*/d2EGFP, designated the Z13, Z14, Z16, Z25-A, Z25-B, Z27-A and Z27-B lines.

The aforementioned transgenic rat models were either provided by collaborators or colleagues (JP and 57C lines, respectively) or created by myself (Y and Z lines), in an attempt to direct the expression of the respective reporter genes to the neuroendocrine system. The purpose of these genetic manipulations was to investigate the regulation of the circadian clock-regulated genes, VP and *Per1*, in the suprachiasmatic nucleus (SCN), and the immediate early gene, *egr-1*, in the SCN and pituitary gland. The present chapter aims to introduce aspects of the neuroendocrine (hypothalamic-pituitary) system, and the use of transgenesis for investigating gene function.

1.2 The Hypothalamus

The hypothalamus is a region of the brain that integrates controls of the endocrine system. It is located at the base of the forebrain, and surrounds the ventral portion of the third ventricle (see Figure 1.1). The optic chiasm is anterior to the hypothalamus, and the inferior part of the hypothalamus (median eminence) gives rise to the pituitary stalk. The hypothalamus receives many inputs regarding the homeostatic state of the body, and responds with the secretion of hormones that either regulate the release of hormones from the anterior pituitary, or are released directly from the posterior pituitary (see 1.3).

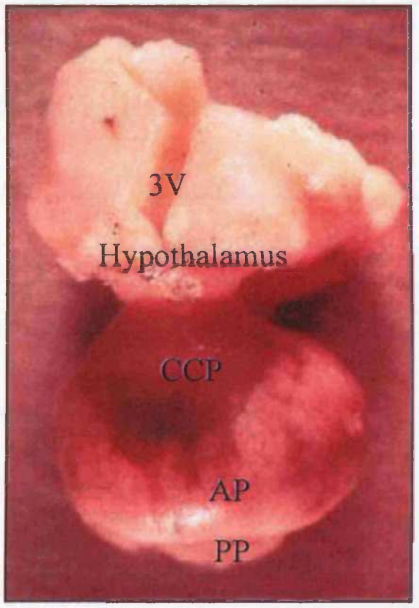


Figure 1.1 A frontal view of a sheep hypothalamus and pituitary gland.
Adapted from http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/hypopit/anatomy.html.
The hypothalamus surrounds the ventral portion of the third ventricle (3V). The pituitary gland is composed primarily of the anterior pituitary and posterior pituitary (AP and PP, respectively; see 1.3). CCP represents the capillary portal plexus (see 1.3.1).

1.2.1 The Suprachiasmatic Nucleus (SCN)

The suprachiasmatic nucleus (SCN) consists of a pair of small, densely packed clusters of neurons, located in the anterior hypothalamus, above the optic chiasm, and adjacent to the third ventricle (see Figures 1.2 and 3.2 for schematic representations). There are 2 subdivisions in the SCN, the dorso-medial (DM) 'shell,' and the ventro-lateral (VL) 'core,' as illustrated by Figure 1.2. Neurons of the DM SCN are primarily characterised by vasopressin (VP) expression, which is co-localised with gamma-aminobutyric acid (GABA). Neurons of the ventral SCN predominantly express vasoactive intestinal polypeptide (VIP) or gastrin-releasing peptide (GRP), which are also co-localised with GABA. In addition, neurotensin (NT) and substance P (SP) are expressed by small populations of neurons of the VL SCN (see by Moore *et al.*, 2002). VIP in the ventral SCN acts through the VIP receptor subtype 2 (VPAC2), which is highly expressed in the SCN; deficiency of this receptor leads to severely disrupted circadian activity (Harmar *et al.*, 2002).

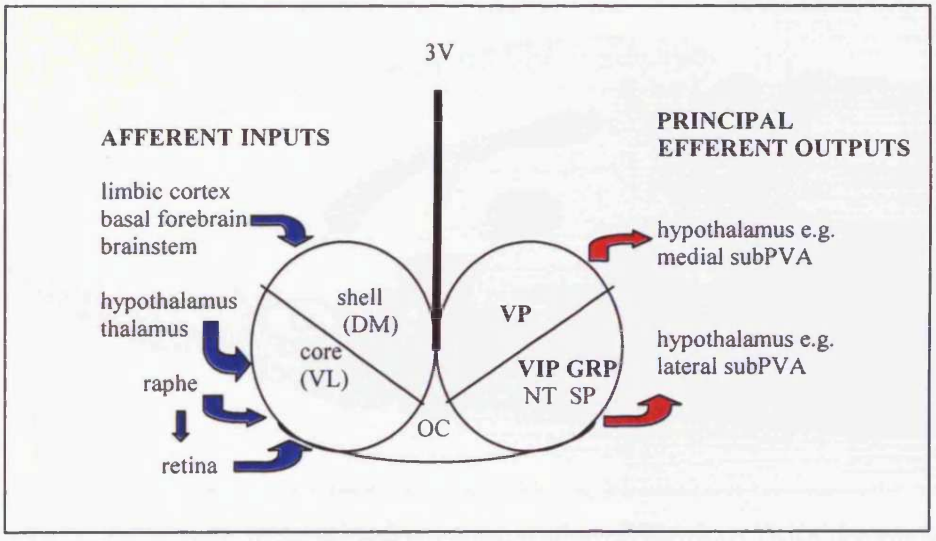


Figure 1.2 Schematic representation of the rat SCN, and its principal projections (see text for further details). (3V: third ventricle; DM: dorso-medial; VL: ventro-lateral; OC: optic chiasm; subPVA: subparaventricular area.) Adapted from Leak and Moore (2001). Also shown are the principal neuropeptides expressed in the SCN: VP, VIP, GRP, NT and SP.

In situ hybridisation (ISH; see 3.2) has been important for localising neuropeptide messenger RNAs (mRNAs) in the SCN (such as those coding for VP, somatostatin (SST), bombesin, VIP, GRP, peptide histidine isoleucine (PHI), and NT; see Ikonomov and Stoynev, 1994; Ibata *et al.*, 1999; van Esseveldt *et al.*, 2000). VP and SST gene expression in the DM subdivision of the SCN show circadian rhythmicity (see 1.2.1.1), with a peak during the subjective day. VIP gene expression in the VL subdivision of the SCN shows rhythmicity only in light-dark conditions, with a peak during the night (see van Esseveldt *et al.*, 2000).

In the rat, afferent projections to the DM SCN include those from the limbic cortex, basal forebrain and brainstem, and to the VL SCN those from the hypothalamus, thalamus, raphe and retina (Moga and Moore, 1997), as illustrated in Figure 1.2 (and Figure 1.4). Extensive studies have discovered numerous efferent projections that arise from each of the subdivisions of the rat SCN (Watts and Swanson, 1987, Watts *et al.*, 1987; Thompson and Swanson, 1998; Leak and Moore, 2001), as illustrated in Figure 1.3.

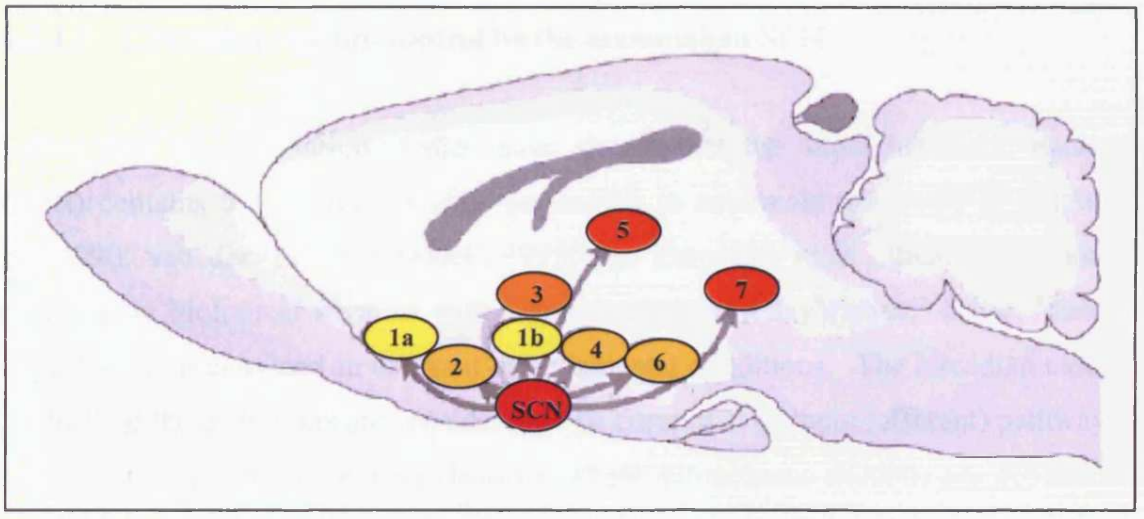


Figure 1.3 Schematic representation of the major projections from the rat SCN (shown here in longitudinal view; key below; see text for further details).

1 neuroendocrine neurons (a: gonadotrophin-releasing hormone (GnRH); b: corticotropin-releasing hormone (CRH))
2 medial preoptic area (POA)
3 paraventricular nucleus hypothalamus (PVH)
4 sub-paraventricular area (subPVA)
5 paraventricular nucleus thalamus (PVT)
6 dorsomedial nucleus hypothalamus (DMH)
7 lateral geniculate nucleus (LGN))

direct connections to neuroendocrine neurons
indirect connections to neuroendocrine neurons
autonomic neurons
extra-hypothalamic structures

Adapted from Buijs and Kalsbeek (2001).

The most dense efferent projections from the DM SCN include the medial preoptic area (POA; see Figure 1.3 [**2**]), the medial sub-paraventricular area (subPVA; see Figure 1.3 [**4**]), and the dorsomedial nucleus hypothalamus (DMH; see Figure 1.3 [**6**]); moderate projections include the paraventricular nucleus thalamus (PVT; see Figure 1.3 [**5**]), the paraventricular nucleus hypothalamus (PVH; see Figure 1.3 [**3**]) and the lateral geniculate nucleus (LGN; see Figure 1.3 [**7**]); sparse projections include the bed nuclei stria terminalis (BNST), zona incerta (ZI), paratenial nucleus (PT), peri-suprachiasmatic area (PSCN), and ventral tuberal area (VTU) (Watts and Swanson, 1987; Watts *et al.,* 1987; Thompson and Swanson, 1998; Buijs and Kalsbeek, 2001; Leak and Moore, 2001). In contrast, the most dense efferent projections from the VL SCN include the PSCN, lateral subPVA, and VTU; moderate to sparse projections include the PVT, PT, and lateral septal nucleus (LS) (Watts and Swanson, 1987, Watts *et al.,* 1987; Leak and Moore, 2001). Neuroendocrine control by the SCN, via these projections is discussed further in 1.2.1.1.

1.2.1.1 Circadian rhythm control by the mammalian SCN

Lesion and transplantation studies have shown that the suprachiasmatic nucleus (SCN) contains the master circadian pacemaker in mammals (reviewed in Ralph *et al.*, 1990; van den Pol and Dudek, 1993; van Esseveldt *et al.*, 2000). Circadian rhythms are biological rhythms with a period of about a day ('circa,' about; 'diem,' day) that are maintained in constant environmental conditions. The circadian clocks controlling these rhythms are considered to be composed of input (afferent) pathways, an oscillator or central clock mechanism (where autonomous rhythms are generated, see 1.2.1.2), and output (efferent) pathways, as illustrated in Figure 1.4.

Regarding afferent pathways, the endogenous circadian oscillator is entrained to environmental light by direct retinal input via the retino-hypothalamic tract (RHT), by indirect photic input (neuropeptide Y input) via the intergeniculate nucleus (IGL), or

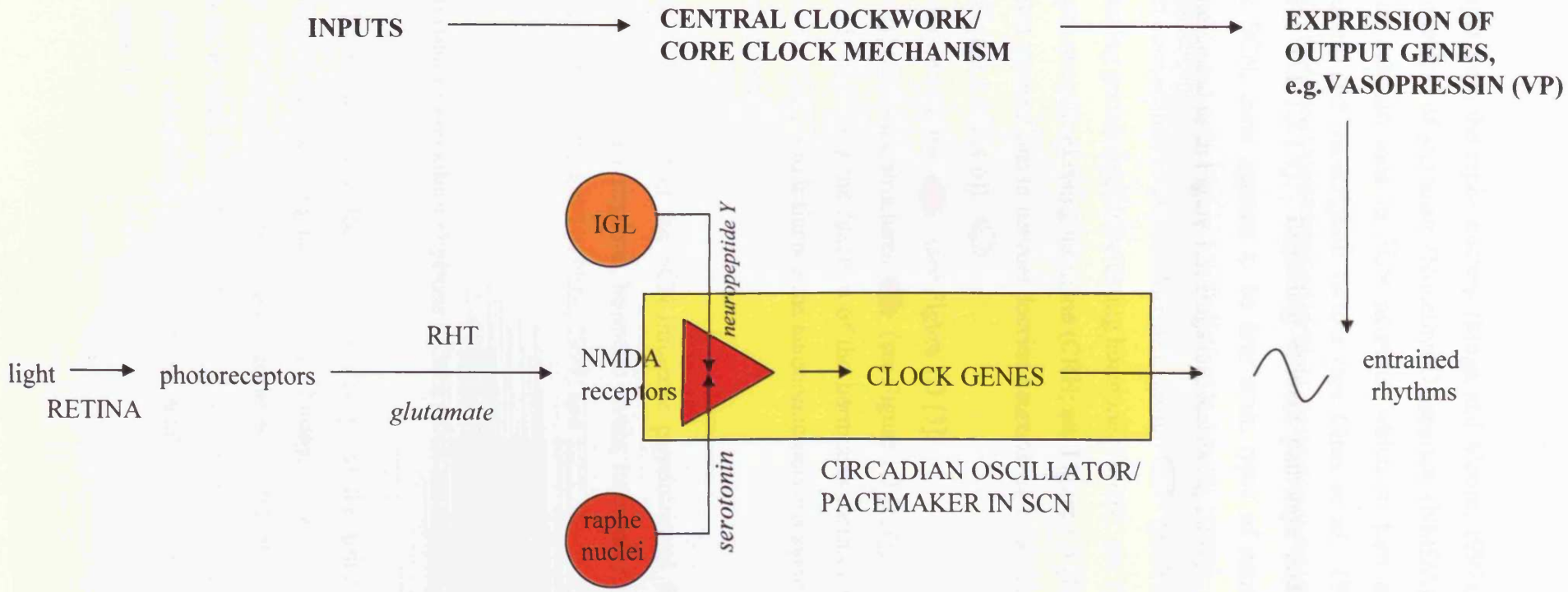


Figure 1.4 Schematic representation of the regulation of circadian rhythms in mammals (see text for further details). Adapted from Ibata *et al.*, 1999.

serotinergic input from the raphe nucleus (Moga and Moore, 1997), as illustrated in Figure 1.4. Activation of glutamate (N-methyl-D-aspartate (NMDA)) receptors leads to an influx of calcium ions in SCN neurons, which in turn activate calcium-dependent kinases, and transcription factors (see Chen *et al.*, 1999; Gillette and Tischkau, 1999; and 1.2.1.4). Regarding efferent pathways and neuroendocrine control by the SCN, there appears to be four main types of neuronal projections involved (colour-coded as in Figure 1.3; Buijs and Kalsbeek, 2001):

i) direct connections to neuroendocrine neurons (such as neurons containing gonadotrophin-releasing hormone (GnRH; see Figure 1.3 [1a]), or corticotropin-releasing hormone (CRH; see Figure 1.3 [1b])
ii) indirect connections to neuroendocrine neurons via hypothalamic structures (see Figure 1.3 [2,4,6])
iii) autonomic neurons (see Figure 1.3 [3])
iv) extra-hypothalamic structures (see Figure 1.3 [5,7])

For further details regarding the function of the hormones mentioned in i) (and ii)), see 1.3.1. The role of iii) is to influence the autonomic nervous system; the role of iv) remains to be elucidated.

Circadian rhythms generated in the SCN influence physiological functions such as body temperature, sleep/wake rhythms, hormone levels, locomotor activity, and food and water intake (see Gillette and Tischkau, 1999; and 1.2.1.3).

1.2.1.2 Regulation of circadian rhythms - Core clock mechanisms within the SCN

Recent molecular studies have led to the discovery of the principal components involved in circadian time-keeping in mammals (Dunlap, 1999; Gilette and Tischkau, 1999; Hastings and Maywood, 2000; Shearman *et al.*, 2000; Reppert and Weaver, 2001). Also, a molecular link between the core clock mechanism and the expression of the output gene vasopressin (VP) has been established (Jin *et al.*, 1999), as illustrated in Figure 1.5.

The core clock mechanism is controlled by the interaction of autoregulatory positive and negative transcriptional/translation feedback loops, which generate time delays in the cyclical transcription of key components (Shearman *et al.*, 2000). CLOCK and BMAL1 are basic-helix-loop-helix (bHLH) / PAS-containing transcription factors, and are paired via protein interactive PAS (PER, ARNT, SIM) domains (Shearman *et al.*, 1999; Gekakis *et al.*, 1998; Whitmore *et al.*, 1998). CLOCK/BMAL1 hetero-dimers drive the rhythmic transcription of three *Period* (*Per*) genes and two *Cryptochrome* (*Cry*) genes, by binding specific regulatory elements (CACGTG (E-boxes)) in their promoters; this leads to the generation of the respective proteins (Gekakis *et al.*, 1998; King and Takahashi, 2000; Shearman *et al.*, 2000). Casein kinase Iε (CKIε) phosphorylates PER proteins in the cytoplasm to regulate their stability and cellular location (Vielhaber *et al.*, 2000). PER-CRY complexes form in the cytoplasm, and translocate to the nucleus, where they act as negative regulators by interacting with CLOCK and/or BMAL1 to inhibit transcription. Thus, PER and CRY regulate their own expression, and form a negative feedback loop. *Per2*, however, is an exception in that it activates a positive *Bmal1* transcription loop (Zheng *et al.*, 1999), as illustrated in Figure 1.5.

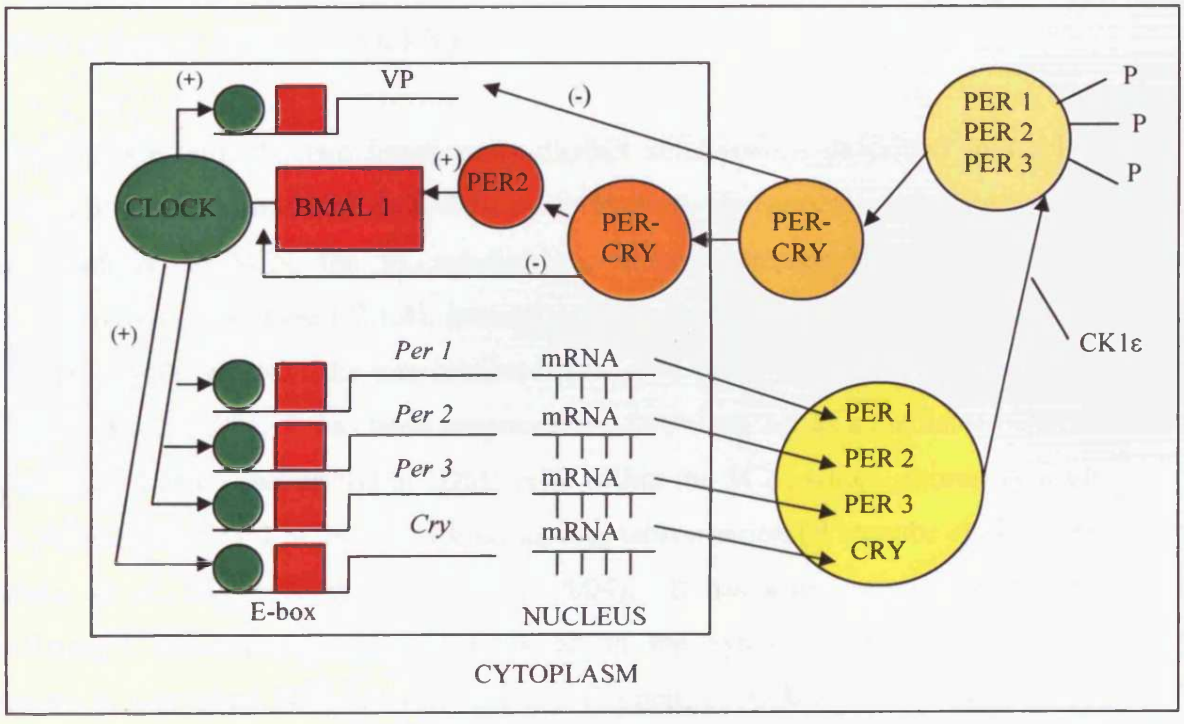


Figure 1.5 Schematic representation of the mammalian circadian clock mechanism (see text for further details).

Recent studies have revealed that the orphan nuclear receptor REV-ERBα is a negative regulator of *Bmal1* transcription, acting through RORE (retinoid-related orphan receptor (ROR) - response element) DNA sequences in the *Bmal1* promoter. The transcription of *Rev-Erbα* is itself negatively regulated by PER and CRY, and positively regulated by BMAL1 and CLOCK (Preitner *et al.*, 2002; Ueda *et al.*, 2002; Kennaway *et al.*, 2003). In addition, there is evidence that DEC (differentially-expressed in chrondrocytes) proteins may be involved in circadian regulation (Honma *et al.*, 2002; Kawamoto *et al.*, 2004). Recent findings indicate that both DEC1 and DEC2 proteins may repress CLOCK/BMAL1-induced activation of the *mPer1* promoter through direct E-box binding (Li *et al.*, 2004), and that DEC1 itself negatively regulates DEC2 through E-box binding in the proximal promoter (Li *et al.*, 2003). The activation of circadian gene expression in the SCN has also been associated with circadian-gated chromatin modification by histone acetylation (Etchegaray *et al.*, 2003). Indeed, a recent study indicates that both rhythmic expression and light induction of *mPer1* and *mPer2* (see 1.2.1.4) are regulated by histone acetylation and deacetylation (Naruse *et al.*, 2004). (The circadian expression of *Per1* is further discussed in 4.1.3.)

In accordance with the two functionally distinct subdivisions described in 1.2.1, a rapid shift in environmental light-dark cycle leads to the desynchronisation of *Per* expression in the SCN; the VL subdivision, which expresses VIP and GRP and receives photic input (see 1.2.1.4), immediately synchronises with the environmental light-dark cycle, whereas the non-retinorecipient DM subdivision slowly phase shifts (Nagano *et al.*, 2003). It has been proposed that GRP may act as a mediator between light-induced (VL) and oscillator (DM) cells within the SCN, which ultimately leads to a phase-shift of DM SCN neurons during resynchronisation (Watanabe *et al.*, 2000; Harmar *et al.*, 2002; Karatsoreos *et al.*, 2004). It has also been suggested that GABAergic transmission may be involved in the synchronisation of circadian rhythms in individual SCN neurons (Liu and Reppert, 2000; Shirakawa *et al.*, 2000). Recent studies indicate that cellular mechanisms involving sodium-dependent action potentials are implicated in the synchronisation of SCN neurons (Honma *et al.*, 2000; Yamaguchi *et al.*, 2003).

1.2.1.3 Regulation of circadian rhythms – Outputs from the SCN

Regarding output genes, VP gene expression in the SCN is positively regulated by E-box elements in its promoter, whilst the PER1 and PER3 proteins negatively regulate VP gene expression, by inhibiting CLOCK-BMAL1-mediated VP transcription (Jin *et al.*, 1999). Rhythmic transcription of the VP gene is associated with an VP peptide rhythm in the SCN (Carter and Murphy, 1991). VP secreted from the SCN is thought to play a role in reducing the stress response whilst concurrently stimulating sexual receptivity (Krajnak *et al.*, 1998). (Circadian VP expression and the role of VP in the SCN are further discussed in 3.1.2 and 3.1.3, respectively).

Recent studies have revealed other circadian output genes from the SCN, such as transforming growth factor (TGF)-α, a signalling molecule that mediates light-induced suppression of locomotion (via action on hypothalamic subPVA receptors; Kramer *et al.*, 2001), and prokineticin (PK)-2, an output molecule that transmits circadian behavioural (locomotor) activity (via receptors in many SCN target sites excluding the subPVA; Cheng *et al.*, 2002). The transcription factor albumin D-element binding protein (DBP) has also been associated with SCN output and locomotor activity (Lopez-Molina *et al.*, 1997). Furthermore, a vast number of rhythmically expressed genes have also been recently identified by DNA microarray studies (in serum-shocked immortalised rat-1 fibroblasts; Duffield *et al.*, 2002, and in the mouse SCN and liver; Panda *et al.*, 2002). These studies revealed that circadian regulation underlies major processes regulated by the SCN and liver, and that rate-limiting steps in these various pathways are key sites of circadian control (Panda *et al.*, 2002). Thus, the value of these studies is the recognition of the fundamental role that circadian clocks play in cellular and organismal physiology.

1.2.1.4 Regulation of circadian rhythms – Input mechanisms to the SCN clock

Light-induced resetting of the circadian clock in the SCN is characterised by an acute induction of expression of the circadian clock genes *Per1* and *Per2* (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997), in addition to several cellular immediate early genes (cIEGs), such as c-*fos* (Kornhauser *et al.*, 1990; Rusak *et al.*,

1990; Sutin and Kilduff, 1992), *jun*-B (Kornhauser *et al.*, 1992; Rusak *et al.*, 1992), and *egr-1* (see 5.1.3). Therefore, these factors may play a role in entrainment of the circadian clock.

Light stimulation during the subjective night activates cyclic adenosine monophosphate (cAMP)-response element (CRE)-mediated gene expression (Obrietan *et al.*, 1999), which in turn stimulates the *Per1* gene in the VL SCN (via CRE in its promoter; Travnickova-Bendova *et al.*, 2002; Tischkau *et al.*, 2003). In response to nocturnal photic stimulation, the transcription factor CRE-binding protein (CREB; Ginty *et al.*, 1993) is phosphorylated at a consensus serine (Ser 133; Gonzalez and Montminy, 1989; or, Ser 142; Gau *et al.*, 2002) phospho-acceptor site, leading to increased transcription of the cIEGs, and the circadian clock genes *Per1* and *Per2*. Similarly, light stimulation during the subjective night is followed by the rapid activation of the p44/42 MAPK (mitogen-activated protein kinase) signalling cascade in the VL SCN (Obrietan *et al.*, 1998), and disruption of signalling via the MAPK pathway curtails both entrainment of the circadian clock (Butcher *et al.*, 2002) and light-induced (CRE-mediated) gene expression (Dziema *et al.*, 2003). These findings suggest that the MAPK signalling cascade may also play an important role in entrainment of the circadian clock.

Thus far, c-*fos* has been the most extensively studied of the cIEGs in the SCN, and has been used for investigating light-responsive signalling pathways in the SCN (see Kornhauser *et al.*, 1996). Recent studies have discovered that the regulation of c-*fos* is different in the core and shell subdivisions of the rat SCN, respectively. For example, a spontaneous c-Fos rhythm has been located in the shell subdivision of the rat SCN, in contrast to nocturnal photic c-Fos induction in the core subdivision of the rat SCN (Sumova *et al.*, 1998; Schwartz *et al.*, 2000; Beaule *et al.*, 2001). Furthermore, since the rhythmicity of the whole SCN is dependent on the photoperiod, it has been suggested that core- to shell- projections may relay information regarding photoperiod from the core- to shell- subdivision of the SCN (Sumova *et al.*, 2000). Taken together, these findings indicate that the circadian function(s) of c-Fos may be cell-specific, and that different parts of the SCN may play distinct roles in photic entrainment. However, the exact function of light-induced cIEGs in the SCN is yet to be elucidated. The further elucidation of the mechanisms

involved in IEG regulation, by light and by the circadian clock, may lead to broader insights into the molecular mechanisms underlying circadian rhythm generation and entrainment.

Recent studies in the mouse have revealed less discrete anatomical subdivisions of the SCN compared to the rat, and are suggestive of a non-conserved SCN organisation. For example, a uniform distribution of retinal inputs (de la Iglesia and Schwartz, 2002) and extensive distribution of light-induced c-Fos (Castel *et al.*, 1997) have been observed throughout the mouse SCN. In addition, a recent study in the mouse and hamster, involving bilateral enucleation (blinding), resulted in elimination of the MAPK rhythm in the 'core' SCN, but not in the 'shell' (Lee *et al.*, 2003). A surprising outcome of another recent study in the mouse was the failure to detect *Period (Per)-1* mRNA, and PER1 and PER2 proteins, at their peak expression time, in the core SCN (LeSauter *et al.*, 2003). A previous study had determined that at its peak, *mPer1* is expressed throughout the SCN (Shigeyoshi *et al.*, 1997). The aforementioned studies therefore suggest a more complex organisation within the (mouse) SCN. Interestingly, other recent studies in mice support the idea (also indicated by c-Fos studies in the rat, above) that the mammalian SCN is arranged into a group of cells that is light-inducible, and another group of cells that is rhythmic (with respect to circadian clock gene expression; Aida *et al.*, 2002; Yan and Silver, 2002; Karatsoreos *et al.*, 2004).

1.3 The Pituitary Gland

Many of the transgenic models studied in this thesis also exhibit expression in the pituitary gland, and therefore some introduction to this system is provided. The pituitary gland (or hypophysis), lies immediately beneath the hypothalamus, and is primarily composed of the anterior pituitary (or adenohypophysis), and the posterior pituitary (or neurohypophysis), as illustrated in Figure 1.1. In addition, an intermediate lobe, between the anterior and posterior pituitary, is present in many mammals, which predominantly secretes α-melanocyte stimulating hormone (MSH) from melanotrophs.

The posterior pituitary does not synthesise the hormone that it stores, and therefore, is not strictly a gland. It is composed largely of the axon terminals of neurons from two hypothalamic magnocellular nuclei, the supraoptic- (SON) and the paraventricular nuclei (PVN), that store two fully processed hormones, VP (also known as anti-diuretic hormone (ADH)) and oxytocin (OT). VP regulates salt and water balance by increasing the permeability of renal collecting tubules and ducts to water. Towards the end of a pregnancy, increased OT stimulates slight contractions of the oestrogen-sensitised myometrium, and consequently initiates labour. OT also causes a contraction of the myoepithelium lining the mammary gland which results in milk-ejection.

The anterior pituitary gland synthesises and secretes many hormones, each with different physiological functions, from 5 different cell types (gonadotrophs, corticotrophs, thyrotrophs, lactotrophs and somatotrophs), as summarised by table 1.1.

Cell type	**Hormone released**	**Function**
Somatotrophs	Growth hormone (GH)	Regulation of post-natal growth
Gonadotrophs	Follicle stimulating hormone (FSH) and luteinising hormone (LH)	Regulation of reproduction
Corticotrophs	Adrenocorticotropic hormone (ACTH)	Regulation of stress
Thyrotrophs	Thyroid stimulating hormone (TSH)	Regulation of metabolism
Lactotrophs	Prolactin (PRL)	Stimulation of post-partum lactation

Table 1.1 A summary of the various cell types of the anterior pituitary gland, the respective hormones released, and their respective functions.

1.3.1 Hypothalamic regulation of the anterior pituitary gland

The synthesis and secretion of hormones from the anterior pituitary is controlled by the release of hypothalamic neuropeptides and neurotransmitters into the external zone of the median eminence, to the anterior lobe, via a capillary portal plexus (see Figure 1.1). The hypothalamic neuropeptides growth hormone-releasing hormone (GHRH) and somatostatin (SST) are synthesised in the arcuate nucleus (Arc) and periventricular nuclei (PeN), and are involved in the regulation of growth hormone (GH) release from somatotrophs.

The neuropeptides CRH (synthesised in the PVN) and VP are involved in the regulation of adrenocorticotrophic hormone (ACTH) release from corticotrophs, and the neuropeptide thyrotropin-releasing hormone (TRH) is involved in the regulation of thyroid-stimulating hormone (TSH) release from thyrotrophs. Dopamine is synthesised in the Arc, and is involved in the regulation of lactation by inhibiting prolactin (PRL).

GnRH is synthesised in rostral hypothalamic neurons, and its release into the portal blood stimulates luteinising hormone (LH) and follicle-stimulating hormone (FSH) release from the gonadotrophs. In the ovary, FSH stimulates oestrogen production and follicular growth, predominantly during the first half of the oestrus cycle, ending with ovulation. The follicle consists of multiple cells containing an oocyte growing within a follicular cyst. A surge in LH causes maturation and eventual rupture of the follicular cyst, releasing the oocyte (ovulation). In addition, LH is involved in the synthesis of androgens and oestrogens, which control gonadal development (Brown and McNeilly, 1999). It has been established that Egr-1 acts as a transcriptional regulator of the pituitary hormone gene that codes for the LH β-subunit, and thus maintains appropriate expression of the LH β-subunit (Lee *et al.*, 1996; Topilko *et al.*, 1998; see also Dorn *et al.*, 1999; Tremblay and Drouin, 1999; Wolfe and Call, 1999; Sevetson *et al.*, 2000; Duan *et al.*, 2002). Further details of the interesting expression pattern and role of Egr-1 in the pituitary are provided in 5.1.2.

1.4 Use of transgenic mammalian models to investigate gene function

Gordon and colleagues were the first to successfully produce a transgenic mammal (mouse) in 1980, which involved the stable integration of foreign DNA into a genome, otherwise known as germline transgenesis. The mouse has become the preferred species of choice for mammalian genetic experiments and transgenesis since there are extensive and well-annotated mouse genomic databases available, and mice are relatively less expensive to maintain compared with rats. Other mammals such as rats (Mullins *et al.*, 1990), sheep (Schnieke *et al.*, 1997), and pigs (Pursel *et al.*, 1990) have also been used as transgenic animal models. In contrast to transgenic animal models, *in vitro* transfection studies in cell lines are unable to reproduce the effects of processes such as developmental cell-to-cell interaction, and endocrine and neuronal communication.

With much refinement, transgenesis has now become a key technique for the production of many animal models. In particular, the generation of transgenic models of human neurological diseases, such as Alzheimer's disease (Games *et al.*, 1995) and Huntington's disease (Mangiarini *et al.*, 1996), has made a substantial impact on the understanding of the molecular basis of human disease, and on the development of novel therapies (Rudolph and Möhler, 1999; Götz, 2001; also see Wong *et al.*, 2002).

1.4.1 Transgenesis Strategies in Rodents

There are a variety of approaches available for rodent transgenesis (see Wells and Carter, 2001). The two major types of strategies commonly used are germline transgenesis and somatic transgenesis. Germline transgenesis refers to transgenesis procedures which involve the stable integration of the transgene into the genome, and subsequent inheritance by progeny. These procedures include:

i) transfection of early embryos with recombinant retroviruses;

ii) intracytoplasmic sperm injection (Perry *et al.*, 1999);

iii) nuclear transfer derived transgenesis (McCreath *et al.*, 2000);

iv) pronuclear injection into fertilised eggs; and,

v) gene targeting in embryonic stem (ES) cells.

Gene targeting approaches using stable ES cell lines have proved to be popular, but are only available for the mouse (Evans and Kaufman, 1981). ES cells offer the advantage of allowing the investigator to manipulate the genome (to target specific genes) by homologous recombination, and therefore allows both gain-of- (knock-in) and loss-of- (knock-out) function analyses of a particular gene to be performed. Since no rat ES cells are available, no 'knock-out' rats have been generated.

Currently, the only reliable and efficient method for generating transgenic rats is by the pronuclear injection of DNA into single cell embryos. The first transgenic rats were reported in 1990 (Mullins *et al.*, 1990; Hammer, *et al.*, 1990), and were followed by the creation of many other transgenic rat lines using pronuclear injection (see Charreau *et al.*, 1996a). The expression of a transgene is affected by its chromosomal location, that is, the site of integration in the host chromosome, a phenomenon known as a position effect (Clark *et al.*, 1994). The outcome of position effects is a lack of consistent transgene expression (in independent lines of transgenic animals which have integrated the same transgene), with low frequencies and levels of expression, lack of tissue specificity and inappropriate expression observed. Since transgene integration is a rare event, and the site of integration is random, both positional effects and multiple integration events can occur. However, the former can be overcome using insulator sequences (naturally occurring DNA sequences that protect transgenes from genomic position effects, thereby establishing independent functional domains within the chromosome; Geyer, 1997), and the latter can be subsequently bred out. Essentially, the procedure involves the pronuclear injection of DNA into fertilised eggs (harvested from superovulated female rats mated with male studs), followed by the surgical transfer of the manipulated eggs into the oviducts of pseudopregnant female rats (oviduct transfer (OVT) mothers mated with vasectomised males).

Somatic transgenesis refers to transgenesis procedures that do not involve the integration of the transgene into the genome (and therefore, progeny do not inherit the transgene), and achieve transient expression of exogenous DNA in the somatic cells of a living organism. Consequently, expression can often be observed a few hours following gene delivery, the transgene can be delivered specifically to a desired cell population or tissue, and the procedure is less time-consuming and less expensive.

Germline transgenesis involving pronuclear injection of sequence-specific transgenes into fertilised eggs was considered to be the most appropriate strategy for creating transgenic rats in the present thesis.

1.4.2 Transgene components

In the context of this thesis, one major aim was to create transgenic rats that overexpressed the particular gene under investigation. In order to direct appropriate expression in a mammalian host, the transgene constructs utilised in this thesis incorporated the following:

i) A mammalian-derived promoter/enhancer capable of driving transcription in the desired tissues/cell populations.
ii) At least one intron (known to increase efficiency of transgene expression) (Brinster *et al.*, 1988).
iii) An appropriate translation initiation sequence and termination codon.
iv) An encoding (genomic or cDNA) sequence for the gene/protein to be overexpressed.
v) A mammalian polyadenylation (poly-A) signal sequence at the 3' end.
vi) A reporter gene divergent from the endogenous transcript to allow monitoring of transgene expression.

Specific details regarding the individual transgene constructs involved in this thesis are discussed in subsequent chapters.

1.4.3 The benefits of using the rat as a transgenic model

The laboratory rat *Rattus norvegicus* is the most commonly used and well-characterised experimental animal in many fields of biomedical research, including behavioural and neurophysiological studies. Consequently, a diverse and detailed knowledge of the rat central nervous system (CNS) is available in the literature and online databases. The larger size of the rat also enables physiological techniques such as microsurgery, multiple blood sampling (in larger volumes), tissue and organ sampling, and analysis of organ function *in vitro* to be more accessible. In addition, the anatomy and reproductive biology of the rat is similar to that of the mouse.

Rats are used as transgenic models of human disease since some disease processes in the rat are closer to human pathology, in terms of their features and origins, than they are in the mouse. With respect to transgenesis, the procedures involved in generating transgenic rats are in essence identical to those involved in generating transgenic mice. One major difference, however, is that micro-injection of rat oocytes can be more difficult since the zona pellucida surrounding the rat oocyte is not as easy to penetrate than that of the mouse (Murphy and Carter, 1993).

As aforementioned, recent studies in mice have indicated less discrete anatomical subdivisions of the SCN compared to the rat (see 1.2.1.4). For this reason, in addition to rat physiology being much more comprehensively understood than that of the mouse, and the rat brain being larger and more completely mapped, it was decided that the rat was the most suitable model for this thesis.

1.5 PRIMARY AIMS OF THESIS

The primary aim of this thesis was to investigate mechanisms that control gene expression in the SCN and other neuroendocrine sites, through the generation and analysis of different transgenic models. Therefore, the aims of this thesis are:

1. To generate transgenic rats (Chapters 4 and 6, respectively) by pronuclear injection of DNA (*mPer1*/d2EGFP (Y) and *egr-1*/d2EGFP (Z) promoter/ reporter transgenes) into fertilised oocytes.

2. To screen for transgene (Y, *egr-1*/d4EGFP (57C) and Z,) incorporation in progeny (Chapters 4, 5 and 6, respectively).

3. To establish and maintain breeding lines of transgenic (rVP/hGH (JP), Y, 57C and Z) rats from founders (Chapters 3, 4, 5 and 6, respectively).

4. To use subsequent generations of transgenic (JP, Y, 57C and Z) rats for analysis of transgene expression (Chapters 3, 4, 5, 6 and 7, respectively). This analysis is principally aimed at investigating the fidelity of transgene expression in the SCN.

5. To investigate the physiological factors that determine both endogenous *egr-1* and transgene expression within the neuroendocrine system of the transgenic rat models (Chapters 5, 6 and 7).

6. To broadly define the sequences required for conferring physiologically-regulated *egr-1* gene expression within the rat brain and anterior pituitary gland (Chapter 7/8).

CHAPTER 2

GENERAL MATERIALS AND METHODS

The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

DNase-, RNase-, DNA and pyrogen-free plasticware [microcentrifuge tubes and pipette tips; Starlabs] were used throughout. Sterile (autoclaved) Elgastat reverse osmosis purified water, is described here as 'sterile water.'

2.1 PREPARATION AND MAINTENANCE OF BACTERIAL CULTURES

2.1.1 Transformation of competent *Escherichia coli (E. coli)* cells

1-5µl of plasmid DNA was added to 50µl of competent JM109 *E.coli* cells (Promega) in a microcentrifuge tube, gently mixed, and the tube was then placed on ice for 20 minutes.

The bacterial suspension was heat-shocked at 42°C for 90 seconds, and then immediately placed on ice for 2 minutes. 100µl LB broth was added to the bacteria, which were then incubated at 37°C for 30 minutes.

The bacteria were then spread onto an LB agar plate containing the appropriate antibiotic, and placed in a 37°C incubator, agar side down, for 20 minutes to allow the suspension to dry. The plates were subsequently inverted (agar on top), and incubated at 37°C overnight, to allow for bacterial growth. The plate cultures of the trans-formants were stored at 4°C (for 2-3 weeks), as required.

2.1.2 Long-term storage of transformed bacteria (glycerol stocks)

A single colony of transformed bacteria was picked (from the plate cultures; 2.1.1), with a sterile transfer loop, and placed in a Universal tube containing 5ml LB broth (supplemented with an appropriate antibiotic). The tube was then incubated in an orbital shaker (225 rpm) at 37°C, overnight, to allow for bacterial growth.

A 0.85ml aliquot of the transformed cells was taken from the Universal tube, and placed in a 1.5ml microcentrifuge tube containing 0.15ml sterile glycerol (i.e. 15% (v/v) glycerol final concentration). The transformed cells were vortexed thoroughly, and stored at -70°C (for up to 3 years), as required.

2.1.3 Recovering transformed bacteria from glycerol stocks

The colonies of bacterial transformants were recovered from frozen glycerol stocks (2.1.2) by immediately streaking a sample (~10µl) on an LB agar plate (supplemented with an appropriate antibiotic), using a sterile transfer loop. The plate was then incubated at 37°C overnight, to allow for bacterial growth.

A single colony was picked from the plate, with a sterile transfer loop, and placed in a Universal tube containing 5ml LB broth (supplemented with an appropriate antibiotic). The tube was then incubated in an orbital shaker (225 rpm) at 37°C, overnight, prior to purification of the plasmid DNA (2.1.4).

2.1.4 Isolation and purification of plasmid DNA

Plasmid DNA was isolated from LB broth medium (2.1.3), and subsequently purified and eluted, using the Wizard® *Plus* SV Mini-Preps DNA Purification System (Promega – see appendix B), and stored at -20°C (for up to a year), as required.

2.2 DNA METHODS

2.2.1 Genomic DNA extraction from rat tail and ear biopsies

Biopsy procedures were performed between post-natal days 10-14, following our laboratory protocol. For each biopsy, 2-3mm of tissue was cut from the tip of the rat's tail or ear, and placed into a 1.5ml microcentrifuge tube. 200µl of (500µg/ml) Proteinase K/STE was added per tube, which was incubated at 55°C overnight, or until the tissue had disintegrated.

For each sample, 1 volume DNA phenol (i.e. 200µl, equilibriated with Tris-HCl, pH 8) was added and mixed by vigorous shaking for 3 minutes. The tubes were then centrifuged at 14,000rpm for 3 minutes, in a bench-top centrifuge. The resultant upper aqueous phase was transferred to a fresh 1.5ml microcentrifuge tube, to which 1 volume phenol/chloroform/isoamyl alcohol (P/C/IAA; 25:24:1) was added. The tubes were shaken vigorously for 2 minutes, and centrifuged again at 14,000rpm for 2 minutes.

The resultant upper aqueous phase was again transferred to a fresh 1.5ml microcentrifuge tube, to which 0.25 volume of 3M NaAc (pH 6) and 2.5 volume of 100% ethanol was added. The tubes were individually inverted several times until the DNA precipitate could be seen, before centrifuging at 14,000rpm for 10 minutes. The supernatant was carefully decanted, to avoid dislodging the white DNA pellet, and excess fluid was blotted on a paper towel. The pellet was then washed twice by adding 1ml of 70% ethanol, mixing by inversion, centrifuging at 14,000rpm for 10 minutes and decanting the supernatant.

Any excess fluid was blotted on a paper towel, before allowing the pellet to dry at room temperature for 10 minutes. The DNA pellet was then resuspended in 50µl TE buffer and left at 4°C overnight to allow the pellet to dissolve. The genomic DNA was then used for genotyping by polymerase chain reaction PCR (2.2.2), and/or detection of DNA sequences by Southern blot analysis (2.2.5).

2.2.2 Genotyping by Polymerase Chain Reaction (PCR) amplification

A standard 25µl PCR reaction consisted of: sterile water, 1x (final concentration) PCR reaction buffer (Promega), 1.5mM (final concentration) magnesium chloride (Promega), 200µM (final concentration) each of dATP, dCTP, dGTP, and dTTP (Promega), 200-400nM (final concentration) of each gene-specific primer (MWG Biotech; see experimental chapters for sequences and concentrations used), 0.5-1µl template DNA and 0.04 units/µl (final concentration) of *Taq* polymerase (Promega).

For multiple reactions, the reagent volumes were scaled up to produce a mastermix (exclusive of template DNA). The mastermix was aliquotted into 0.5ml sterile microcentrifuge tubes, prior to the addition of the DNA template. The PCR Express (Hybaid) machine was used for all PCR reactions, using various thermal cycling conditions (see experimental chapters).

2.2.3 Nucleic acid fractionation by agarose gel electrophoresis

Nucleic acids can be resolved according to mass by agarose gel electrophoresis. Agarose gels were prepared by first dissolving 1% (w/v) agarose in 1x TAE buffer, using a microwave oven. The gel was cooled for 10-20 minutes prior to adding 100ng/ml ethidium bromide.

A casting tray was made by placing masking tape around either end of the gel tray, to generate a 'wall,' and a well-forming comb was placed at one end of the gel tray. The gel solution was then poured into the prepared gel tray, and left to set for 15-20 minutes, after which the tape was removed.

The gel tray was then submerged in 1x TAE in the electrophoresis tank (various models). 1x Orange-G loading buffer was added to each sample prior to pipetting into the agarose gel wells (cathode side). An appropriate size marker was also loaded on the end. Electrophoresis was conducted at 90-120V, until the nucleic acid was sufficiently resolved.

The ethidium bromide-stained bands in the agarose gel were then visualised and photographed using the GeneGenius™ Imaging System (Syngene). Images were stored as compressed TIFF files.

2.2.4 Quantification of nucleic acid concentration using OD_{260} spectrophotometry

1-2μl of nucleic acid samples were diluted in sterile water (to a final volume of 500μl) and quantitated by spectrophotometry at a wavelength of 260nm. A 500μl water blank was pipetted into a 1ml quartz cuvette (Amersham Pharmacia Biotech), and read at 260nM on the GeneQuant spectrophotometer (Amersham Pharmacia Biotech). This provided a reference for sample comparison.

From the OD_{260} readings, the concentration of the nucleic acid could be calculated since the absorbance of 1 OD_{260} is equivalent to 50μg/ml double-stranded DNA (or 40μg/ml single stranded RNA).

2.2.5 Southern Blot Analysis (summarised by Figure 2.1)

2.2.5.1 Genomic DNA digestion

A standard 20μl restriction endonuclease digestion reaction consisted of sterile water, 10-15μg (final concentration) of genomic DNA (2.2.1), 1x (final concentration of appropriate) reaction buffer and 20-30 units (final concentration) of the appropriate restriction enzyme. Digests were incubated overnight in a 37°C waterbath.

2.2.5.2 Electrophoresis of digested, genomic DNA

1x (final concentration) Southern loading dye was added per digest (2.2.5.1), prior to loading on a 0.7-1.5% agarose gel in 1× TAE (see 2.2.3 for preparation), with the appropriate markers (e.g. molecular weight marker and/or transgene). Electrophoresis was conducted at 100-120V, for 1.5 - 2 hours, and then gels were treated for Southern blotting (2.2.5.3).

2.2.5.3 Southern Blotting

Gels (2.2.5.2) were first gently agitated in depurination buffer for 15 minutes, then denaturation buffer for 15 minutes and finally transfer buffer for 10 minutes, as described by Sambrook *et al.*, (1989). The DNA was then transferred from the gels onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) via capillary action of transfer buffer, as illustrated in Figure 2.1, for at least 12 hours. The membrane was rinsed in 50mM NaPi, left to air-dry for 10 minutes, and baked at 80°C for 30-60 minutes. The DNA was then UV cross-linked onto the membrane (Stratalinker, Stratagene; Autocrosslink mode), and stored at 4°C, ready for hybridisation (see 2.2.5.5).

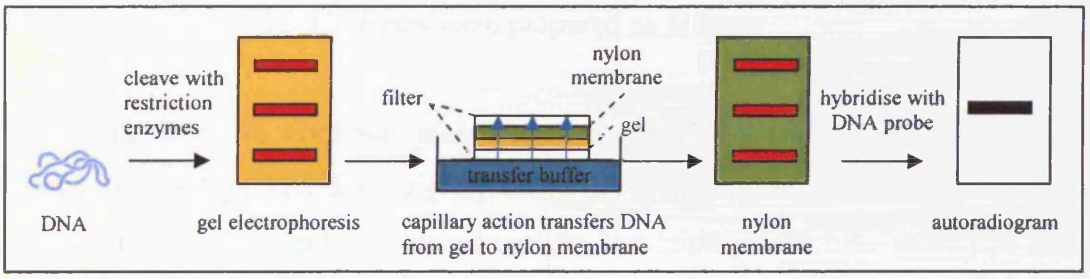


Figure 2.1 Schematic representation of the main processes involved in Southern blot analysis

2.2.5.4 DNA probe preparation

DNA probes were obtained by gel-purifying appropriate fragments from restriction digests of DNA, using the Qiaex II Agarose Gel Extraction Kit (Qiagen – see appendix B), quantitated (2.2.4), and radio-labelled by the random primer method (2.2.5.4.1).

2.2.5.4.1 Radio-labelling DNA probes – the OLB (random primer) method

20-50ng of purified DNA probe (2.2.5.4) was mixed with sterile water, to a final volume of 32µl, in a 1.5ml microcentrifuge tube, and placed in boiling water for 10 minutes. The probe was immediately cooled on ice for 1 minute and centrifuged briefly (to collect the liquid in the bottom of the tube).

10µl OLB 5x buffer and 2µl BSA (10mg/ml, Promega) were added to the tube, which was then incubated at 37°C for 45 minutes. Following a brief centrifugation, 5µl α^{32}P-dCTP (≡1.85MBq or 50µCi, Amersham Pharmacia Biotech) and 1µl Klenow large fragment DNA polymerase (5U/µl, Promega) were added to the reaction tube, mixed gently, and incubated overnight at room temperature. 50µl OLB-C Stop solution was added the following day, prior to purification of the probe through a Sephadex G-50 spin column (2.2.5.4.2).

2.2.5.4.2 Size exclusion chromatography - Sephadex G-50 spin column

To separate the radio-labelled probe from unincorporated nucleotides, conventional size-exclusion chromatography through 1ml columns of Sephadex G-50 was used (Sambrook *et al.*, 1989). Columns were prepared as follows:

A ball of sterile glass wool was placed in the bottom of a 1ml syringe barrel (up to ~0.1ml level) and packed down tightly using the syringe plunger, and then a pasteur pipette. The syringe barrel was placed upright in a 15ml plastic tube and filled to the top with well-shaken Sephadex G-50 suspension in TE (pH 8.0), using a pasteur pipette. The Sephadex G-50 column was then centrifuged at 3,000rpm for 3 minutes at room temperature.

A ball of tissue paper was placed into a 15ml plastic tube (up to ~1ml level), and an open 0.5ml microcentrifuge tube was placed above it, using a pair of forceps. The Sephadex column was then inserted into the 15ml falcon tube so that the end of the syringe barrel was inside the open microcentrifuge tube.

The labelled probe was applied to the surface of the spun Sephadex, and allowed to absorb into the column, before centrifuging at 3,000rpm for 3 minutes. The column was carefully discarded, and the 0.5ml microcentrifuge tube containing the purified labelled probe was carefully removed. Probes were used immediately, or stored in a lead pot at -20°C until required.

2.2.5.5 Southern Hybridisation

Southern blot membranes (2.2.5.3) were rinsed in wash buffer, rolled in nylon mesh, and placed in a 100ml hybridisation tube. 15ml of pre-warmed (65°C) pre-hybridisation buffer was added to the membrane(s) in the hybridisation tube, which was then incubated at 65°C, for 30 minutes, in a rotisserie oven (Stuart Scientific, 5120H).

50-100µl of probe (prepared as described in 2.2.5.4) was denatured by boiling for 10 minutes, and quenching on ice for at least 1 minute. The probe was collected in the bottom of the tube by brief centrifugation, and added to 10-15ml of pre-warmed (65°C) hybridisation buffer. The probe/hybridisation buffer mix was used to replace the pre-hybridisation buffer in the hybridisation tube, which was then incubated at 65°C, overnight.

2.2.5.6 Stringency Washes

The membrane(s) were first rinsed in pre-warmed wash buffer, and then washed at room temperature, on a rotary shaker (Stuart Scientific rocking platform; 35 rev/min), for 5 minutes. The buffer was replaced with fresh pre-warmed (65°C) wash buffer, and incubated at 65°C, whilst shaking, for 30 minutes. The background radioactivity on the membrane was checked, and the buffer replaced with fresh pre-warmed (65°C) wash buffer, as necessary.

2.2.5.7 Detection and visualisation of the probe

The membrane was heat-sealed in polythene and exposed either to X-ray film (Kodak MXB film) at -70°C, or a storage phosphor screen (Kodak K) at RT, for appropriate periods prior to development (X-ray film, X-ray film processor, Agfa Gevaert) or imaging (Phosphor screen, Personal FX, BioRad), under safe-light conditions.

2.3 RNA METHODS

To minimise RNase contamination, and thus RNA degradation, solutions and lab-ware were pre-treated by vigorous shaking with 0.1% (v/v) diethylpyrocarbonate (DEPC), to inactivate RNases. The DEPC-treated solutions and lab-ware were left at room temperature overnight, and then autoclaved for 15 minutes at 121°C the following day.

Laboratory benches, pipettes and racks were cleaned by spraying and wiping down with RNaseAway (Ambion) or 70% (v/v) IMS. All pipette tips and microcentrifuge tips were purchased RNase-free (Starlabs). Gloves were worn to reduce the chance of RNase contamination from fingers.

2.3.1 RNA extraction from rat tissues

Male and female adult Sprague-Dawley (SD) rats, of various ages (see experimental chapters), were killed by stunning and decapitation according to UK Home Office Regulations (Schedule 1 humane killing methods).

Various tissues (see experimental chapters) were immediately dissected out (~ 20mg), placed into a 1.5ml microcentrifuge tube and placed on dry ice for prompt freezing. The tissues were transferred on dry ice, and either placed in a -70°C freezer for temporary storage, or immediately used for total RNA extraction (2.3.1.1).

2.3.1.1 Total RNA extraction from rat tissues

Tubes containing the tissue samples were wiped and placed on wet ice, ready for total RNA extraction using a guanidium isothiocyanate (GTC; deproteinisation reagent)-phenol-chloroform extraction method (modified from Chomczynski and Sacchi, 1987).

Tissue samples were homogenised in 400µl GTC-A solution prior to addition of 0.1 volume NaAc (3M, pH 5.5), 1 volume RNA phenol and 0.2 volume chloroform-isoamyl alcohol (49:1). The mixture was shaken for 10 seconds, incubated on ice for 10 minutes, and centrifuged at 14,000rpm for 10 minutes at 4°C.

The upper aqueous layer was transferred by pipette to a fresh 1.5ml microfuge tube. RNA was precipitated by the mixing with 2 volumes (~800µl) of ice-cold absolute ethanol, freezing at -70°C for at least 1 hour, and centrifuging at 14,000rpm for 10 minutes at 4°C.

The pellet was resuspended in 120µl ice-cold GTC-A solution, and 2 volumes of absolute ethanol were added. The mixture was briefly vortexed prior to incubating at -70°C for a further 30 minutes. Tubes were then centrifuged at 14,000rpm for 10 minutes at 4°C. The RNA pellet was washed with 120µl of 95% ethanol, and the tubes were centrifuged for a further 2 minutes at room temperature. The supernatant was removed with a pipette, and the pellet resuspended in an appropriate volume of DEPC-treated water by pipetting, brief vortexing, and brief centrifugation.

1µl of RNA was quantitated (2.2.4.), prior to reverse-transcription-PCR (RT-PCR) or Northern Blot analysis (2.3.2 and 2.3.3, respectively). The integrity of the RNA preparation was also checked by electrophoresis of 1µl of the RNA sample on a fresh agarose gel (see 2.2.3 for preparation). RNA samples were stored at -70°C.

2.3.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.3.2.1 DNase-treatment of total RNA samples

DNase I treatment was performed on total RNA samples to degrade any potentially contaminating DNA that may be present in the RNA samples.

A 10µl 'DNase I' reaction consisted of: DEPC-treated water, 1-7µg total RNA, 1x (final concentration) DNase I buffer (Promega), 0.1 units (final concentration) of RQ1 DNase I (Promega; 1U/µl), and 2 units (final concentration) of RNasin (Promega; 40U/µl). The reaction was incubated at 37°C for 30 minutes and then briefly centrifuged. 1µl of 10x RQ1-DNase termination solution (Promega) and 30µl of DEPC-treated water were added to stop the reaction, and the RNA was purified by P/C/IAA extraction (2.3.2.1.1).

2.3.2.1.1 RNA purification (phenol/chloroform/isoamylalcohol extraction)

For each sample, 1 volume P/C/IAA (25:24:1) was added, and tubes were vortexed thoroughly, and centrifuged at 14,000rpm for 10 minutes at 4°C. The resultant upper aqueous phase was transferred to a fresh 1.5ml microfuge tube, 1 volume of chloroform was added, and the tube was vortexed thoroughly, and centrifuged at 14,000rpm for 10 minutes at 4°C.

The resultant upper aqueous phase was again transferred to a fresh 1.5ml microfuge tube, and 0.1 volume 3M NaAc (pH 5.8) and 2.5 volumes (pre-chilled) 95% ethanol were added. Tubes were thoroughly vortexed, and incubated on ice for 10 minutes prior to centrifuging at 14,000rpm for 15 minutes at 4°C.

The supernatant was carefully removed by pipette, and the pellet was then washed in 500µl (pre-chilled) 80% ethanol. The tubes were centrifuged at 14,000rpm for 5 minutes at 4°C, and the resultant supernatant pipetted off. The RNA pellet was air-

dried at room temperature for 10 minutes and resuspended in 10µl DEPC-treated water. 1µl of RNA was quantitated (for RT-PCR; 2.3.2.2) as described in 2.2.4.

2.3.2.2 First–strand cDNA synthesis

First strand cDNA was synthesised using the Superscript™ II reverse transcriptase enzyme of the Superscript™ Pre-amplification System (Invitrogen – see appendix B). An initial 12µlreaction was set up consisting of: DEPC-treated water, 1µg total RNA, 1µl oligo (dT) 12-18 (0.5µg/µl; Amersham Pharmacia Biotech) and 1µl dNTP mix (10mM of each of dATP, dCTP, dGTP, and dTTP; Promega). The reaction was incubated at 65-70°C for 5 minutes, quenched on ice for at least 1 minute, and briefly centrifuged.

7µl of pre-mix was then added to the initial reaction, consisting of 4µl 5x reaction buffer (Invitrogen), 2µl 0.1M DTT (Invitrogen) and 1µl RNasin (40U/µl; Promega), and incubated at 42°C for 2 minutes. The tubes were centrifuged briefly, and 1µl Superscript™ II (200U/µl) was added to each reaction. All reactions were then incubated at 42°C for 50 minutes, and then at 70°C for 15 minutes (to terminate cDNA synthesis).

1µl of cDNA was used per polymerase chain reaction, as described in 2.2.2. In this case, 1.25 units of *Taq* polymerase (Promega) were used per reaction. All remaining cDNA was stored at -20°C.

2.3.3 Northern Blot analysis

2.3.3.1 RNA preparation

RNA samples (2.3.1) were allowed to thaw on ice (~30 minutes), if required, prior to preparation for northern blot hybridisation.

3 volumes of 3:1 LB (stored at -20°C) was added to 5-20μg RNA, and the mixture was heated at 65°C for 15 minutes, and then quenched on ice. 0.1 volume of NLB (containing 0.1 volume ethidium bromide) was added per RNA sample, prior to loading the samples into a 1% (v/v) formaldehyde agarose gel (2.3.3.2).

2.3.3.2 RNA Electrophoresis

A 1% formaldehyde agarose gel was prepared as follows: 2.5g agarose was boiled in 190ml distilled water. 12.5ml 20× MAE, 6ml DEPC-treated water and 42ml formaldehyde was added to the hot agarose mixture (in fume cabinet). The gel mixture was immediately mixed by swirling, and allowed to cool at room temperature for 15-30 minutes.

A gel tray was prepared, and an appropriate thick well-forming comb inserted. The gel mixture was poured, allowed to set for 20-30 minutes, and the gel was then placed in a tank (containing 1x MAE). RNA samples were loaded into the gel, which was electrophoresed at 70-100V for 2-3 hours, and then northern blotted (2.3.3.3).

2.3.3.3 Northern Blotting

The RNA was transferred from the gel (2.3.3.2) onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) via capillary action of 20x SSPE buffer, for at least 16 hours.

The membrane was rinsed in 1× SSPE, left to air-dry for 10 minutes, and then baked at 80°C for 30 minutes. The RNA was then UV cross-linked onto the membrane (see 2.2.5.3) prior to wetting the membrane in 1xSSPE. The membrane was placed (RNA side down) briefly on a transilluminator where 18S and 28S RNA bands were pencil-marked for future reference. The blot was either hybridised immediately (2.3.3.4.), or heat-sealed in polythene and stored at -20°C until required.

2.3.3.4 Northern Hybridisation / Stringency washing

The northern blot was hybridised, washed, exposed and imaged as described for Southern Hybridisation (2.2.5.5 and 2.2.5.6, respectively).

2.3.4 *IN SITU* HYBRIDISATION (ISH)

2.3.4.1 Tissue preparation

2.3.4.1.1 Fresh frozen tissue sampling

Male and female adult Sprague-Dawley rats, of various ages (see experimental chapters), were killed by stunning and decapitation, according to UK Home Office Regulations (Schedule 1 humane killing methods).

Various tissues (see experimental chapters) were immediately dissected out with RNase-free instruments, and loosely wrapped in aluminium foil. The tissue was then submerged in a metal beaker of cold isopentane (maintained at -40°C with dry ice), using forceps, and frozen by agitation for 1~2 minutes. The samples were then placed on dry ice, and transferred to a light-tight container into a -70°C freezer for temporary storage, or immediately placed in a cryostat for sectioning (2.3.4.1.2).

2.3.4.1.2 Embedding/sectioning of tissue

A hotplate (Bibby HC502) was set at 40°C, and the cryostat chamber (Bright OTF) at -20°C. The frozen tissue sample (2.3.4.1.1) was transferred to the freezing platform of the cryostat. The tissue was then left for 20 minutes to equilibrate to the platform

temperature. An embedding medium (Bright Cry-M-Bed) base was made on the chuck, and the tissue immediately mounted (with an appropriate orientation). Medium was built up to surround the target tissue, and the chuck was then placed on the freezing platform area of the cryostat (~2 minutes). The chuck was then placed into the microtome of the cryostat. The blade was cleaned, moved and/or changed, as required.

Initially, the mounted tissue was approached with thick cuts (20-30μm) by moving the tissue towards the blade, using the cutting wheel of the cryostat. The thickness control was then adjusted to 12μm to cut thin sections, prior to approaching the desired region. A fine paintbrush was used to clean the blade in between cuts. 2-3 sections were collected by freeze-thaw mounting, that is, by placing frozen, RNase-free, electrostatically-charged microscope slides (Superfrost plus slides, BDH) over frozen sections, and thawing on the palm of the gloved hand. The sections were dried by placing the slides on the hotplate for ~30 seconds. The slides were placed in a slide box (containing dessicant) on the freezing platform, and then transferred to a -70°C freezer at the end of the session, until required (2.3.4.3.1).

2.3.4.2 Probe preparation for ISH

2.3.4.2.1 Oligonucleotide probe labelling - the 3'-end labelling reaction

Oligonucleotide probes were labelled by the 3'-end labelling reaction (Sambrook *et al.*, 1989). An initial 22.5μl reaction was set up in a 1.5ml microcentrifuge tube on ice, and comprised: DEPC-treated water, 200ng of the oligonucleotide (MWG Biotech), 1x (final concentration) tailing buffer (pH7.2, Amersham Pharmacia Biotech) and 1.85MBq or 50μCi α^{35}S-dATP (Amersham Pharmacia Biotech). The reagents were mixed and then microcentrifuged at 8,000rpm for 10 seconds at room temperature.

2.5µl terminal deoxynucleotidyl transferase (14U/µl, Amersham Pharmacia Biotech) was then added, to bring the final volume of the reaction mixture up to 25µl. The reaction was mixed by pipetting, and incubated at 37°C for 1 hour. To stop the reaction, the tube was placed on ice. The probe was then purified through a Sephadex G-50 spin column (2.3.4.2.1.1).

2.3.4.2.1.1 Size exclusion chromatography - Sephadex G-50 spin column

To separate the radio-labelled probe from unincorporated nucleotides, conventional size-exclusion chromatography through 1ml columns of Sephadex G-50 were used, and were prepared as described in 2.2.5.4.2. In addition, the column was equilibrated with TE buffer (pH 7.6) by adding 100µl TE buffer to the surface of the Sephadex and centrifuging at 3,000rpm for 3 minutes, 3 times.

The labelled probe (25µl – see 2.3.4.2.1) was applied to the surface of the Sephadex, and allowed to absorb into the column, before centrifuging at 3000rpm for 3 minutes. The column was then rinsed with 25µl of TE (total volume = 50µl) and centrifuged at 3,000rpm at room temperature for 3 minutes.

The column was discarded, and the 0.5ml microfuge tube containing the purified labelled probe was carefully removed. 1µl 1M dithiothreitol (DTT; reducing agent) was added to the labelled probe to maintain the integrity of the probe. The probe activity was then determined (2.3.4.2.1.2).

2.3.4.2.1.2 Determination of probe activity

2µl of probe (2.3.4.2.1.1) was added to 1ml of scintillation fluid, and counted in a scintillation counter (Beckman Coulter LS6500), using the ^{35}S channel. The ^{35}S-labelled probe was diluted to a final concentration of $1x10^7$cpm/ml hybridisation buffer II and stored at -20°C until required.

2.3.4.2.2 Riboprobe labelling

2.3.4.2.2.1 Linearising plasmid template

Plasmid DNA was linearised by digestion with a suitable restriction endonuclease. A standard 40μl restriction endonuclease reaction consisted of: DEPC-treated water, ~1.2μg plasmid, 1x (final concentration of appropriate) reaction buffer and 1 unit (final concentration) of the appropriate restriction enzyme. Digests were incubated in a 37°C waterbath for 90 minutes, and linearised plasmid DNA was extracted (see 2.3.4.2.2.2).

2.3.4.2.2.2 Extracting linearised plasmid

Linearised plasmid DNA (2.3.4.2.2.1) was extracted using P/C/IAA (25:24:1), as follows: 280μl TE and 300μl P/C/IAA were added per digest. The mixture was vortexed for 10 seconds, and centrifuged at 13,500rpm, for 5 minutes. The resultant upper aqueous phase was transferred to fresh tubes containing 300μl P/C/IAA, and vortexed briefly. The tubes were then centrifuged at 13,500rpm for 1 minute. The resultant upper aqueous phase was again transferred to fresh tubes. 30μl 3M NaAc and 750μl 100% ethanol were then added to each tube. The tubes were inverted 5 times, and the linearised plasmid DNA was allowed to precipitate at -70°C for 30 minutes. The tubes were centrifuged at 13,500rpm, for 20 minutes, at 4°C. The subsequent supernatant was poured off, and the pellet in each tube was allowed to air-dry (~10 minutes). The pellet was then resuspended in 4.5μl of DEPC-treated water. A 0.5μl aliquot of the purified plasmid DNA was then loaded on a 1% agarose gel (see 2.2.3 for preparation), to check that the templates were linear prior to proceeding to *in vitro* transcription (2.3.4.2.2.3).

2.3.4.2.2.3 *In vitro* transcription of linearised plasmid DNA template

RNA probes were generated from linearised plasmid DNA templates (2.3.4.2.2.2 using the Riboprobe® *In Vitro* Transcription System (Promega – see appendix B).

20µl reactions were set up at room temperature for each of the probes (anti-sense and sense), using components from the Riboprobe® kit, and comprised: DEPC-treated water, 1x (final concentration) transcription optimised buffer, 10mM DTT (final concentration), 1-2 units (final concentration) of Recombinant RNasin® RNase Inhibitor, 0.5mM (final concentration) of each of rATP, rCTP and rGTP, 12µM UTP, 0.2-1µg linearised template DNA, ~50µCi α^{35}S-rUTP (Amersham Pharmacia Biotech) and 1 unit (final concentration) of either T7 or SP6, as appropriate.

The reagents were mixed gently and incubated at 37°C for 1 hour, and then incubated at 65°C for 5 minutes. The probe was then purified through a Sephadex G-50 spin column (2.3.4.2.2.4).

2.3.4.2.2.4 Size exclusion chromatography - Sephadex G-50 spin column

To separate the radio-labelled probe from unincorporated nucleotides, conventional size-exclusion chromatography through 1ml columns of Sephadex G-50 were used, and were prepared as described in 2.2.5.4.2. In addition, the column was equilibrated in with DEPC-treated water by adding 100µl DEPC-treated water to the surface of the Sephadex and centrifuging at 3000rpm for 3 minutes, 3 times.

The labelled probe (20µl – see 2.3.4.2.2.3), 79µl DEPC-treated water and 1µl 1M DTT was applied to the surface of the Sephadex, and centrifuged at 3000rpm for 4 minutes, at room temperature. The column was discarded, and the 0.5ml micro-centrifuge tube containing the purified labelled probe was carefully removed. The probe activity was then determined (2.3.4.2.2.5).

2.3.4.2.2.5 Determination of probe activity

2µl of probe (2.3.4.2.2.4) were quantitated (see 2.3.4.2.1.2) and diluted to a final concentration of $1x10^7$cpm/ml hybridisation buffer I. 10µl 1M DTT was then added to the $1x10^7$cpm/ml probe and the probe was stored at -20°C until required.

2.3.4.3 ISH Protocol

2.3.4.3.1 Pre-treatment – fixation of tissue sections

Tissue sections (2.3.4.1.2) were taken from -70°C and directly immersed into DEPC-treated plastic jars (each with the capacity for 5 slides) containing PAF, and incubated at room temperature for 10 minutes. The PAF was poured off, and the slides rinsed by filling the jars with DEPC-treated PBS (~35 ml), twice, for 5 minutes each. The PBS was then replaced with a fresh mix of DEPC-treated 0.25% acetic anhydride / 0.1M triethanolamine for 10 minutes.

To dehydrate the sections, the slides were transferred through the following ascending concentrations of fresh ethanol (used to reduce osmotic shock which may loosen thick sections):- 70%, 90%, and 100% ethanol, for 1 minute each. To delipidate the sections, the slides were incubated in chloroform for 5 minutes. The sections were then transferred through 100%, 90% and 70% ethanol, for 1 minute each.

The ethanol was poured off, replaced with either 2x SSC (for hybridisation with an oligonucleotide probe) or 4x SSC (for hybridisation with a riboprobe), and incubated for 2 minutes, or until ready for probe hybridisation.

2.3.4.3.2 Hybridisation of oligonucleotide probes to tissue

The $1x10^7$cpm/ml ^{35}S-labelled probe (see 2.3.4.2.1 - 2.3.4.2.1.2) was incubated at 42°C for 5 minutes. The back of the slide and area surrounding the sections were wiped with tissue. 70µl of probe was applied to each slide and covered with a Nescofilm coverslip, ensuring that there were no air bubbles present over the sections, and that the coverslip did not overlap the edge of the slide.

The slides were incubated at 42°C overnight, in a humidified container, prior to washing (2.3.4.4.1).

2.3.4.3.3 Hybridisation of riboprobes to tissue

The $1x10^7$cpm/ml ^{35}S-labelled probe (see 2.3.4.2.2 – 2.3.4.2.2.5) was incubated at 65°C for 10 minutes. 40µl of nucleic acid mix was heated to 65°C for 5 minutes, per ml of probe, then quenched on ice for 2 minutes. 40µl nucleic acid mix was added to each probe (sense and anti-sense), and then vortexed and centrifuged briefly.

The back of the slide and area surrounding the sections were wiped with tissue. 70µl of probe was applied to each slide and covered with a Nescofilm coverslip, ensuring that there were no bubbles over the sections, and that the coverslip did not overlap the edge of the slide.

The slides were incubated at 56°C overnight, in a sealed a humidified container, prior to washing (2.3.4.4.2).

2.3.4.3.4 Washing and dehydration

2.3.4.3.4.1 Washing and dehydration of oligonucleotide-probed sections

Small glass staining dishes (each with a capacity for 10 slides) were washed with acid cleaning solution for 5 minutes, and rinsed thoroughly under running tap water. Each glass staining dish holds ~100ml of solution, therefore for 6 washes, 600ml of 1x SSC was prepared by diluting the 20x SSC stock with DEPC-treated water. 300ml of this was pre-warmed to 56°C.

The slides (2.3.4.3.2) were rinsed in small plastic beakers of 2x SSC, to remove the Nescofilm coverslips. The slides were then washed in thoroughly cleaned small glass staining dishes (as described above) containing 100ml 1x SSC, by incubating on a rotary shaker (Stuart scientific rocking platform; 35 rev/min) for 10 minutes, at room temperature.

The slides were subsequently washed in 1x SSC at 56°C, on a shaker, 3 times, for 20 minutes each. This was followed by 2 washes in 1x SSC at room temperature, on a rotary shaker, for 10 minutes each. Finally, the sections were rinsed with DEPC-treated water, and dehydrated in 70% ethanol for 1 minute. The slides were then left to dry at near vertical, with the frosted ends orientated down, for ~30 minutes.

2.3.4.3.4.2 Washing and dehydration of riboprobe-probed sections

The slides (2.3.4.3.3) were rinsed in small plastic beakers of 4x SSC, to remove the Nescofilm coverslips. The slides were then rinsed in small glass staining dishes (washed as described in 2.3.4.4.1) containing 100ml 4x SSC, and transferred to the pre-prepared RNase A solution at 37°C, for 30 minutes.

The slides were then washed in 2x SSC/1mM DTT at room temperature, on a rotary shaker (Stuart Scientific rocking platform; 35 rev/min), twice, for 5 minutes each. The slides were then washed in 1x SSC/1mM DTT at room temperature, on a rotary shaker, for 10 minutes, followed by a wash in 0.5x SSC/1mM DTT at room temperature, on rotary shaker, for 5 minutes. The slides were subsequently washed in (pre-warmed) 0.1x SSC/1M DTT at 60°C, on a shaker, for 30 minutes, and then rinsed with 0.1x SSC/1mM DTT at room temperature.

The sections were dehydrated in ascending concentrations of ethanol:- 50% ethanol in 0.08x SSC and 1mM DTT, 70% ethanol in 0.08x SSC and 1mM DTT, 95% ethanol and 100% ethanol (twice), for 3 minutes each, and then left to dry at near vertical, with the frosted ends of the slides orientated down, for ~30 minutes.

2.3.4.3.5 Detection and visualisation of probes

2.3.4.3.5.1 Film autoradiography

The dried sections (see 2.3.4.3.4.1) were taped to cardboard (to ensure an even pressure of all the slides against the X-ray film). The slides were loaded into an X-ray film cassette, and exposed either to X-ray film (Kodak MXB film), at -70°C for 7 days, or a storage phosphor screen (Kodak K), at room temperature for 3 days, prior to development or imaging (2.2.5.6).

2.3.4.3.5.2 Phosphor screen imaging

The dried sections (see 2.3.4.3.4.2) were taped to cardboard (to ensure an even pressure of all the slides against the Phosphor screen). The slides were loaded into an X-ray film cassette, and exposed to a storage Phosphor screen (Kodak K) at room temperature for 3 days, prior to imaging (2.2.5.6).

2.3.4.3.5.3 Emulsion autoradiography

Following exposure to X-ray film or Phosphor screen, the slides (2.3.4.3.5.1 and 2.3.4.3.5.2) were removed from the cardboard backing, rinsed in absolute ethanol and dried at a near vertical, at room temperature, for 30 minutes (with frosted ends orientated down). The dried slides were then taken into the dark room ready for dipping in a photographic emulsion.

The dark room was set up with minimal safelight conditions, by turning a red safelight towards the corner of the room. A fan heater, drying box, tissue and 45°C waterbath (water level at ~1 inch from metal gauze) were taken into the dark room. A slide mailer was inserted into a glass Coplin jar half-filled with distilled water, and then placed in the waterbath to equilibrate (~15 minutes).

The power to the waterbath was switched off (to minimise light exposure from lights in the waterbath). LM-1 emulsion (Amersham Pharmacia Biotech) was opened under safelight conditions in the dark room, and melted by placing in the 45°C waterbath for 15 minutes.

The glass Coplin jar (containing the slide mailer and warmed water) was removed from the waterbath, and the emulsion was poured into the slide mailer. The slides were briefly dipped in the emulsion, wiped on the back with tissue, and then placed at a near vertical in the drying box (frosted ends up).

The slide drying box was closed, and the fan heater was turned on. The slides were left to dry for at least 1 hour, in the warmth, and in complete darkness. The remaining emulsion was returned to the bottle, sealed in its box, and returned to the 4°C refrigerator.

Fresh dessicant (stored at 55°C) was placed in the lid of a black slide box. Under safelight conditions, the slides were placed into the black box, and sealed with black electrical tape. The slides were then placed at 4°C for a month, prior to development

(2.3.4.5.3.1). (Exposure time to autoradiographic emulsion is ~4 times longer than exposure time to X-ray film.)

2.3.4.3.5.3.1 Development of Emulsion

The D19 developer and fixer solutions were prepared according to the manufacturer's instructions (and stored at 4°C). The fixer was poured into a large (400ml) glass staining dish, and placed in the dark room. Elgastat osmosis purified water was poured into two other dishes, and placed in the dark room. The D19 was poured under safelight conditions, in the dark room, ready for developing the slides.

The box of slides (2.3.4.5.3) was taken from the 4°C refrigerator, and opened in total darkness in the dark room. The rack of slides was transferred to the fresh D19 developer and incubated for 3.5 minutes (avoiding agitation). The slides were transferred to the stop solution (Elgastat osmosis purified water) for 15 seconds, and then to the fresh fixer solution for 6 minutes.

The slides were rinsed gently under running water for 5 minutes, and counterstained by dipping in fresh, filtered 1.2% neutral red for 2 minutes, or in Mayer's haematoxylin for 1 minute. The sections were rinsed in water for ~2 minutes, and then dehydrated rapidly by incubating in ascending concentrations of ethanol:- 70%, 90% and 100% (twice) ethanol for 15 seconds each. The slides were then cleared in xylene, twice, for 2 minutes each. A drop of DPX was placed onto the sections, and a glass coverslip was applied.

2.3.4.3.5.3.2 Image capture

Slides (2.3.4.5.3.1) were viewed with a Leica DM-RD microscope, and images were captured using a Spot camera (1.30) and Spot Advanced Image software (Spot software 2.2; Diagnostic Instruments, Sterling Heights, MI, USA), and imported into Adobe Photoshop (4.0).

CHAPTER 3

A TRANSGENIC RAT MODEL FOR STUDYING CIRCADIAN VASOPRESSIN EXPRESSION IN THE SUPRACHIASMATIC NUCLEUS

3.1 OVERVIEW AND AIMS OF STUDY

The aim of the present study was to characterise expression in 'JP' transgenic rat models, and define the sequences required for conferring VP expression in the SCN.

3.1.1 The rat VP gene

The coding region of the rat VP (rVP) gene consists of 3 exons and 2 introns, spanning a total length of 2.2kb, (Figure 3.1; Schmale, 1983).

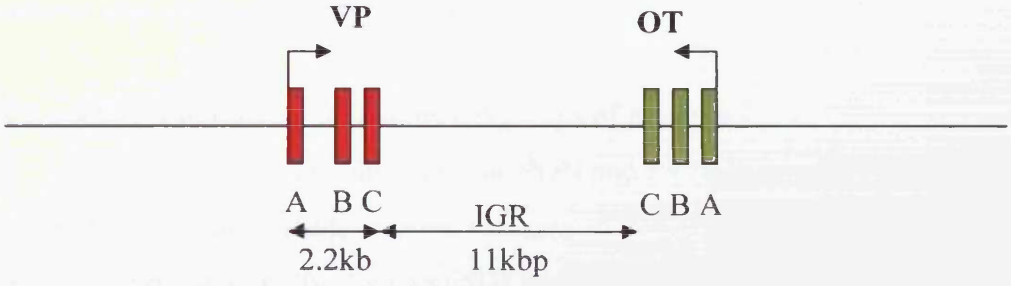


Figure 3.1 The rVP/OT gene locus. VP and OT are transcribed toward each other from opposite DNA strands. (not to scale; vertical bars represent exons; IGR : intergenic region.)

Exon A of the rVP gene encodes a 5' untranslated region, a signal sequence, the hormone VP, and an N-terminal region of neurophysin. Exon B encodes the central part of neurophysin (NPII). Exon C encodes the C-terminal part of neurophysin, the

glycopeptide, and the 3' untranslated region (Ivell and Richter, 1984; Rehbein *et al.*, 1986). In the rat, the VP gene is found in close proximity (11kbp) to the oxytocin (OT) gene, (Figure 3.1). VP and OT possess similar overall structural organisation, and have considerable neurophysin sequence homology at the nucleotide and amino acid levels (Ivell and Richter, 1984).

3.1.2 VP gene expression in the rat brain

VP is primarily synthesised in the magnocellular neurons of the SON and PVN (Figure 3.2), is projected to the posterior pituitary gland, and released into the peripheral circulation to regulate salt and water balance in mammals (see Burbach *et al.*, 2001). In addition, VP is synthesised in the parvocellular neurons of the PVN (Figure 3.2), and is projected via the median eminence to the portal circulation, where it regulates ACTH secretion from the anterior pituitary, under chronic stress conditions (see Burbach *et al.*, 2001). Parvocellular and magnocellular refer to the relative size of the different neurons.

VP mRNA is expressed in distinct subgroups of neurons:-

i) in the magnocellular neurons of the SON and PVN, (Sherman *et al.*, 1986; Robinson *et al.*, 1988);
ii) at a lower level in the parvocellular neurons of the SCN and PVN (Sherman *et al.*, 1986; Uhl and Reppert, 1986; Burbach *et al.*, 1988);
iii) in neurons of the bed nucleus of the stria terminalis (BNST; Figure 3.2) (Brot *et al.*, 1993; Szot and Dorsa, 1993; Wang *et al.*, 1993);
iv) in neurons of the medial amygdaloid nucleus (MA; Figure 3.2) (Szot and Dorsa, 1993; Wang *et al.*, 1993).

The VP gene is differentially regulated in different brain regions. In the SON, VP gene expression is under osmotic (not circadian) control (Zingg *et al.*, 1986; Ang *et al.*, 1993; Grant *et al.*, 1993a and b; Cagampang *et al.*, 1994). In the SCN, VP gene expression is under circadian control, and is subject to circadian variations in mRNA

levels, mRNA poly (A) tail length, and VP protein levels (Earnest and Sladek, 1986; Uhl and Reppert, 1986; Robinson *et al.*, 1988; Carter and Murphy, 1989; Yamase *et al.*, 1991; Tominaga *et al.*, 1992; Cagampang *et al.*, 1994; Kalsbeek *et al.*, 1995; Gillette, 1997). Thus, VP mRNA rhythms are present exclusively in SCN cells (Reppert and Uhl, 1987; Burbach *et al.*, 1988; Cagampang *et al.*, 1994; Jac *et al.*, 2000) and reach a peak level late in the light phase of 12 hour light: 12 hour dark conditions (Cagampang *et al.*, 1994; Carter and Murphy, 1991).

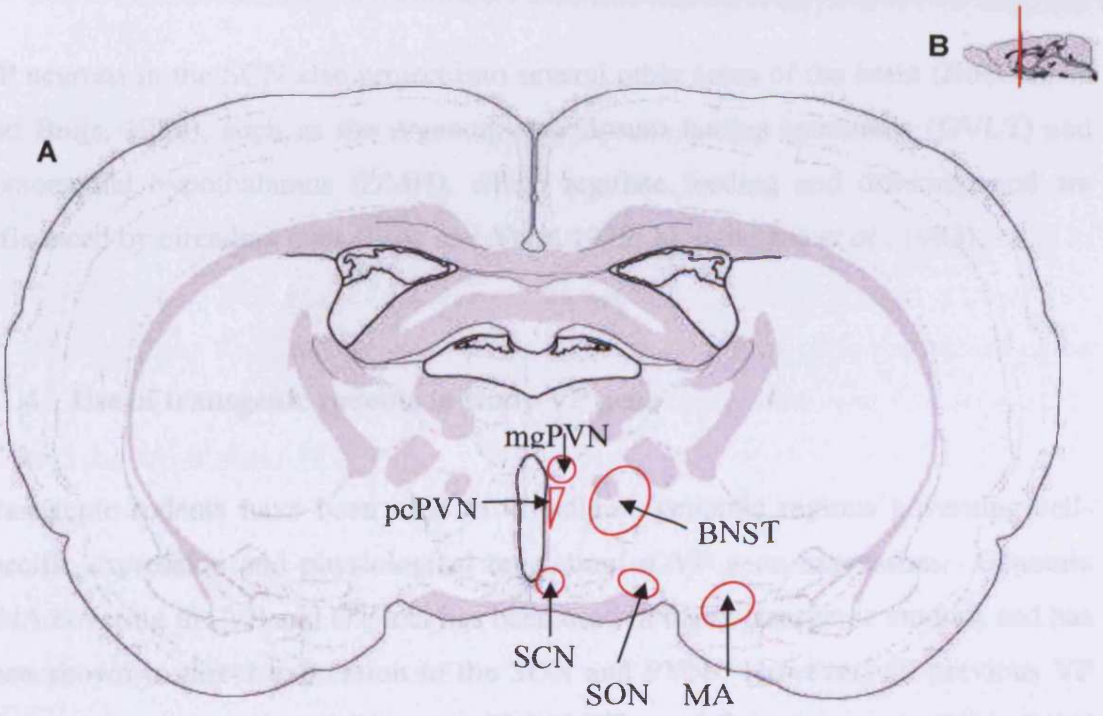


Figure 3.2 Schematic representation of VP gene expression in different regions of the rat brain (unilaterally shown here in coronal section (**A**)). Arrows and circles indicate the magnocellular PVN (mg PVN), parvocellular PVN (pcPVN), bed nucleus of the stria terminalis (BNST), suprachiasmatic nucleus (SCN), supraoptic nucleus (SON) and medial amygdaloid nucleus (MA). The position of the coronal section in the rat brain is indicated (red line) in the parasagittal view (**B**). Modified from Swanson (1998/1999).

3.1.3 The role of VP in the SCN

VP appears to play a role in conveying output from the SCN; VP derived from the SCN plays an inhibitory role in corticosterone secretion (Buijs *et al.*, 1993), in addition to a stimulatory role on the gonadotrophin axis of female rats (Palm *et al.*, 1999; 2001). Given that corticosterone is induced by stress, and that GnRH controls LH levels, it appears that the SCN can concurrently reduce the stress response while stimulating sexual receptivity (Krajnak *et al.*, 1998).

VP neurons in the SCN also project into several other areas of the brain (Hoorneman and Buijs, 1982), such as the organum vasculosum lamina terminalis (OVLT) and dorsomedial hypothalamus (DMH), which regulate feeding and drinking, and are influenced by circadian cues (Bray and York, 1979; Mangiapane *et al.*, 1983).

3.1.4 Use of transgenic rodents to study VP gene

Transgenic rodents have been used to investigate genomic regions governing cell-specific expression and physiological regulation of VP gene expression. Genomic DNA covering the VP and OT loci has been used in many transgenic studies, and has been shown to direct expression to the SON and PVN. However, all previous VP transgene constructs, containing up to 13.4 kb (Figure 3.3**g**) of genomic DNA, failed to direct significant levels of expression to the SCN, as summarised in Figure 2.2. Thus, it is apparent that additional sequences are required to direct VP gene expression to the SCN.

Murphy *et al.* (1987) initiated the VP transgenic studies by fusing 1.25kb of the bovine VP (bVP) promoter to sequences encoding the SV40 virus transforming large T-antigen (Tag; Figure 3.3**a**). Russo *et al.* (1988) fused 1.5kb of rVP with the human growth hormone (hGH) reporter gene (Figure 3.3**b**), and Ang *et al.* (1993) fused 1.25kb of the bVP promoter to the bacterial chloramphenicol acetyltransferase (CAT) gene (Figure 3.3**e**).

As a result of incorporating these chimaeric transgene constructs into murine hosts, significant widespread ectopic expression was observed (Figures 3.3**a**, **b** and **e**). Thus, it can be deduced that these constructs, containing genomic DNA 1.25-1.5kb 5' upstream of the VP gene and no endogenous 3' downstream sequences, did not contain the correct sequences to produce cell-specific expression in transgenic mice.

Studies by Ang *et al.* (1993) involved a construct consisting of the bVP gene flanked by 1.25kb upstream and 0.2kb downstream sequences, and another flanked by 9kb upstream and 1.5kb downstream sequences (Figures 3.3**f** and **g**). The former confined transgene expression to the nervous system, and the latter to discrete groups of hypothalamic neurons and ovary. Thus, by increasing the size of the transgene construct up to 13.4kb, this group were able to further restrict VP gene expression in transgenic mice. This suggests that there are additional silencer elements present in the bVP gene and flanking regions, and that the elements restricting expression to the hypothalamus are located 1.25-9kb from the transcription initiation site and/or 0.2-1.5kb downstream from VP.

Grant *et al.* (1993) also produced a construct consisting of a larger genomic fragment containing the rVP gene (including all exons and introns) and 3kb each of 5' and 3' flanking sequences (Figure 3.3**d**). Zeng *et al.* (1994) generated the first homologous rat transgenic system (designated 3-VCAT-3) by incorporating a construct consisting of the rVP gene, with 72bp of exon III replaced by sequences from the CAT reporter gene, and 3kb each of 5' and 3' flanking sequences, into rat hosts (Figure 3.3**h**). Zeng *et al.* (1994) also generated '5-VCAT-3' transgenic rats in a similar way, extending the 5' upstream sequences to 5 kb (Figure 3.3**i**).

As a result of extending the VP 3' downstream sequences to 3kb (Figures 3.3**d**, **h** and **i**), cell-specific VP expression was observed in the hypothalamus. Thus, it can be deduced that these constructs, containing 3-5kb 5' upstream sequences and 3 kb 3' downstream sequences, contained the elements required to restrict expression to the hypothalamus. However, additional sequences are required to direct significant expression to the SCN.

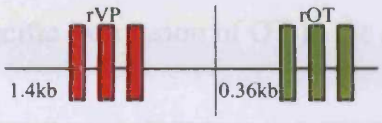
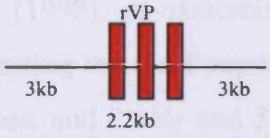
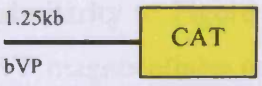
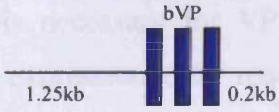
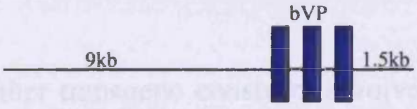
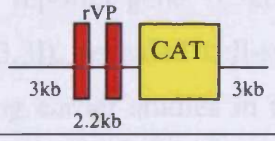
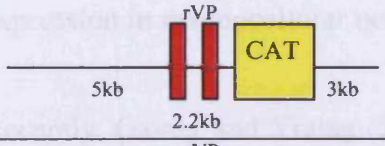
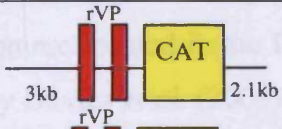
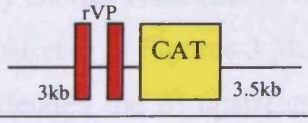
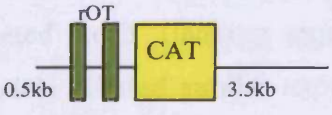
TRANSGENE	SITE OF EXPRESSION	REFERENCES
a 	tumours in pancreas and anterior pituitary	Murphy *et al.* (Am J. Pathol., 1987)
b 	ectopic	Russo *et al.* (Neuron, 1988)
c 	no rVP expression; rOT gene expression in mag-nocellular SON and PVN	Young *et al.* (J. Neuroend., 1990)
d 	highest expression in hypothalamus; lower expression in CNS	Grant *et al.* (Mol. End., 1993; Ann. N.Y.Acad.Sci,'93)
e 	reporter expression ubiquitous	
f 	CNS neurons	Ang *et al.* (EMBO J., 1993)
g 	more restricted in brain; ovary	
h 	SON and PVN; (few cells of SCN)	Zeng *et al.* (J. Neuroend., 1994)
i 	magnocellular SON and PVN	Waller *et al.*, (Endocrinol.,1996)
j  **k** 	equivalent expression of CAT in VP magnocell-ular neurons; identical ectopic CAT expression patterns in brains; no expression in OT neurons	Gainer, H. (Adv.Exp.Med. Biol., 1998)
l 	cell-specific expression of CAT only in OT neurons	

Figure 3.3 Transgene constructs used in previous studies of the VP gene. Vertical bars indicate exons.

Young *et al.* (1990), hypothesised that VP expression was partially regulated by sequences found present in the OT gene, and therefore, created a transgene by fusing the rVP and rat OT (rOT) genes (Figure 3.3**c**), which was then incorporated into murine hosts. It was found that rOT gene expression was directed to the magnocellular SON and PVN; but no rVP gene expression was detected. This indicates that the intergenic region (IGR) may contain the elements required for cell-specific expression of OT in the magnocellular neurons.

Gainer (1998) re-examined this theory, with respect to VP expression, by incorporating the CAT reporter gene in the rVP gene, and including 3kb of upstream sequences, and 2.1kb and 3.5kb of 3' flanking sequences, respectively (Figures 3.3**j** and **k**; note similarity to Figure 3.3**h**). Using these constructs, CAT expression was observed in VP magnocellular neurons, but was absent in OT neurons. This indicates that the IGR is necessary for VP gene expression in VP neurons, and that full magnocellular expression only requires 2.1kb of downstream sequences (and 3kb upstream sequences).

Another transgene construct involved in Gainer's study, consisting of the rOT gene, the CAT reporter gene, 0.5kb upstream sequences and 3.5kb downstream sequences (Figure 3.3**l**), revealed cell-specific expression of CAT only in OT neurons, thus, supporting earlier studies in recognising the importance of the IGR for cell-specific expression in magnocellular neurons.

Recently, Gainer and Young (2001) suggested that the cell-specific enhancers for VP expression are located in the IGR, 0.5-3kb downstream of the VP gene. Most recent studies by Davies *et al.* (2003) involved the generation of transgenic rodents similar to those illustrated in Figures 3.3**h** and **i**. Based on the original '3-VCAT-3' model, this group extended the 5' upstream sequences to 11kb, to give a '11-VCAT-3' model, and truncated the 3' flanking sequences to 0.2 kb, to give a '3-VCAT-0.2' model. These models elicited similar expression patterns as those seen in the '5-VCAT-3' and '3-VCAT-3' models. Hence, it can be deduced from these studies that only 3kb of 5' upstream sequences and 0.2kb 3' downstream sequences are required for full magnocellular expression.

To summarise, the elements required to direct high level VP expression to the SCN were not present in the sequences used in all previous VP transgene constructs. Thus, it appears that additional sequences are required to direct VP gene expression to the SCN.

3.1.5 Generation of transgenic rats using a rVP/OT locus cosmid construct

The requirement for additional sequences that direct VP expression to the SCN may be satisfied by the use of a genomic construct that incorporates the entire VP/OT locus.

A 44kb rVP/hGH transgene construct, consisting of the *Not*I fragment of the cVO14 construct (Figure 3.4), was designed, generated and micro-injected into fertilised rat oocytes in the Laboratory of ICAF Robinson, National Institute for Medical Research, Mill Hill, London. The transgene construct includes 8kb 5' of rVP, 24kb 5' of rOT, and 11kb of intergenic sequences.

The present study involves a line of homozygous JP-59 (copy number 1) and JP-17 (copy number 5) transgenic rats, kindly supplied by the Laboratory of ICAF Robinson.

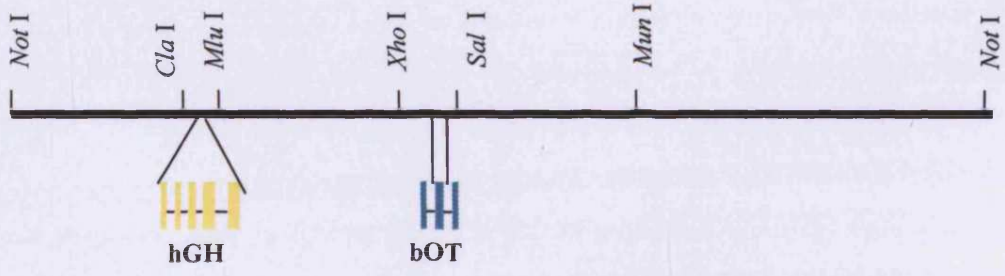


Figure 3.4 The *Not*I fragment of the cVO14 construct (44kb). The hGH reporter gene was inserted into the 5' untranslated region of the rVP gene. The rat OT homologue was replaced by the bovine OT (bOT) gene. Vertical bars indicate exons. Adapted from Wells *et al.*(2003).

The use of a homologous rat transgenic system, will facilitate interpretation because the anatomy and functioning of the rat hypothalamo-neurophyseal and SCN system is better understood than that of the mouse, and the rat is the preferred species for neuroscience and physiological studies, due to its larger size.

3.1.6 Experimental aims

The aims of the present study are:

1. To confirm localisation of VP mRNA in the SCN of 'JP' rats, using established ISH protocols.

2. To localise hGH reporter gene expression in the JP transgenic rat brain.

3. To investigate the circadian rhythm of hGH reporter gene expression in the SCN of JP transgenic rats.

4. To define the sequences required for directing VP gene expression to the SCN.

3.2 METHODS - *In situ* hybridisation (ISH)

Gene expression is manifested initially by RNA transcript production within cells. *In situ* ('at the site of') hybridisation (ISH) can be used to localise these transcripts within biological material. The technique used in the present study involves the specific annealing of a radioactively-labelled nucleic acid probe to complementary sequences immobilised in fixed tissue. ISH allows one to identify which cells in a population contain the mRNA for a given protein, and also to quantify the level of gene expression in that cell.

The procedure consists of 5 stages:-

i) tissue preparation;
ii) probe preparation;
iii) hybridisation of the probe to the tissue;
iv) washing and dehydration;
v) detection and visualisation of the probe.

RNase contamination could lead to target degradation before and during hybridisation, therefore, solutions and lab-ware were pre-treated by vigorous shaking with 0.1% diethylpyrocarbonate (DEPC), to inactivate RNases. The DEPC-treated solutions and lab-ware were left at room temperature (RT) overnight, and then autoclaved for 15 minutes at 121°C the following day. Gloves were worn to reduce the chance of RNase contamination from fingers.

The major factor that limits the sensitivity of ISH is the amount of non-specific background and thus, the conditions of hybridisation and washing are critical.

The ISH protocol used in the present study is a modification of various published methods (Krajnak *et al.*, 1998; Ang *et al.*, 1993; McNiff Funkhouser, 1993; Simmons *et al.*, 1989; Mazjoub *et al.*, 1983). The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

3.2.1 Tissue preparation

3.2.1.1 Fresh frozen sampling

Rats were housed in 14 hour light – 10 hour dark conditions (with lights on at 05:00 = zeitgeber time (ZT) 0), with food and water available *ad libitum*. Male adult JP Sprague-Dawley (SD) rats were killed by stunning and decapitation, according to UK Home Office Regulations (Schedule 1 humane killing methods). Sampling times used in the present study were based upon those used in previous studies (see 3.4).

Dissections of JP-17 brains were performed at ZT 11 ± 10 minutes (n=4; age 2 years) and ZT 19 ± 10 minutes (n=4; age 2 years) by Dr. Randip Bains (Laboratory of ICAF Robinson, Mill Hill, London), and were gratefully received (on dry ice) for further analysis in our laboratory.

JP-59 brains were immediately dissected out at ZT 4 ± 5 minutes (n=3; age 6 weeks) and ZT 18 ± 5 minutes (n=3; age 6 weeks), and ZT 11 ± 5 minutes (n=3; age 4 months) (on different days), and loosely wrapped in aluminium foil (RNase-free). The tissue was then submerged in a metal beaker of cold isopentane (maintained at -40°C with dry ice), using forceps, and agitated for ~1 minute.

The frozen sample (surrounded by foil) was placed on the bench, with the base of the brain turned uppermost. A section containing the SCN was isolated as follows: The brainstem was promptly cut off with a razor blade and discarded, and a vertical cut was made just rostral to the optic chiasm; the remains of the brain (containing the optic chiasm) were wrapped in fresh aluminium foil and placed on dry ice. The frozen brain sample was then transferred to a container into a -70°C freezer for temporary storage, or immediately placed in a cryostat for sectioning (see 2.3.4.1.2.).

3.2.1.2 Embedding/sectioning of tissue

The frozen brain sample (see 3.2.1.1) was mounted in embedding medium (Bright Cryo-M-Bed), as described in 2.3.4.1.2, with the third ventricle positioned vertically and the ventral surface of the brain facing down. The section thickness control was adjusted to 12μm to cut thin sections, prior to approaching the SCN region (identified using rat brain maps: Paxinos and Watson, 1986; Palkovits and Brownstein, 1988).

2-3 sections per slide were collected by freeze-thaw mounting, that is, by placing frozen, RNase-free, electrostatically-charged microscope slides (Superfrost plus slides, BDH) over frozen sections, and thawing on the palm of the gloved hand. SCN sections were dried as described in 2.3.4.1.2, and then placed in a slide box (containing dessicant) on the freezing platform. The slides were then transferred to a -70°C freezer at the end of the session, until use.

3.2.2 Probe preparation

3.2.2.1 Oligonucleotide probes

Oligo(deoxy)nucleotide probes are short nucleotide sequences (~10-50 bp long) that are made using a DNA synthesiser, and can be used for the detection of RNAs. The single-stranded DNA probes (oligonucleotide probes) are less sensitive than single-stranded RNA probes (riboprobes) in ISH, because their small size limits the amount of label that can be incorporated into the probe. Oligonucleotide probes, however, have the advantage of being designed specifically to correspond to a defined RNA sequence, give good probe penetration (due to their small size), and no molecular cloning is required.

3.2.2.1.1 Analysis of VP gene expression by *in situ* hybridisation (ISH) with ^{35}S-labelled VP oligonucleotide probe

The oligonucleotide sequence of the probe used for analysing VP gene expression in the present study, was complementary to the coding region of the endogenous rat VP mRNA (bp 825-872, 5' GTA GAC CCG GGG CTT GGC AGA ATC CAC GGA CTC TTG TGT CCC AGC CAG 3'), and was labelled by the 3'-end labelling reaction described in 2.3.4.2.1.

The reducing agent, dithiothreitol (DTT), was added to solutions containing ^{35}S-labelled probe, to protect the sulphur from oxidising. DTT is an anti-oxidant which stabilises ^{35}S attachment to the probe by maintaining the sulphur molecule in a reduced state. DTT also reduces the possibility of ^{35}S forming disulphide bonds with sulphur molecules in the tissue, thus, eliminating another source of non-specific probe binding.

3.2.2.2 RNA probes (Riboprobes)

^{35}S-labelled single-stranded RNA probes (riboprobes) are more sensitive than oligonucleotide probes. Riboprobes form RNA-RNA hybrids, which are more stable than DNA-RNA hybrids.

Riboprobes are generated by inserting a fragment of the gene of interest into a plasmid vector containing promoter sites for specific RNA polymerases, for example, pGEM-5Zf+ (see appendix C). The gene fragment is inserted into the multiple cloning site (MCS) which has a T7 polymerase promoter site at one end, and an SP6 polymerase promoter site at the other. Anti-sense probes are transcribed from one promoter, whilst sense probes are transcribed by the other. In order to prevent the RNA polymerase from transcribing the plasmid sequences, the plasmid is cut with a restriction enzyme at the end of the gene fragment. Thus, two alternatively linearised plasmids are required to make sense and anti-sense probes.

3.2.2.2.1 Analysis of VP/hGH transgene expression by ISH with ^{35}S-labelled hGH riboprobe

In the present hGH riboprobe study, the plasmid, pThGHc, is a 751 bp fragment of the human hGH cDNA corresponding to nucleotides 55-806, in pGEM-5Zf+ (vector map in appendix C), orientation SP6...5'-3'...T7 (Laboratory of ICAF Robinson). The pThGHc plasmid was received as lyophilised DNA. In order to enable long-term storage of the plasmid as glycerol stocks, competent *E.coli* bacterial cells were transformed with the reconstituted circular DNA plasmids (see 2.1). For the anti-sense probe, the plasmid was linearised with *Mlu*I (and transcribed with T7 RNA polymerase), and for the sense probe, the plasmid was linearised with *Nco*I (and transcribed with SP6 RNA polymerase).

Anti-sense and sense restriction endonuclease digests were set up in 1.5ml microfuge tubes, as described in 2.3.4.2.2.1, using *Mlu*I and *Nco*I enzymes (New England Biolabs, NEB), with buffers 3 and 4 (NEB), respectively. Following purification of the linearised templates (see 2.3.4.2.2.2), ^{35}S-labelled RNA probes were generated using the Riboprobe® *In Vitro* Transcription System (Promega), as described in 2.3.4.2.2.3.

3.2.3 Hybridisation of the probes to the tissue

Tissue samples were pre-treated as described in 2.3.4.3.1.

JP-59 brains removed at ZT18 were hybridised with a ^{35}S-labelled VP oligo-nucleotide probe (see 3.2.2.1.1), as described in 2.3.4.3.2. In addition, some slides were treated with 70µl of 100x cold oligonucleotide as a control (acts as a competitor).

JP-59 brains removed at ZT 11 and ZT 4, and JP-17 brains removed at ZT 11 and ZT 19, were hybridised with ^{35}S-labelled hGH riboprobes (see 3.2.2.2.1) respectively, as described in 2.3.4.3.3. A sense probe was used as a control.

3.2.4 Washing and dehydration

Following hybridisation of the ^{35}S-labelled VP oligonucleotide probe, the tissues were washed to remove non-specifically bound probe, as described in 2.3.4.3.4.1.

Following hybridisation of the ^{35}S-labelled hGH riboprobe, the tissues were treated with RNase A solution (0.02mg RNase A in 1x RNase buffer), to digest any remaining single-stranded probe (thus reducing background), and then washed to remove non-specifically bound probe, as described in 2.3.4.3.4.2.

3.2.5 Detection and visualisation of probes

Hybridised probes are visualised by exposing the sections to a radiation sensitive material. A low resolution signal was obtained by placing the slide adjacent to either an X-ray film or a storage phosphor screen (see 2.3.4.3.5.1 and 2.3.4.3.5.2).

For higher resolution analysis and detection of low abundance hybrids, the slides were dipped in photographic emulsion, dried and exposed as described (see 2.3.4.3.5.3) and then developed, stained and coverslipped as described (see 2.3.4.3.5.3.1). VP oligo-nucleotide-probed sections were stained with neutral red, and hGH riboprobed sections were stained with haematoxylin. Images of slides were captured as described (see 2.3.4.3.5.3.2). Signals from the hybridised probe could thus be analysed microscopically, by evaluating the number of silver grains per unit area of tissue (or the number of silver grains over labelled cells, compared to background).

3.3 RESULTS

3.3.1 ISH analysis of VP gene expression in JP-59 rats using ^{35}S-labelled VP oligonucleotide probe (sampling time ZT 18)

ISH analysis of VP gene expression in JP-59 rats revealed that the ^{35}S-labelled VP oligonucleotide probe hybridised to neurons of both the SCN and SON, at levels comparative to the endogenous VP levels previously reported, in other strains of rat (Jac *et al.*, 2000; Cagampang *et al.*, 1994; Burbach *et al.*, 1988; Uhl and Reppert, 1986), with higher levels present in the magnocellular neurons of the SON compared with the SCN (see Figures 3.5 and 3.7).

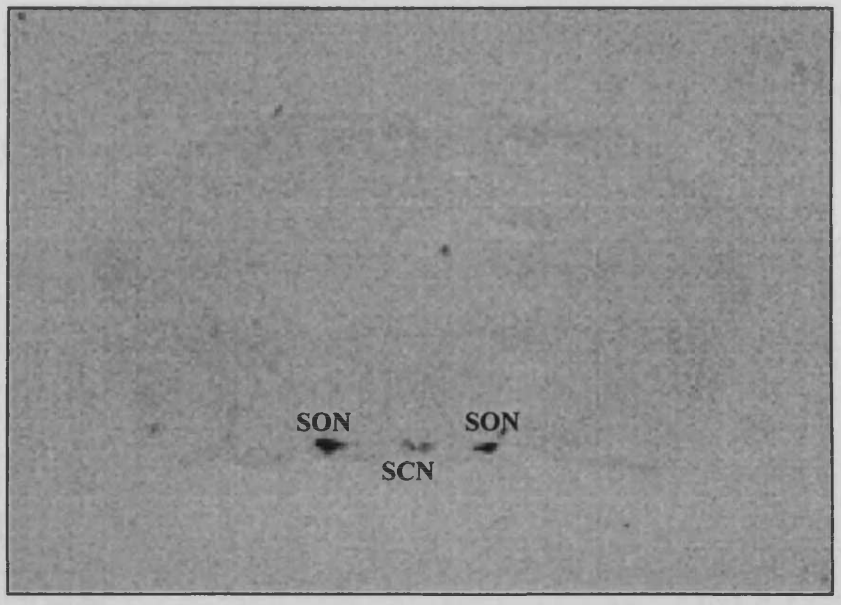


Figure 3.5 Film autoradiograph of VP mRNA in coronal section (12μm) of JP-59 rat hypothalamus, following ISH with an VP-specific oligonucleotide probe. Brains were sampled at ZT 18.

Analysis at a higher magnification indicated that the ^{35}S-labelled VP oligonucleotide probe hybridised specifically to the DM SCN (see Figure 3.6). In control experiments, using 100x cold oligonucleotide, there was no evidence of any hybridisation (images not shown).

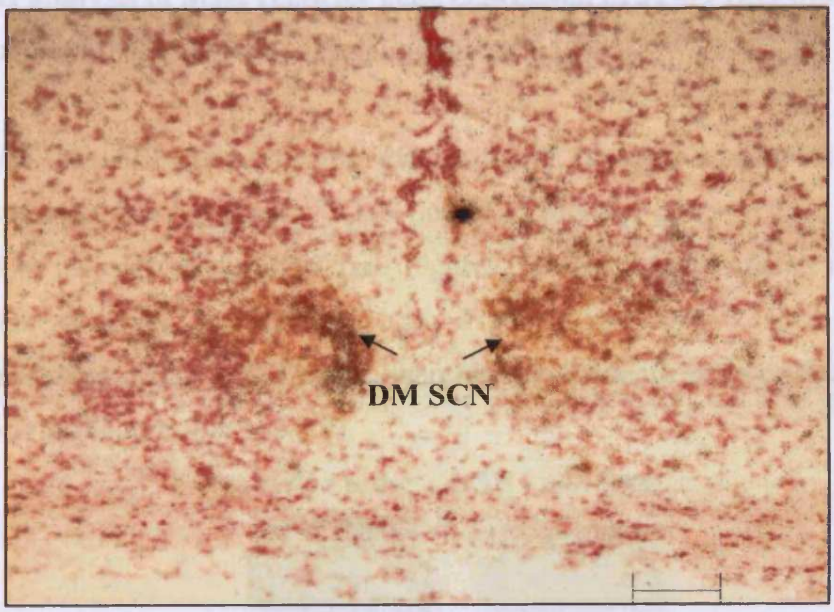


Figure 3.6 Emulsion autoradiograph of VP mRNA in coronal section (12µm) of JP-59 rat hypothalamus, following ISH with an VP-specific oligonucleotide probe. Silver grains (representing hybridisation signal) are visible in the DM SCN (indicated by arrows). Brains were sampled at ZT 18. Scale bar = 100µm.

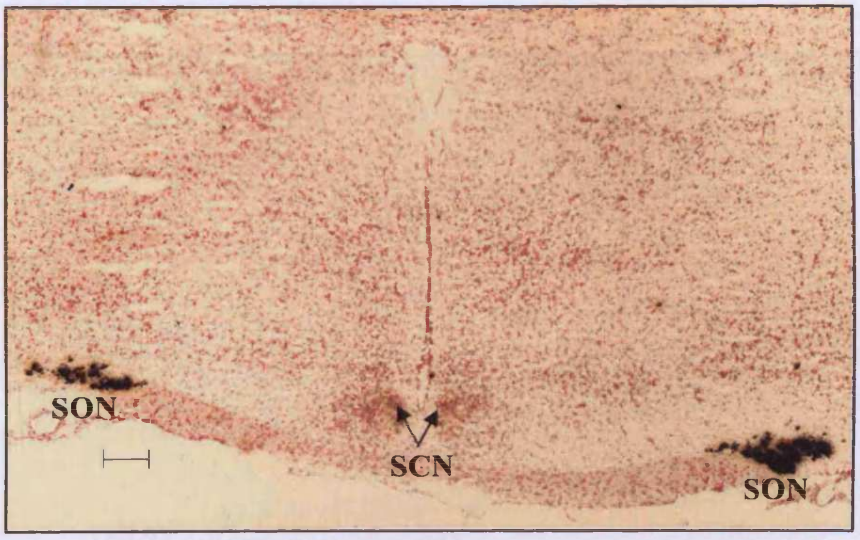


Figure 3.7 Emulsion autoradiograph of VP mRNA in coronal section (12µm) of JP-59 rat hypothalamus, following ISH with an VP-specific oligonucleotide probe. Silver grains are visible in the SCN and SON. (Arrows indicate the location of the SCN.) Note the abundance of silver grains over the magnocellular neurons of the SON, and the relatively lower labelling over the parvocellular neurons of the SCN. Brains were sampled at ZT 18. Scale bar = 200µm.

3.3.2 ISH analysis of hGH reporter gene expression in JP-59 rats with ^{35}S-labelled hGH riboprobe (sampling time ZT 11)

ISH analysis of hGH reporter gene expression in JP-59 rats sampled at ZT 11 demonstrated that the anti-sense ^{35}S-labelled hGH reporter gene-specific probe only hybridised to neurons of the SON (see Figures 3.8 and 3.10), with no evidence of hybridisation observed in the SCN (see Figures 3.10 and 3.11). In control experiments, using a sense hGH probe, there was no evidence of hybridisation above background (see Figures 3.8 and 3.9).

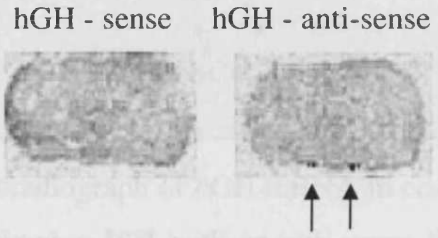


Figure 3.8 Phosphor screen images of hGH mRNA in coronal sections (12μm) of JP-59 rat hypothalamus, following ISH with an hGH riboprobe. Hybridisation signals are visible in the SON of sections hybridised with the anti-sense probe (indicated by arrows). Brains were sampled at ZT 11.

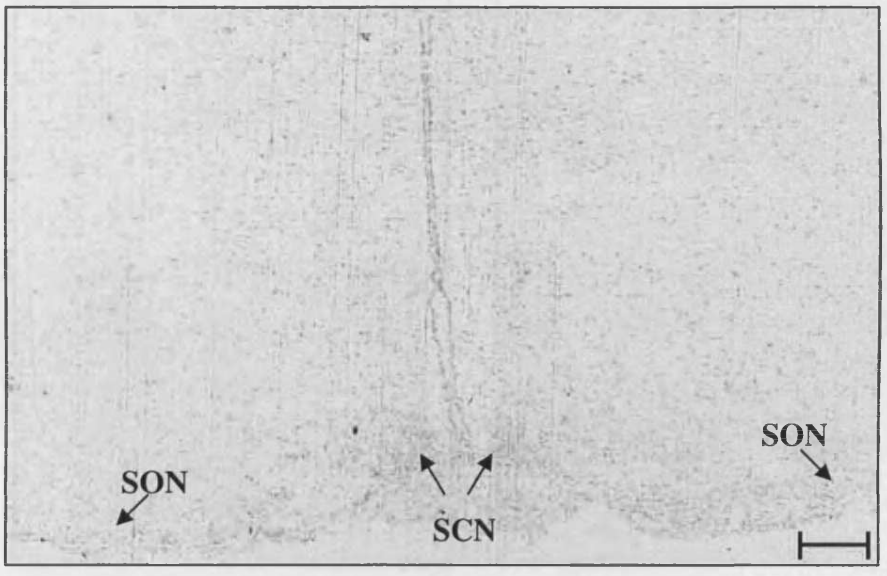


Figure 3.9 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-59 rat hypothalamus, following ISH with a sense hGH riboprobe. No hybridisation signals are visible. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 11. Scale bar = 200μm.

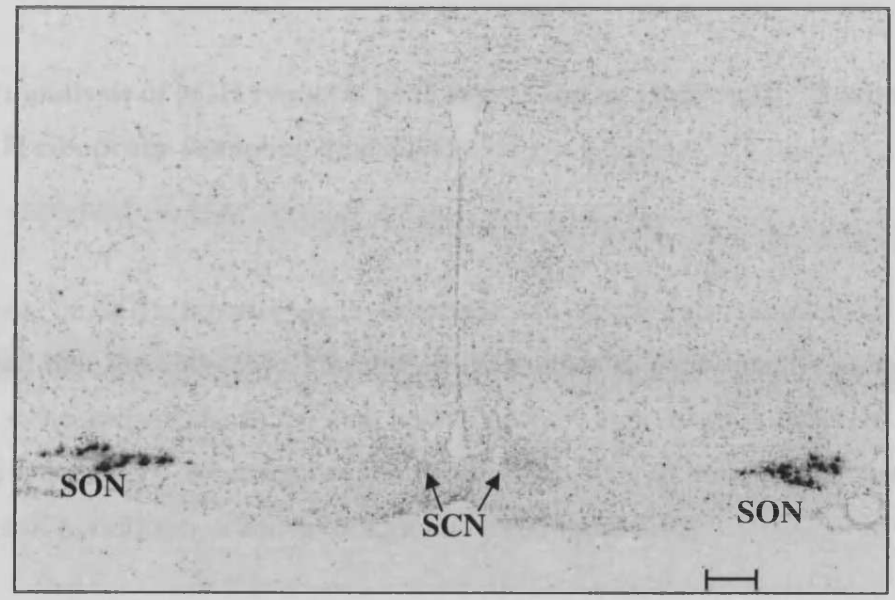


Figure 3.10 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-59 rat hypothalamus, following ISH with an anti-sense hGH riboprobe. Silver grains are visible in the SON. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 11. Scale bar = 200μm.

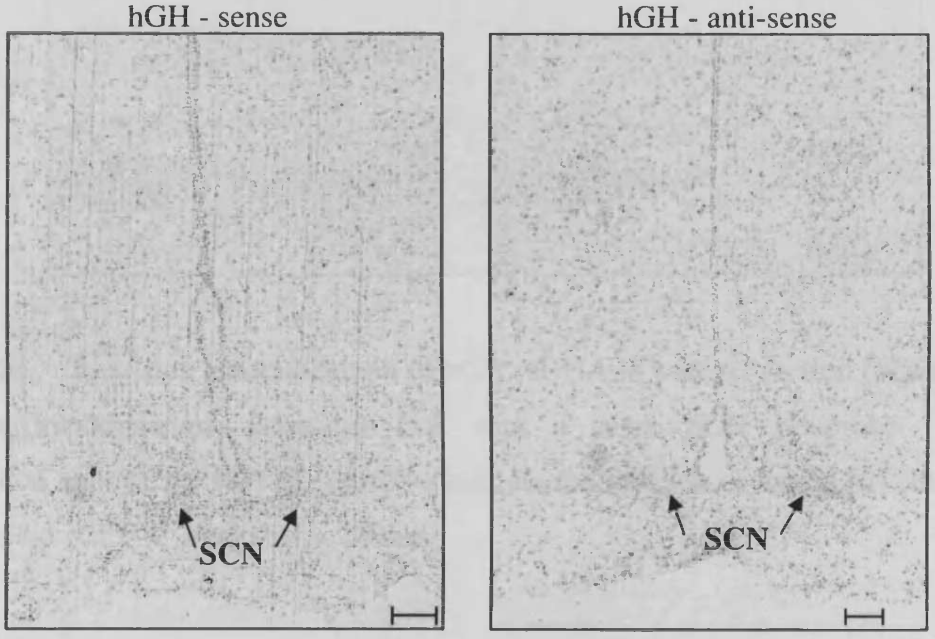


Figure 3.11 Higher resolution emulsion autoradiographs of hGH mRNA in coronal sections (12μm) of JP-59 rat hypothalamus, following ISH with an hGH riboprobe. No hybridisation signals are visible. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 11. Scale bar = 100μm.

3.3.3 ISH analysis of hGH reporter gene expression in JP-59 with ^{35}S-labelled hGH riboprobe (sampling time ZT 4)

ISH analysis of hGH reporter gene expression in JP-59 rats sampled at ZT 4 demonstrated that the anti-sense ^{35}S-labelled hGH reporter gene-specific probe only hybridised to neurons of the SON, with no evidence of hybridisation observed in the SCN (see Figure 3.13). In control experiments, using a sense hGH probe, there was no evidence of hybridisation above background (see Figure 3.12).

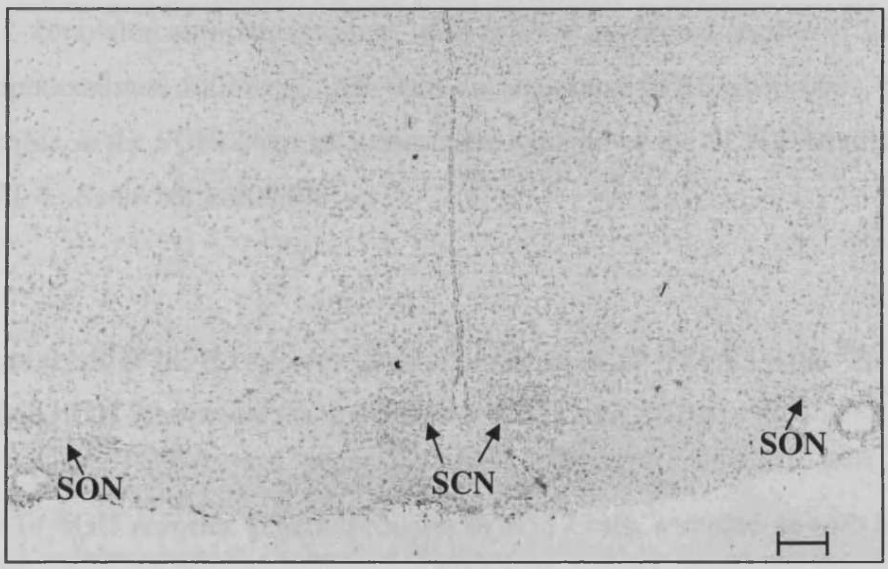


Figure 3.12 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-59 rat hypothalamus, following ISH with a sense hGH riboprobe. No hybridisation signals are visible. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 4. Scale bar = 200μm.

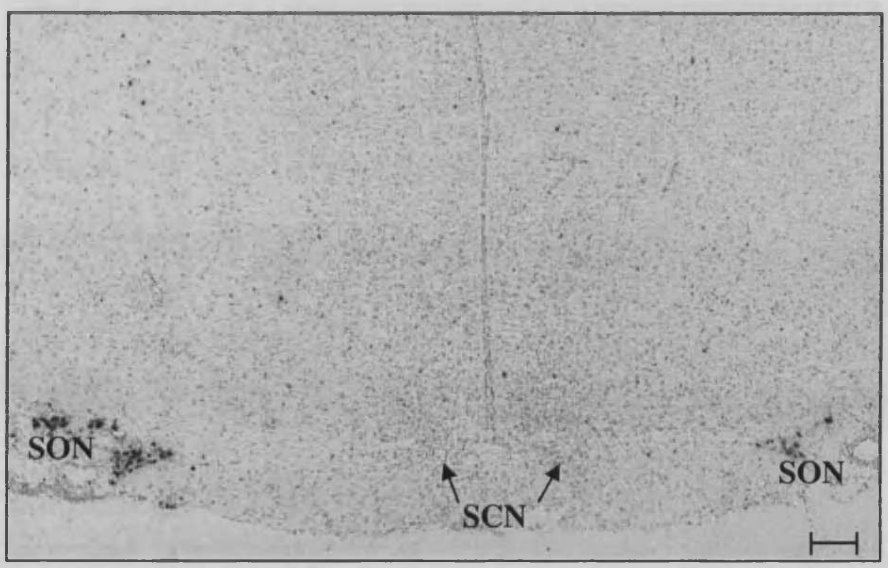


Figure 3.13 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-59 rat hypothalamus, following ISH with an anti-sense hGH riboprobe. Silver grains are visible in the SON. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 4. Scale bar = 200μm.

3.3.4 ISH analysis of hGH reporter gene expression in JP-17 rats with ^{35}S-labelled hGH riboprobe (sampling times ZT 11 and ZT19)

ISH analysis of hGH reporter gene expression in JP-17 rats, sampled at both ZT 11 and ZT19, demonstrated that the anti-sense ^{35}S-labelled hGH reporter gene-specific probe only hybridised to neurons of the SON, with no evidence of hybridisation observed in the SCN (see Figures 3.14 and 3.15). In control experiments, using a sense hGH probe, there was no evidence of hybridisation above background (images not shown).

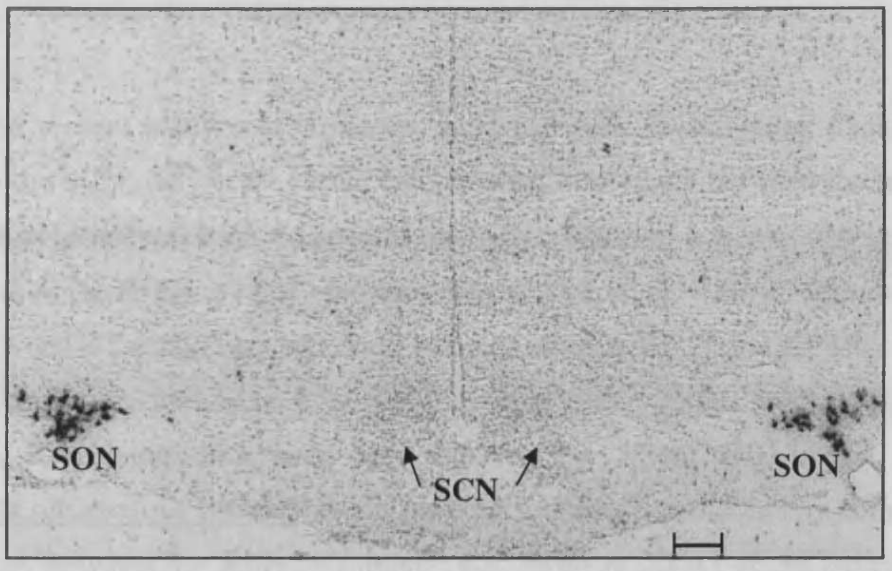


Figure 3.14 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-17 rat hypothalamus, following ISH with an anti-sense hGH riboprobe. Silver grains are visible in the SON. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 11. Scale bar = 200μm.

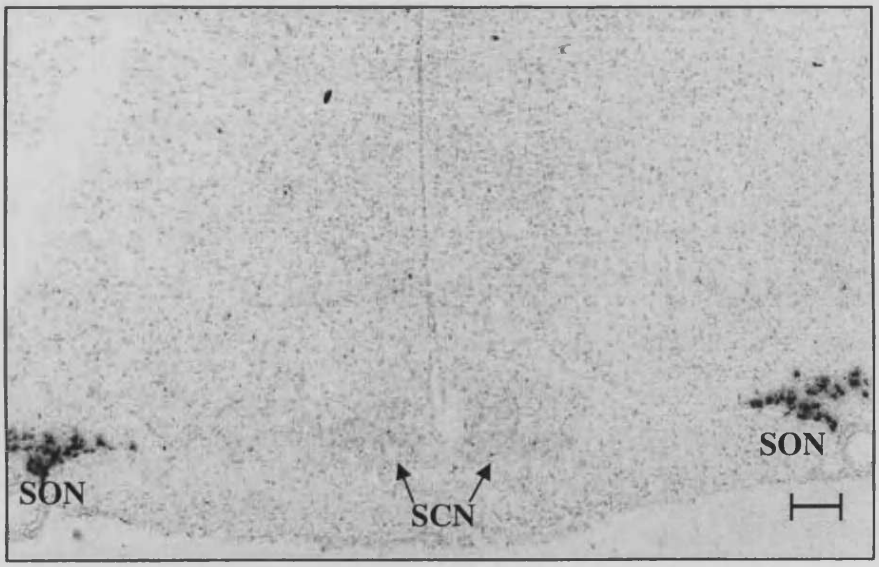


Figure 3.15 Emulsion autoradiograph of hGH mRNA in coronal section (12μm) of JP-17 rat hypothalamus, following ISH with an anti-sense hGH riboprobe. Silver grains are visible in the SON. (Arrows indicate the location of the SCN.) Brains were sampled at ZT 19. Scale bar = 200μm.

Given that the present study was primarily concerned with investigating transgene expression to the SCN, only crude visual comparisons were made between the levels of hGH reporter gene expression detected in the SON. Thus, no apparent differences were observed, in the levels of hGH reporter gene expression, between JP-59 samples taken at ZT 11 and ZT 4, and between JP-17 samples taken at ZT 11 and ZT 19.

If transgene expression had been directed to the SCN, NIH-Image (see http://rsb.info.nih.gov/nih-image/) would have been used to quantitatively compare specific activity between the different samples, e.g. levels of ZT 11 JP-59 samples v levels of ZT 4 JP-59 samples.

3.4 DISCUSSION

It was considered important to analyse the targeting of transgene expression to the SCN because this would have many implications for future studies. For example, studies could involve the exploration of gene regulatory elements that silence or enhance VP gene expression in the SCN, and the study of other intracellular processes within these cells.

The findings of the present study confirm that the VP gene is expressed in neurons of both the SCN and SON of the JP-59 rat brain (at ZT 18), with higher levels present in the magnocellular neurons of the SON compared with the SCN (see 3.3.1). The present study also reveals that hGH reporter gene expression (transgene expression) is localised in the SON of both the JP-59 and JP-17 rat brains (at ZT 11 and 4, and ZT 11 and 19, respectively; see 3.3.2 - 3.3.4).

However, the findings of the present study were negative with respect to the SCN since ISH analysis of samples from both lines of rats (JP-59 - copy number 1; JP-17 - copy number 5) failed to detect any hGH reporter gene expression in this area of the hypothalamus. It is possible that levels of transgene expression were too low for detection by ISH, but other techniques such as *in situ* RT-PCR were not attempted. Therefore, the circadian rhythm of transgene expression could not be investigated. In addition, the sequences required for expression of the VP gene in the SCN, could not be defined.

The sampling times chosen for the present study were based upon previous studies. Burbach *et al.* (1988) observed a peak of VP mRNA expression in the SCN of Wistar rats at ZT 11, and a nadir at ZT 18 (ZT 0 = 06:00; 14 hour light: 10 hour dark regimen. Furthermore, Cagampang *et al.* (1994) observed a peak at ZT 8, and a nadir at ZT20 (ZT 0 = 09:00; 12 hour light: 12 hour dark regimen). In addition, a recent study by Yambe *et al.* (2002) observed peak VP mRNA levels at ZT9 (ZT 0 = 09:00; 12 hour light: 12 hour dark regimen), in the SCN of SD rats.

Given that the light regimen used in the present study is similar to that used in Burbach's study, (ZT 0 = 05:00; 14 hour light: 10 hour dark), ZT 4, ZT 11 and ZT 19 were chosen as sampling times.

Using a similar approach to that used in the JP rat study, Waschek *et al.*, (1999) used a large transgene construct in an attempt to direct VIP expression to the SCN. VIP is highly expressed in SCN neurons (see Waschek *et al.*, 1999). The construct consisted of 16.5kb of the murine VIP (mVIP) gene, including 12kb of 5' flanking sequences, fused (in exon 4) to the β-galactosidase (lacZ) reporter gene (see Figure 3.16).

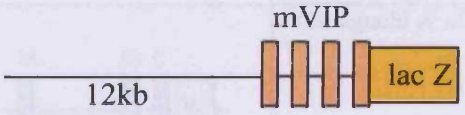


Figure 3.16 The VIP transgene construct which failed to express in the SCN. Vertical bars indicate exons (from Waschek *et al.*, 1999).

In theory, the use of large genomic fragments, including all exons, introns and extensive 5' and 3' flanking sequences, should enable transgene expression at a level comparable to that of the endogenous gene. However, Waschek's large transgene construct failed to direct expression to the SCN. There is therefore a precedent for large genomic transgenes failing to express in the SCN.

Nevertheless, several groups have successfully conferred rhythmic expression of other (trans)genes in the SCN, as summarised by Figure 3.17. The transgenic models (Figure 3.17) recapitulate both tissue-specific constitutive expression and inducible expression in the brain, similar to that of the respective endogenous gene.

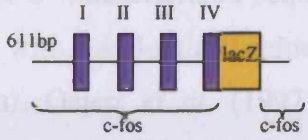
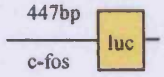
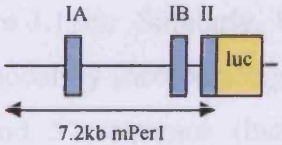
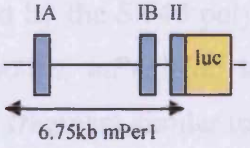
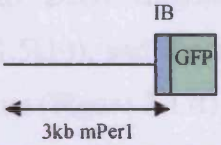
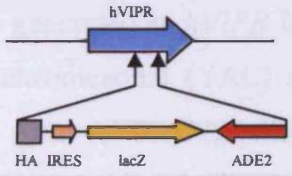
TRANSGENIC MODEL	TRANSGENE	REFERENCES	REFERENCES OF FURTHER STUDIES
c-fos-lacZ mice (and rats)	a 7.75 kb  Schilling *et al.*, 1991	Smeyne *et al.*, 1992a (Kasof *et al.*, 1995a)	Smeyne *et al.*, 1992b, Smeyne *et al.*, 1993a,b Robertson *et al.*, 1995 (Kasof *et al.*, 1995b Kasof *et al.*, 1996)
c-fos-luc mice	b 3.75 kb  	Geusz *et al.*, 1997	
mPer1-luc mice	c  d  	Yamaguchi *et al.*, 2000 Wilsbacher *et al.*, 2002	
mPer1-luc rats	e	Yamazaki *et al.*, 2000	Abe *et al.*, 2002
mPer1-GFP mice	f 4.1 kb  	Kuhlman *et al.*, 2000	Kuhlman *et al.*, 2003 Kriegsfield *et al.*, 2003 LeSauter *et al.*, 2003 Witkovsky *et al.*, 2003 Karatsoreos *et al.*, 2004 Kuhlman and McMahon, 2004
hVIPR-lacZ mice	g  	Shen *et al.*, 2000	King *et al.*, 2003

Figure 3.17 Transgene constructs which confer (rhythmic) expression in the SCN. Vertical bars indicate exons.

Smeyne *et al.* (1992a) and Kasof *et al.* (1995a) generated *c-fos-lacZ* transgenic rodent models by incorporating a 7.75kb DNA fragment (Figure 3.17**a**) containing a *c-fos-lacZ* fusion gene (Schilling *et al.*,1991; comprises the entire murine *c-fos* gene [including 611bp of 5' untranscribed sequences encompassing all known regulatory elements] together with the *ß*-galactosidose (*lacZ*) gene fused in exon 4, and the 3' untranslated region). Geusz *et al.* (1997) generated a *c-fos-luc* transgenic mouse model by incorporating a 3.75kb DNA fragment containing 447bp of the human *c-fos* promoter, the *luciferase* (*luc*; Figure 3.17**b**) reporter gene, the SV40 small-t intron and the poly(A) signal.

Yamaguchi *et al.* (2000) generated an *mPer1-luc* transgenic mouse model by incorporating a DNA fragment containing 7.2kb of the *mPer1* promoter, and the *luc* reporter gene (Figure 3.17**c**). Similarly, Yamazaki *et al.* (2000) generated an *mPer1-luc* transgenic rat model by incorporating a DNA fragment containing 6.75kb of the *mPer1* promoter and 5' sequence (including *mPer1* exon 1, intron 1 and the untranslated portion of exon 2 up to the mPER1 initiator ATG codon), and the *luc* reporter gene, flanked by the SV40 poly(A) signal (Figure 3.17**e**). Wilsbacher *et al.* (2002) generated another *mPer1-luc* transgenic mouse model (Figure 3.17**d**) by incorporating a DNA fragment similar to that of Yamazaki's group.

Kuhlman *et al.* (2000) generated an *mPer1-GFP* transgenic mouse model by incorporating a 4.1kb DNA fragment containing 3kb of the *mPer1* promoter (excluding intron 1 (1.5kb)), and the destabilised enhanced green fluorescent protein (d2EGFP) reporter gene (Figure 3.17**f**).

Shen *et al.* (2000) generated an *hVIPR-lacZ* transgenic mouse model by incorporating a yeast artificial chromosome (YAC) clone containing ~117kb of the human VIP receptor (*hVIPR*) gene (VPAC2), with extensive 5' and 3' flanking sequences, integrated with the hemagglutinin (HA) epitope tag sequence (at the C-terminus of the VPAC2 protein) and a *lacZ* reporter gene (flanked by a viral internal ribosomal entry site (IRES) and poly(A) sequences from SV40, and the yeast ADE2 gene; (Figure 3.17**g**)).

3.4.1 Conclusions

It has been shown (see 3.3) that cell-specific expression of VP is dependent on the presence of *cis*-acting gene regulatory sequences in (gene) flanking DNA that enhance and restrict its expression (enhancer and silencer elements, respectively). Since these elements bind sequence-specific *trans*-acting factors, transcription is therefore regulated by the presence of transcription factors within a particular cell.

The findings of the present study show that the larger JP transgene construct (compared to those used in previous studies) failed to direct detectable reporter gene expression in the SCN. Therefore, the sequences in the JP transgene probably lack the elements required to direct VP expression to the SCN. Thus, the full complement of *cis*-acting sequences mediating the regulation of the VP gene remain elusive, and additional studies are required to define the sequences that direct VP gene expression to the SCN.

3.4.2 Future Directions

Transgenesis is a powerful technique for studying gene function *in vivo*. The expression of a transgene, however, can be affected by its chromosomal location, that is, the site of integration in the host chromosome, a phenomenon known as a position effect (Clark *et al.*, 1994). The outcome of position effects is a lack of consistent transgene expression (in independent lines of transgenic animals which have integrated the same transgene), with low frequencies and levels of expression, lack of tissue specificity and inappropriate expression observed for the smaller (plasmid-based) transgene constructs (see 3.3).

Genes are organised on chromosomes as expression domains which are thought to contain all the regulatory elements required for correct gene expression (see Montoliu, 2002). Thus, transgene constructs lacking most or some of these regulatory sequences are sometimes subject to position effects when randomly integrated within the host genome (Giraldo and Montoliu, 2001).

Previous studies have progressively added regulatory regions of genomic DNA in an attempt to overcome position effects, and localise appropriate expression of VP transgene constructs in the SCN (see 3.3). In theory, the incorporation of all regulatory elements that are associated with a given expression domain in a transgenic construct should enable optimal expression levels in the host, regardless of position of integration (Giraldo and Montoliu, 2001). Therefore, future studies may involve the incorporation of much larger DNA fragments (compared to that of the present study), containing all regulatory elements (including distal key regulatory sequences), into artificial chromosome-type vectors, such as yeast artificial chromosomes (YACs; Larin *et al.*, 1991; see Figure 3.17**g** for example), bacterial artificial chromosomes (BACs; Monaco and Larin, 1994), and P1-derived artificial chromosomes (PACs; Skorupski *et al.*, 1992), which all have large cloning capacities. YAC vectors have the ability to clone DNA fragments of up to 1-2Mb, whilst BAC and PAC vectors have the capacity to clone up to 300kb (see Giraldo and Montoliu, 2001). The generation of transgenic mice, carrying large fragments of DNA in YACs, have revealed expression levels comparable to that of the endogenous gene, and tissue-specific expression matching that of the endogenous gene (see Forget, 1993).

CHAPTER 4

A TRANSGENIC RAT MODEL FOR STUDYING THE REGULATION OF MPER1 GENE EXPRESSION

4.1 OVERVIEW AND AIMS OF STUDY

The aim of the present study was to generate an *mPer1*/d2EGFP promoter/reporter transgenic rat model, and characterise *mPer1*/d2EGFP transgene expression.

4.1.1 The mouse *Period 1* (*mPer1*) gene

The *Period 1* (*Per1*) gene is a key molecule involved in the translation-transcription feedback loop that regulates the mammalian circadian clock, located in the SCN (see 1.2.1.2).

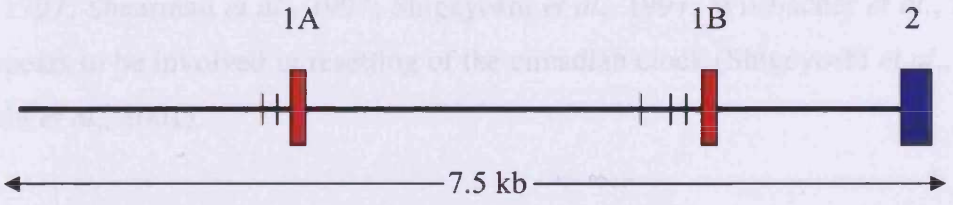


Figure 4.1 Structural organisation of the 5' coding region of the *mPer1* gene (not to scale). Vertical bars and lines represent exons and E-boxes, respectively.

The 5' upstream and coding region of the mouse *Per1* (*mPer1*) gene incorporates 3 exons and 2 introns, spanning a total length of 7.5 kb, (Figures 4.1 and 4.4; Yamaguchi *et al.*, 2000).

Yamaguchi *et al.* (2000) identified 2 alternative forms of *mPer1* mRNA (incorporating exons 1A and 1B, respectively), each associated with its own functional promoter (located upstream of each of the alternative first exons). Both promoters are utilised during circadian rhythms and during light-induction of *mPer1* expression.

4.1.2 The role of *Per1* in the SCN

PER1 appears to play a role in the regulation of circadian rhythms in mammals by interacting with other clock proteins in a transcriptional/translation feedback loop in the SCN (Dunlap, 1999; Jin *et al.*, 1999; Shearman *et al.*, 2000; King and Takahashi, 2000; see 1.2.1.2). Furthermore, *mPer1* null mutant studies in mice have revealed that the circadian period in these animals is shorter than that of wild-type animals (Cermakian *et al.*, 2001). The *mPer1* gene also appears to play a central role in conveying the light-entraining information to the SCN, since it is the only clock gene that has convincingly been shown to be induced rapidly by light stimulation (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997; Wilsbacher *et al.*, 2002), and appears to be involved in resetting of the circadian clock (Shigeyoshi *et al.*, 1997; Albrecht *et al.*, 2001).

4.1.3 *Per1* gene expression and induction in mammals

Extensive studies have revealed that *Per1* mRNA is widely expressed throughout the mammalian brain with highest levels in the SCN, pineal gland, Arc, and PVN, (in the mouse - Shearman *et al.*, 1997; Sun *et al.*, 1997; Abe *et al.*, 2002; and in the rat - Shieh, 2003; and Asai *et al.*, 2001 (SCN, PVN and pineal gland only)). In addition, studies have indicated the presence of *mPer1* RNA expression in mammalian peripheral tissues such as the testis, skeletal muscle, heart, lung, liver, kidney and brain (Sun *et al.*, 1997; Tei *et al.*, 1997; Yamazaki *et al.*, 2000).

Circadian expression of *Per1* is exhibited in the SCN, with peak levels of *Per1* mRNA at mid-day (in the mouse - Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Sun *et al.*, 1997; Shigeyoshi *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; LeSauter *et al.*, 2003; and in the rat - Yan *et al.*, 1999a; Miyake *et al.*, 2000; Yamazaki *et al.*, 2000), and of PER1 protein about 6 hours later (in the mouse - Hastings *et al.*, 1999; LeSauter *et al.*, 2003). Furthermore, *Per1* mRNA appears to be rapidly induced by light during the early and late subjective night (in the mouse - Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997; Zylka *et al.*, 1998; and in the rat - Yan *et al.*, 1999a). In addition, circadian expression of *Per1* has been demonstrated in the mouse PVN (Asai *et al.*, 2001) and the rat pineal gland (see Simonneaux *et al.*, 2004). There is evidence that indicates that upregulation of the *mPer1* gene in PVN CRH neurons is suggestive of a mechanism in which stress signals affect corticosterone secretion (Takahashi *et al.*, 2001; also see Buijs *et al.*, 2003). The significance of *Per1* expression in the pineal gland still remains to be determined. Circadian expression of *mPer1* has also been demonstrated in the retina, with peak levels of *mPer1* mRNA occurring late in the subjective day (Shearman *et al.*, 1997; Sun *et al.*, 1997; Witkovsky *et al.*, 2003) and of mPER1 protein about 5 hours later (Witkovsky *et al.*, 2003).

4.1.4 Use of transgenic rodents to study the *mPer1* gene

Several groups have successfully generated *mPer1* transgenic models that recapitulate both tissue-specific constitutive expression and inducible expression, similar to that of the endogenous gene (see Figure 4.2; reiterated from 3.4). Yamaguchi *et al.* (2000) generated an *mPer1-luc* transgenic mouse model by incorporating a DNA fragment containing 7.2 kb of the *mPer1* promoter, and the *luc* reporter gene (Figure 4.2**a**). Yamazaki *et al.* (2000) generated an *mPer1-luc* transgenic rat model by incorporating a DNA fragment containing 6.75 kb of the *mPer1* promoter and 5' sequence (including *mPer1* exon 1, intron 1 and the untranslated portion of exon 2 up to the mPER1 initiator ATG codon), and the *luc* reporter gene, flanked by the SV40 poly(A) signal (Figure 4.2**c**). Wilsbacher *et al.* (2002) generated another *mPer1-luc* transgenic mouse model (Figure 4.2**b**) by incorporating a DNA fragment similar to that of Yamazaki's group.

Kuhlman *et al.* (2000) generated an *mPer1*-GFP transgenic mouse model by incorporating a 4.1 kb DNA fragment containing 3 kb of the *mPer1* promoter (excluding intron 1 (1.5 kb)), and the destabilised (d2) enhanced green fluorescent protein (EGFP) reporter gene (Figure 4.2**d**).

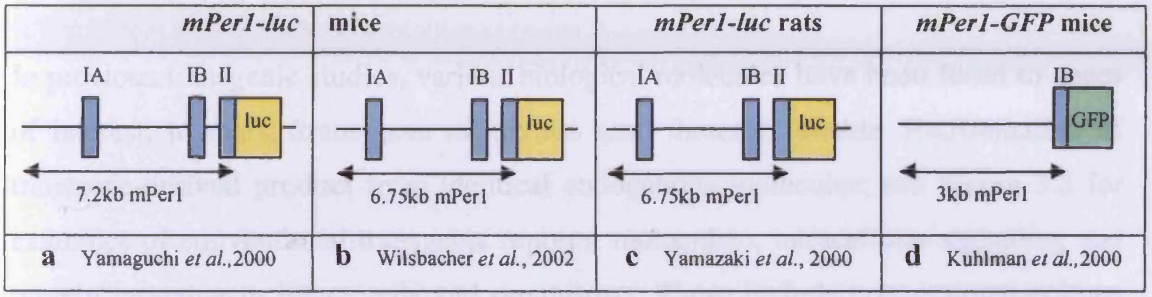


Figure 4.2 Transgene constructs used in previous studies of the *mPer1* gene (not to scale). Vertical bars indicate exons.

As a result of incorporating these transgene constructs into murine hosts (Figures 4.2**a**, **b** and **d**; Yamaguchi *et al.*, 2000; Wilsbacher *et al.*, 2002; Kuhlman *et al.*, 2000, respectively), both rhythmic and inducible expression in the SCN, similar to that of the endogenous gene, was observed. Extensive further studies of the *mPer1-GFP* transgenic mouse generated by Kuhlman *et al.*, (2000) have confirmed tissue-specific rhythmic and inducible expression (rhythmic expression and light induction of transgene mRNA in the SCN, Kuhlman *et al.*, 2000 and 2003; rhythmic expression of transgene protein in the SCN and Arc, Kriegsfeld *et al.*, 2003; circadian expression of transgene mRNA and protein in the SCN, LeSauter *et al.*, 2003; circadian expression of transgene mRNA and protein in the retina, Witkovsky *et al.*, 2003). Thus, it can be deduced that the sequences of the 3 kb *mPer1* promoter fragment (Figure 4.2**d**), were sufficient to confer both circadian and photic regulation *in vivo*, in transgenic mice.

As a result of incorporating the 6.75 kb *mPer1* promoter fragment (Figure 4.2**c**) into rat hosts, both rhythmic and inducible expression, similar to that of the endogenous gene, was observed (in the SCN, liver, lung and skeletal muscle, Yamazaki *et al.*, 2000; and in the SCN, Arc, pituitary gland and pineal gland and PVN, Abe *et al.*,

2002). Thus, it can be deduced that the sequences of the 6.75 kb *mPer1* promoter fragment (Figure 4.2**c**), were sufficient to confer both circadian and photic regulation *in vivo*, in transgenic rats.

4.1.5 Transgene reporters

In previous transgenic studies, various biological molecules have been fused to genes of interest, to mark (trans)gene expression (and therefore enable discrimination of transgene-derived product from identical endogenous molecules; see Figure 3.3 for examples of conventional transgenic reporter molecules), intracellular signalling and protein targeting in intact cells and organisms. These include conventional epitope tags (e.g. streptavidin, polyaspartic acid, calmodulin-binding peptide), enzyme-based reporter genes (e.g. CAT, luciferase, lacZ, β-lactomase), the hGH reporter system, and more recently, fluorescence-based reporter genes (e.g. enhanced green fluorescent protein [EGFP], enhanced cyan fluorescent protein [ECFP], enhanced blue fluorescent protein [EBFP]; Tsien, 1998; Tavare *et al.*, 2001). Numerous transgenic animals have been successfully created that possess either enzyme-based or fluorescence-based reporter genes for gene promoter analysis and physiological studies (see Spergel *et al.*, 2001).

Unlike the conventional reporter molecules (see above), which require downstream processing using antibody conjugates and/or enzyme assays for detecting transgene protein expression, fluorescence-based reporter genes are capable of direct fluorescence. Consequently, fluorescent proteins do not require any downstream processing, and enable expression to be directly visualised and measured in fixed or living tissues using a fluorescence microscope.

4.1.6 Destabilised and enhanced green fluorescent protein (dEGFP)

The GFP gene was initially cloned from the hydromedusan jellyfish *Aequorea victoria* in an attempt to elucidate its fluorescent properties (Prasher *et al.*, 1992). The

green fluorescent protein (GFP) is a single polypeptide of 238 amino acids, forming a monomeric 27kDa protein. The fluorescence emitted by GFP (green light; 507nm) is produced subsequent to electron transfer from the aequorin complex, as it degrades to the ground state, following excitation by UV (395nm) light (Chalfie *et al.*, 1994). The GFP protein has become a widely used reporter in gene expression and protein localisation studies in intact cells and organisms (Chalfie *et al.*, 1994; Tsien 1998).

To improve detection in mammalian systems, several GFP variants have been designed by modifying the excitation spectrum of GFP. One variant, enhanced GFP (EGFP), contains 2 mutations which red-shift the spectral excitation peak to 489nm (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994). Thus, EGFP can be excited by the 488nm argon-ion laser found in most fluorescence microscopes. EGFP provides significantly higher fluorescence intensities in mammalian cells than wild-type GFP. However, red-shifted variants of GFP exhibit long half-lives and rapidly accumulate in mammalian cells, and therefore limit their use in transient promoter activity and gene expression studies. Consequently, destabilised and enhanced GFP (dEGFP) variants, with altered half-lives of 1 to 4 hours, have been developed (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994). The implications of dEGFP are immense in biological research, in that they can be used for studying protein transport, and measuring transient gene expression, under the control of an inducible promoter. Furthermore, fusion proteins comprising a gene of interest and a dEGFP gene can be overexpressed without rapidly accumulating to potentially toxic intracellular levels. Moreover, dEGFPs can be used for studies that require rapid reporter turnover, such as the periodic expression of genes that regulate circadian rhythms.

pd2EGFP-1 (Clontech; see appendix C) is a promoterless d2EGFP vector that encodes a destabilized, red-shifted variant of wild-type GFP (Excitation maximum = 488 nm, emission maximum = 507 nm; Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994), and has been optimized for brighter fluorescence and higher expression in mammalian cells. d2EGFP is derived from EGFP (GFPmut1; Cormack *et al.*,1996), and with its half-life of approximately two hours, is suitable as an *in vivo* reporter of dynamic changes in gene expression (Chalfie *et al.*, 1994), for a given functional promoter of interest.

4.1.7 Generation of an *mPer1*/d2EGFP promoter/reporter transgenic rat model

A 4.1 kb *mPer1*/d2EGFP promoter/reporter transgene construct, consisting of ~3 kb of *mPer1* gene promoter fragment and 1.1 kb of coding sequence for destablised and enhanced GFP (derived from the pd2EGFP-1 vector, Clontech) was designed and generated by the Laboratory of DG McMahon, Department of Physiology, University of Kentucky, Lexington, USA (see Figures 4.2**d** and 4.3). The transgene has already been successfully integrated in murine hosts (Kuhlman *et al.*, 2000), and has demonstrated appropriate expression in the respective transgenic mouse model (Kuhlman *et al.*, 2003; Kriegsfeld *et al.*, 2003; LeSauter *et al.*, 2003; Witkovsky *et al.*, 2003; see 4.1.4).

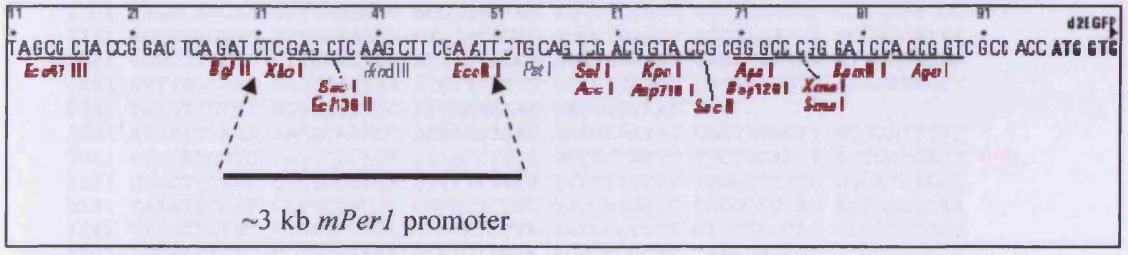


Figure 4.3 Generation of the *mPer1*/d2EGFP promoter/reporter transgene construct (Kuhlman *et al.*, 2000). A ~3 kb *Xho*I-*Bgl*I fragment of the *mPer1* promoter (see Figure 4.4) was ligated into the *Xho*I-*EcoR*I sites of the pd2EGFP-1 vector (Clontech; restriction map and cloning site shown above). The 4.1 kb *mPer1*/d2EGFP promoter/reporter transgene construct was released by *Xho*I-*Afl*II digestion (see Figure 4.5 and 4.2.1.2).

The use of the rat as a circadian transgenic model will facilitate interpretation because the anatomy and functioning of the rat SCN system is better understood than that of the mouse, and the rat is the preferred species for neuroscience and physiological studies, due to its larger size.

Thus, the present study aims to generate an *mPer1*/d2EGFP transgenic rat model, using the 4.1 kb *mPer1*/d2EGFP promoter/reporter transgene described (see Figure 4.2**d** and above).

***mPer1* sequence (nucleotides 1141-5580; accession number AF223952)**

```
1141 CTTGGGAGTC TGCAAGAAGG GGCTGGCCAGCC'AGGCCCAA AGAAAATAAA TAGGAGGCGA  BglI
1201 CTGTCATTTC ACCGTCATTG TCATGCAATC TAATGATATC TTCAGTGTAG TGCTCAGATT
1261 TTTAAGCTTT GGGGATCACT AGAGACACAA AGCTCAGGCA CCAGAAACCT CTTGTAATGC
1321 CAGAGTCTCC AAAGTATGCC CACTACGCCA TAGCCTGTCA GATCTACGCC TGCGTACAAG
1381 TCGCTCGGCA CGCCTGCGCA GACTTCGCAG TATTGGGTAA GTGTCGTCAA GGAAAATCCC
1441 CAGCTTCTGG GTAAACAAGT TGCCGCGTGA GCCAGCCTGC ACGTGTTCCC TACAGCTCTG
1501 AGCCCTCTCA GCCTATGAGA AAGTTTTTAG GGCAGGGCTG GCATTTGCGT CACTGATTTA
1561 GGCAGGGCGG GGTTGTCTCT GCAGCCAGTA AAAATGTTGA GAGTGGGTGG GGGTGTGGCT
1621 TCCGGTCAGA TGTCCAATCA ACGAGTTGGG GCGCCCCCGC CCTCACGTGG CTCTATCACT
1681 ACCGGTTGTC ATATTGTCCA ATGGGAAAGT GACTAAGTTC TGAAATGCCA ATGGTTGCTT
1741 TACAAGGGCT TGCCGGCTGC ACGCGGAGCG GGGCATGGGT TGGCGTCTCT GAGCCAATAA
1801 GCTAGGGAGG CGTGACAGAA CGGGCGGTGG[GAGGGCC'TCGAGGATTCTGT GGAGGAGCCC  XhoI
1861 CAGGAGTGAA GAAATTTGGA GTCTACGTGC GCTCGGAACT]GTGAGTAACC CCTTACTCTG
1921 GAGCCGTCGA ACTTGTGTTT TTGGGGAGGT GCGCTCGACC AAAAGGGGTG TCAGTGGCGT
1981 GGTGTGTGTG AGGGATCATG CAAATGTGAA TTAGCTGCAG AACAAATGCC TAATGGTTGT
2041 GGAGAAAGAG ACCAAAAGTT CAGCTCTAGG GGAACATGAA TGAGAGTTAC CAGCAACAGA
2101 TCTCTCACCA CTCTAGGGTC CCTGGACACT AATGCCTAGC GCAGGGTGCT CAGAAGCCCC
2161 TAGCCATCCG AGGGAGCCGA AGCGCCTGCG ATATGACCCC TGAGAGCTGG TTATGGTTTG
2221 GGGTAGGCTC CCTAAACACT TGGAAGAGAC CGCCCTGGTA GGGCGGGCGG GAACGTAGTA
2281 TGGTGATCTG AGAAAGGCTG GTGTGTTGAT TTGACCGAAA GGCTGTCTCA TCACGACTAT
2341 AGGTGGTATC GTGGCCTCAG GGACATGCTC GAAAATAGGA ATGGGCTAGT AATTTGTTGG
2401 ATTCTGAAAG GGAGGATAGG GTGGAGGGTT TCTGTACACC GTTTCGTCTA CTCTTCTTAA
2461 GCTGCTAGAG GTGGAGTTAC CAAAGGACTG CTCTGGTGTC TAGACGGTCG GTCGGGCAGA
2521 GGTGAGGGAG GAGAGGGAGG AAGTGAGATT CTTAGGAAAGCAGTGTGCCTGCAGGCTTC{'T  XhoI
2581 CGAGTGATCT CGGGTTTTGA GCATAGAAAC GAACCATGAT GGTGCGCACG GAGCACAAAC
2641 AATGCCTTGA GATCAGGCTA AGGGGAGAGA AGGAAAAGGA ATTGGATATA GCCTGTATTC
2701 TAGGCACCAA GCTTAGAAAC AGGGGAATAC TCCCCCCGCA GTCCTACGGT GCTGGAATGC  →F2
2761 AGTGTAGAGG TTTGGTAACA CTTTACAGGG TCCCTAAGCC AGCAGGACAA GCCATCATTA
2821 GGGCTTGAAC CTTGGGCTCC ATTATTAGGA AGCTGAGAAT GAAGCAGACA ATTAAGGCAA
2881 GTTTAGTAAC TACCTACTAA GTCTTAGTTT TTCCTTATGA CATCAGGGTG ATACTTACCT
2941 TCAGTTGTTT GGGGGCTTAC ATGAGAACAA TATGTGTAAA GTTCTTAGCA CAGCGCTTGG  →F3
3001 ATCATTTACA AACACAAACT ATAAGGTAAC AAAAGAATAG CGATAGAGTT GGGGATTTAT
3061 GTGCATGCTG TGGCCAGAGA GCAATCTCCA TCTTCTGTTT TTCTGCAAGT TTTGATGACT  ←R1
3121 CCTCTTTTCC CTCTCCCTGA TTTTTTTTTT TTTTTTTTTT TTGGTCTTCG GGCACCAGCC
3181 CAAATTCCAT CATTCCTCCC TGTATTCCTC TCCCAAGATC TGGGCCCCAG ACTGAGGCAA
3241 TTTGCTGTGT CCCAGTTTGA CTTCATCTTA AATCCATTCT CTCTGACCAC CCCCCCCCCC
3301 ACCTATTCTG TGCTGTAATA GTAACTAGAA AGTCTGCCTC TAAAGAGGGG CCCCAGGGAA
3361 GGAGCAAAGG TGCGTTCTCT CTCAGAACCT AGGCCTCCAA TTTACCCGCC CTAGCTCTTT
3421 GGTACCTGGC CAGCAACCGT GTACAGTCTG GTGACGGGGA AAAGCCAATG CCAGGAGTTT  →F4
3481 TAAGACCCAG CTGAGGGAGG GTAAAAGTAG GTCCCGCAAA GAGAACCAGG CATGGGGGCG
3541 GGGGAGGGGG ACCCCCTTCC TCCTAACTGT CTCTCCAGGA ATTTTGGCT TTTGTACAGG
3601 ACCGCTGTCG TTGGGTTGGG GGAGGCGCCA AGGCTGTGTG CATGTCCTGG GCCATCAGCC
3661 AAGAGAACAC GATGTTCCCT AGTGCGCTGG CCGCCGCCCT CTGGGGCTGG CCGCCTCCCA
3721 AACCGCTGCC TCTCCAGCCT CCCTGCCCCA CATTCCCCAG CTGCCTCGCC CCGCCTCCTG
3781 CCTCCGCTTT GACGTCACCT CCCTCTCCTG CCCCCGCTTC TCCATTGACG GCAGCAGAGC  →F5
3841 CTGGTTACTG TGGGGGACTG ATGAGGCCCG ACAGCCTGGG CCTTGGGATC AGGTTGGGGC  ←R2
3901 TGTTTGGAGT GCTGAAACCT TTTGTCTGTG TAAATGACAG ATAGGGAAGT GGGCGAGCAA
3961 TGGCTGCTTG GGTCAGAGGA ATCACACCTA AATCCTTGAG AGCTGTGGAA AGAGAAAGGG
4021 GTCTGGAAAA AAAAAAAGGA CAGCACACGG TCACAACGCA GTACGAGGGG GCAGGAGAGC
4081 AGCATCATTT TCAGGAGGAG GAAGCTGAGC ACTCAGCCTC CCGTGTCTTT TGTTTTCTGT
4141 GTTTCCCTGC TTCTGTTTTT CTGGGTTATT TTATAACAGG TCTGTGTCCC AGCATTTCTA
4201 TAGAACCTTG TCTCGCCGCC TCCTCTAAGG GAAACACCAT TGTTAAGGAA AGCTTTAGCC
4261 ACGTGACAGT GAGGGGCGTG CACTTAACAG CTGATTATGT CAGCCGCTGC GTGTTGGCCT  →F6
4321 CTTCACTTGC CACCTACGTT TGCAGGGAAG CCTGAGAACT ACTCAGCCCA GGCCTAGGGA
4381 AGGGGACTCT GTCACTGTAG CTCTCCTGGA CCCAGACTGG CATGTTGTCC CCCCCTCCCC
4441 AAAATCGAGT CTCTATTCTC TTTTTATGAA TCAATGCTCT ACTTTTCTTT TGAAAACATA
4501 ATTTGTTGCC TCCTCTCCTG AGGCTCTCTC TCCCCTACCT ATCCTTAATA GAAACAGAGC
4561 CATCCTGTTT ACCGAGCATC TACTGTCAGT CCTGACGCTG AGACGTACTT ACCTTACTGC  ←R3
4621 TGAGAATACA GTAAATGAGA CAGATGGGCT TGCTGCTGGG TTCATGAACC TTGGCTTTTG
4681 TATTCTAAGC AGCAGGAATA AAAAGCCCAA TAAAAAGCCC TTGCCACCCA GCCACCCATC
4741 CTTTTTCTGC CTACTGCTGC AACTGCCCTC TCTGCTGCCC CTTTTGTAGT ACTGGCTTCC
4801 TGGTCCCCAC TTTTGGGGCA GCCCTAAATG TGGGTGGCTG CATCCTCTGC AGAGGCAGCA
4861 CTAGTCACCA AGTAGGCTTC CTGGAGACCT TTTCTCTGAT TGGCTAGTGA AGCACCTGCT
4921 TTTGTTTCCT TTCACAGTAG CCATTGGCTT TCCCTGTCCT TTCCGTTTGT CCGTATCCTT
4981 CCATCACCCA CTCACCCCTT AACGACACGT GGGCCCTCAA TTGCCCTTCT CTCAGGATCT
5041 GAAGGGTCAG AGGAAAGGGT TGGATTCTTT ATAACAAGGC TGGGGAGAGG CCAGGGAATG
5101 TCAGTCTAGG TTTTTCTCTC TCCCACTTCC CTTGGGTAGC AGACATTTCA TTCACCCGGC
5161 ACCAGGACAG GTGTCTTGTT CTGCCAAGCT GGTCAGTTTA GGAAGTAGGT TTCTCTTGAG
5221 CACTTCCTGT GGCCCAGGTA TCCTCCCTGA AAAGGGGTAG TTTCCCTCCC TCACTTCCCT  →F7
5281 TTCATTATTG ACGGTGTGAG ACATCCTGAT CGCATTGGCT GACTGAGCGG TGTCTGAGGC  ←R4
5341 CCTTCAGCCC AGCACCAGCA CCCAAGTCCA CGTGCAGGGA TGTGTGTGAC ACAGCCCTGA
5401 CCTCAGTGGG GGCCAGTAGC CAATCAGATG CCAGGAAGAG ATCCTTAGCC AACCGGGGGC
5461 GGGGCCTGCG GCTCTTCGGG CAGAAGGCCA ATGAGGGGCA GGGCCTGGCA TTATGCAACC
5521 CGCCTCCCAG CCTCGCGGAG CTTCTGGGTT[GCGGGCCGAAAC'}GGCAAGCGGATGGAGGGC  BglI
```

Figure 4.4 The *mPer1* sequence (www.ncbi.nlm.nih.gov/entrez/ accession number AF223952) above comprises 5' end and 3' end *Bgl* I restriction sites (nucleotides 1172-5562), annotation for the ~3 kb *Xho*I - *Bgl*I *mPer1* promoter used in the *mPer1*/d2EGFP transgene ({ }; nucleotide positions 2580-5562), and the oligonucleotide primer sequences (see 4.2.6.1) used to sequence the transgene DNA (highlighted and labelled appropriately within the sequence. F indicates 'forward' primers, and R indicates 'reverse' primers (the reverse complement of the sequences highlighted)). [ ] indicate exons within the sequence above, and the continued *mPer1* sequence below, respectively.

***mPer1* sequence (nucleotides 5581-7502; accession number AF223952)**

```
5581 GCTCGAACGGCCAG]GTAGGG ATCCCTGCTG CAATCCTAGG GTTAGTCTTT GTCCCGGAGC
5641 TTGGCCGCCA CTACTTCCAA CGTGATGGGT CGCTTTCAGC TCTCAGAGAG CAAAACAATA
5701 ATCTGCCTTT CCTGTCACTA TCCACCCTTC CCCGCCCCTG GGCCCTAGGT GTTCAACTCA
5761 ACCCGTCACG GGTTGTCTGC GTTTGTTATT CGTTCACCTA ATCTCCTCCT CTGCTCCTCC
5821 AGAGCAGCCA TCCTGAACCT AAGAGACCTT TAGCGAACAC GACCCCTTTA CACATTGCTC
5881 GACACTCGGG AGTCCATGGG TTTGCCCTAG CCCAAAGACC CCCTTCAGGC TTCTGCGCTC
5941 CCTGTCTTCC TCCCTCCAAT TCCTGGCCTC GTGCCGGTCG TGATGTCAAC CGCTTCAGGC
6001 TGGAACATCC TGTTCTCAGC GCTAGTTCTT GCTGTTGGCC ACAGCCTTCC TTCCCTTTTC
6061 TCCTGGCGCT CAGAAAATAC TTGGGATGGG GGTGTGGTTA GACAGGGAGT AGAGGAAAAA
6121 CTATATATGC TGGTTGTTGT GTGTCCCCTA TCTATGTGGT AGTAGGGTTA ACTAGAAAAG
6181 TAAGGGACGA AGGAAGATGC CTGAGTCGTT GCCTATGGCA AGTGGTAACT CCAGTCCTGG
6241 TGTTTGGGAG TGGGCAGGGG CTTGAGAAAG AAAAGCAGTG TCTTGATCAG AATAATGTTC
6301 GAGGCAAGAG CGAGGATGGG GGCGTTTCCA CAAAGAGCAG AGGCCGAGTG GGGAAGCTAG
6361 GACTTGCTCC TGGAGTTCCT CTAGTTTGTT ACTCTTCACA TGGCTCCTAG GCTCTTTGGG
6421 CCCTGGGAAT TTGTTATGGT GGGTGTTCTT CTCCTCCCCG CTGCCCTGAA CCTTGTTAGC
6481 CAGTATGAGG GTGTGTTGGC CAGTATAGGA CTGGGTCTGT TTCCCACTTC CACGAAGATG
6541 GGGATTGGGG GAGGAGTCGT TCCTGCCCTC CTGTGGTCCC TCCAGCAACC GCTGAGCTCA
6601 GCGGCTGACG TCGGTTTCCC TGGCGACCGC GGCTGTGGCG GAAGCGCGTG GTGGGGCCAG
6661 GCACATCGGC GCGCATGTGC AGCGGGGGTG GCACCGCCCC CGGATAAAAT TAGCCCGGAA
6721 GCCTAAATAT AGGAGGCGAT CAGCTCACCC CCTGCTCCGA GGCCTCAGAG TCCCAGACCA
6781 GGTGGGGACC TGATGAGAAT TTGGGCATAG GAAACCTGCA AGCTTTGACC CTCAGCTACT
6841 GTTCTAGTCG ATTGTTCAGG CTGTACTCAT TCCACACTGG CAAGGGGTGT AAGAGATGGC
6901 CTACGAGAGC TGCCTTTCTA CCTGTGGTAT CCTTAGGTCC CCCTAAGGAA ATAGAACATA
6961 TTTCTATTGC AAGCCCCAGG CCTGAGTCAC AACAGTGAGG GGCAGGCAGA GGAAGGACTG
7021 GGTGTAGCCA GCAGATGCTG TGGGGTTAAT AGCTCAGCTT TTGCTAAACA TTCCTTTTTG
7081 GTTTCTTTTT CTAG[GTGTCGTGATTAAATT AGTCAGCCCT CAGAGACAGG CGTCCTACCT
7141 CCTTTATCCA GACCTCAAAA GCCCCGTTGT GCACCCGTGG TGGCTTCTTC ACCTTCCCTG
7201 TTTCGTCCTC CACTGTATGG CCCAGACATG AGTGGTCCCC TAGAAGGGGC CGATGGGGGA
7261 GGAGACCCCA GGCCCGGAGA ACCTTTTTGT CCTGGAGGAG TCCCATCCCC TGGGGCCCCG
7321 CAGCACCGGC CTTGTCCAGG CCCCAGCCTG GCTGATGACA CTGATGCAAA CAGCAATGGC
7381 TCAAGTGGCA ATGAGTCCAA CGGACCCGAG TCCAGGGGCG CATCTCAGCG GAGTTCTCAT
7441 AGTTCCTCTT CTGGCAATGG CAAGGACTCA GCTCTGCTGG AGACCACTGA GAGCAGCAAG
7501 AG]
```

Figure 4.5 The d2EGFP sequence (Clontech) below comprises the ~1.1 kb fragment of d2EGFP used in the *mPer1*/d2EGFP transgene ({ }; 5' end *Xho*I and 3' end *Afl*II restriction sites; nucleotide positions 32-1187), and the oligonucleotide primer sequences used to sequence the transgene DNA (highlighted in blue and labelled appropriately within the sequence; see 4.2.6.1).

pd2EGFP-1 sequence (Clontech)

```
                                                                                    XhoI  HindIII
1       TAGTTATTAC TAGCGCTACCGGACTCAGATC{'TCGAGCTCA'AGCTTCG'AATTCTGCAGTCG           EcoR1  BamHI
61      ACGGTACCGC GGGCCCGG'GATCCACCGGTC GCCACCATGG TGAGCAAGGG CGAGGAGCTG           EGFP-F1
121     TTCACCGGGG TGGTGCCCAT CCTGGTCGAG CTGGACGGCG ACGTAAACGG CCACAAGTTC           ←mPER1-R5
181     AGCGTGTCCG GCGAGGGCGA GGGCGATGCC ACCTACGGCA AGCTGACCCT GAAGTTCATC
241     TGCACCACCG GCAAGCTGCC CGTGCCCTGG CCCACCCTCG TGACCACCCT GACCTACGGC
301     GTGCAGTGCT TCAGCCGCTA CCCCGACCAC ATGAAGCAGC ACGACTTCTT CAAGTCCGCC
361     ATGCCCGAAG GCTACGTCCA GGAGCGCACC ATCTTCTTCA AGGACGACGG CAACTACAAG
421     ACCCGCGCCG AGGTGAAGTT CGAGGGCGAC ACCCTGGTGA ACCGCATCGA GCTGAAGGGC
481     ATCGACTTCA AGGAGGACGG CAACATCCTG GGGCACAAGC TGGAGTACAA CTACAACAGC
541     CACAACGTCT ATATCATGGC CGACAAGCAG AAGAACGGCA TCAAGGTGAA CTTCAAGATC           EGFP-F2
601     CGCCACAACA TCGAGGACGG CAGCGTGCAG CTCGCCGACC ACTACCAGCA GAACACCCCC
661     ATCGGCGACG GCCCCGTGCT GCTGCCCGAC AACCACTACC TGAGCACCCA GTCCGCCCTG
721     AGCAAAGACC CCAACGAGAA GCGCGATCAC ATGGTCCTGC TGGAGTTCGT GACCGCCGCC
781     GGGATCACTC TCGGCATGGA CGAGCTGTAC AAGA'AGCTTAGCCATGGCTT CCCGCCGGAG           EGFP-R
841     GTGGAGGAGC AGGATGATGG CACGCTGCCC ATGTCTTGTG CCCAGGAGAG CGGGATGGAC           HindIII
901     CGTCACCCTG CAGCCTGTGC TTCTGCTAGG ATCAATGTGT AGATGCGCGG CCGCGACTCT
961     AGATCATAAT CAGCCATACC ACATTTGTAG AGGTTTTACT TGCTTTAAAA AACCTCCCAC
1021    ACCTCCCCCT GAACCTGAAA CATAAAATGA ATGCAATTGT TGTTGTTAAC TTGTTTATTG
1081    CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT AAAGCATTTT
1141    TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAATGTATC'}TTAAGGCGTAAATT           AflII
1201    GTAAGCGTTA ATATTTTGTT AAAATTCGCG TTAAATTTTT GTTAAATCAG CTCATTTTTT
1261    AACCAATAGG CCGAAATCGG CAAAATCCCT TATAAATCAA AAGAATAGAC CGAGATAGGG
1321    TTGAGTGTTG TTCCAGTTTG GAACAAGAGT CCACTATTAA AGAACGTGGA CTCCAACGTC
1381    AAAGGGCGAA AAACCGTCTA TCAGGGCGAT GGCCCACTAC GTGAACCATC ACCCTAATCA
1441    AGTTTTTTGG GGTCGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG GAGCCCCCGA
1501    TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA AGGAAGGGAA GAAAGCGAAA
1561    GGAGCGGGCG CTAGGGCGCT GGCAAGTGTA GCGGTCACGC TGCGCGTAAC CACCACACCC
1621    GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TCAGGTGGCA CTTTTCGGGG AAATGTGCGC
1681    GGAACCCCTA TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA
1741    TAACCCTGAT AAATGCTTCA ATAATATTGA AAAAGGAAGA GTCCTGAGGC GGAAAGAACC
1801    AGCTGTGGAA TGTGTGTCAG TTAGGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA
1861    GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCAGGTG TGGAAAGTCC CCAGGCTCCC
1921    CAGCAGGCAG AAGTATGCAA AGCATGCATC TCAATTAGTC AGCAACCATA GTCCCGCCCC
1981    TAACTCCGCC CATCCCGCCC CTAACTCCGC CCAGTTCCGC CCATTCTCCG CCCCATGGCT
2041    GACTAATTTT TTTTATTTAT GCAGAGGCCG AGGCCGCCTC GGCCTCTGAG CTATTCCAGA
2101    AGTAGTGAGG AGGCTTTTTT GGAGGCCTAG GCTTTTGCAA AGATCGATCA AGAGACAGGA
2161    TGAGGATCGT TTCGCATGAT TGAACAAGAT GGATTGCACG CAGGTTCTCC GGCCGCTTGG
2221    GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC
2281    GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA CCTGTCCGGT
2341    GCCCTGAATG AACTGCAAGA CGAGGCAGCG CGGCTATCGT GGCTGGCCAC GACGGGCGTT
2401    CCTTGCGCAG CTGTGCTCGA CGTTGTCACT GAAGCGGGAA GGGACTGGCT GCTATTGGGC
2461    GAAGTGCCGG GGCAGGATCT CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC
2521    ATGGCTGATG CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC ATTCGACCAC
2581    CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG AAGCCGGTCT TGTCGATCAG
2641    GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG AACTGTTCGC CAGGCTCAAG
2701    GCGAGCATGC CCGACGGCGA GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT
2761    ATCATGGTGG AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG
2821    GACCGCTATC AGGACATAGC GTTGGCTACC CGTGATATTG CTGAAGAGCT TGGCGGCGAA
2881    TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CCGATTCGCA GCGCATCGCC
2941    TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT GGGGTTCGAA ATGACCGACC
3001    AAGCGACGCC CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT
3061    TGGGCTTCGG AATCGTTTTC CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA
3121    TGCTGGAGTT CTTCGCCCAC CCTAGGGGGA GGCTAACTGA AACACGGAAG GAGACAATAC
3181    CGGAAGGAAC CCGCGCTATG ACGGCAATAA AAAGACAGAA TAAAACGCAC GGTGTTGGGT
3241    CGTTTGTTCA TAAACGCGGG GTTCGGTCCC AGGGCTGGCA CTCTGTCGAT ACCCCACCGA
3301    GACCCCATTG GGGCCAATAC GCCCGCGTTT CTTCCTTTTC CCCACCCCAC CCCCCAAGTT
3361    CGGGTGAAGG CCCAGGGCTC GCAGCCAACG TCGGGGCGGC AGGCCCTGCC ATAGCCTCAG
3421    GTTACTCATA TATACTTTAG ATTGATTTAA AACTTCATTT TTAATTTAAA AGGATCTAGG
3481    TGAAGATCCT TTTTGATAAT CTCATGACCA AAATCCCTTA ACGTGAGTTT TCGTTCCACT
3541    GAGCGTCAGA CCCCGTAGAA AAGATCAAAG GATCTTCTTG AGATCCTTTT TTTCTGCGCG
3601    TAATCTGCTG CTTGCAAACA AAAAAACCAC CGCTACCAGC GGTGGTTTGT TTGCCGGATC
3661    AAGAGCTACC AACTCTTTTT CCGAAGGTAA CTGGCTTCAG CAGAGCGCAG ATACCAAATA
3721    CTGTCCTTCT AGTGTAGCCG TAGTTAGGCC ACCACTTCAA GAACTCTGTA GCACCGCCTA
3781    CATACCTCGC TCTGCTAATC CTGTTACCAG TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC
3841    TTACCGGGTT GGACTCAAGA CGATAGTTAC CGGATAAGGC GCAGCGGTCG GGCTGAACGG
3901    GGGGTTCGTG CACACAGCCC AGCTTGGAGC GAACGACCTA CACCGAACTG AGATACCTAC
3961    AGCGTGAGCT ATGAGAAAGC GCCACGCTTC CCGAAGGGAG AAAGGCGGAC AGGTATCCGG
4021    TAAGCGGCAG GGTCGGAACA GGAGAGCGCA CGAGGGAGCT TCCAGGGGGA AACGCCTGGT
4081    ATCTTTATAG TCCTGTCGGG TTTCGCCACC TCTGACTTGA GCGTCGATTT TTGTGATGCT
4141    CGTCAGGGGG GCGGAGCCTA TGGAAAAACG CCAGCAACGC GGCCTTTTTA CGGTTCCTGG
4201    CCTTTTGCTG GCCTTTTGCT CACATGTTCT TTCCTGCGTT ATCCCCTGAT TCTGTGGATA           →mPer1-F1
4261    ACCGTATTAC CGCCATGCAT
```

4.1.8 Experimental aims

The aims of the present study are:

1. To generate an *mPer1*/d2EGFP transgenic rat model using established transgenesis techniques.

2. To confirm transgene integration (and stable inheritance of the *mPer1*/ d2EGFP transgene in further generations) using established Southern blot hybridisation protocols.

3. To investigate transgene expression in the *mPer1*/d2EGFP transgenic rat using reverse-transcriptase polymerase chain reactions (RT-PCRs) and fluorescence microscopy.

4. To verify the sequences of the *mPer1*/d2EGFP transgene.

4.2 METHODS

The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

4.2.1 Preparation of transgene DNA (for micro-injection)

4.2.1.1 Plasmid preparation

The plasmid, P1PG #4, containing a ~3kb fragment of the *mPer1* gene promoter in pd2EGFP-1 (vector map in appendix C; see Figure 4.2**d** and 4.3), was received from the Laboratory of D Klein, Section on Neuroendocrinology, National Institute of Health, Bethesda, USA. In order to enable long-term storage of the plasmid as glycerol stocks, competant *E.coli* bacterial cells were transformed (see 2.1).

Fresh aliquots of transgene DNA were prepared on a monthly basis using the following procedure. The plasmid (P1PG #4) containing the *mPer1*/d2EGFP transgene (see Figures 4.4 and 4.5 for sequences) was recovered from glycerol stocks, as described in 2.1.3 (where 50μg/ml (final concentration) kanamycin (Sigma) was used to supplement LB), and isolated and purified, as described in 2.1.4.

4.2.1.2 Extraction and purification of transgene DNA

The transgene plasmid (4.2.1.1) was subjected to *Xho*I-*Afl*II restriction digestion (50µl DNA, 30µl sterile water, 10µl 10x buffer 2 (NEB; final concentration 1x), 5µl *Xho*I (NEB; final concentration 0.5 unit), and 5µl *Afl*II (NEB; final concentration 0.5 unit)), at 37°C, for 2 hours. Subsequent restriction fragments were resolved by 1% 1xTAE agarose gel electrophoresis (see 2.2.3), to give a 4.1 kb band (transgene DNA fragment), and a 3.2 kb band (pd2EGFP vector fragment). The transgene DNA fragment (4.1 kb) was briefly visualised under UV, excised using a scalpel, and then

extracted from the gel using the QIAEX II gel extraction kit (Qiagen, see appendix B). The resultant pellet (transgene DNA) was eluted in 50µl MITE buffer, and then purified through a Sephadex G-50 spin column (prepared as described in 2.2.5.4.2; equilibrated with 100µl of MITE buffer, centrifuged at 3000g for 3 minutes, 5 times, prior to the application of the transgene DNA to the surface of the Sephadex), centrifuged at 3000g for 4 minutes.

4.2.1.3 Quantitation and further purification of transgene DNA

The concentration of the eluted DNA (4.2.1.2) was determined by comparison against the Hyperladder I (Bioline) quantitative marker following fractionation by 1% 1xTAE agarose gel electrophoresis (see 2.2.3). The transgene DNA concentration was diluted to 2-5ng/µl with MITE buffer, filter-sterilised through into a 0.45µM VectaSpin PVDF filter (Whatman; microcentrifuged for 1 minute at 12,000 rpm), and then stored at -20°C in 20µl aliquots, until required for micro-injection (4.2.2.4.2). Each aliquot of transgene DNA was thawed on ice, and microcentrifuged at 14,000rpm for 3 minutes immediately before use.

4.2.2 Rat transgenesis

The procedures involved in rat transgenesis are basically identical to those used for generating transgenic mice (Murphy and Carter, 1993). Since rat transgenesis has now become a routine, well-documented procedure, only an overview of the transgenesis procedures and equipment used will be described here. The transgenesis protocol used in the present study is a modification of various published methods (Glover and Hames, 1995; Murphy and Carter, 1993), optimised to improve efficiency. Essentially, transgenic rats were generated by: i) pronuclear injection of transgene (*mPer1*/d2EGFP) DNA into fertilised one-cell eggs, and ii) surgical transfer of cultured oocytes into recipient pseudopregnant mothers, as summarised by Figure 6.4.

4.2.2.1 Animal procedures

All animal procedures were conducted according to UK Home Office regulations (Schedule 1 humane killing methods), and local ethical review. SD rats were maintained in approved laboratory conditions on a 14 hour light - 10 hour dark cycle, with food and water available *ad libitum.*

4.2.2.1.1 Anaesthetising the rat for surgery

General inhalation anaesthesia was used for all surgical procedures. For each procedure, the rat was placed in an induction chamber supplied with a gaseous mixture of 1 litre O_2/minute and 0.5 litre NO_2/minute, carrying 4% vaporised halothane. When fully anaesthetised, the rat was placed on the operating table with its nose and mouth placed in a facemask, whereby anaesthesia was maintained for the duration of surgery by a 2-2.5% halothane gaseous supply. Several minutes prior to completion of surgery, the halothane concentration was reduced gradually to 0%, followed by the complete removal of the facemask. The rat was allowed to recover in a gently heated observation cage with insulation material, and then returned to normal caging. A fluovac extraction system (Harvard, IMS) was used with all anaesthetic procedures.

4.2.2.1.2 Vasectomy of the male rat

A colony of 25 individually-caged vasectomised male SD rats was established, maintained and used to induce pseudopregnancy in sexually mature female rats. Fresh (7-8 week old) vasectomised male rats were introduced to the colony on a monthly basis and animals older than 1 year of age were killed.

Each vasectomy subject was anaesthetised (see 4.2.2.1.1) and laid on its back, before swabbing the lower abdomen and scrotum with 70% alcohol. The scrotal skin was lifted using a pair of blunt forceps, and a 2cm incision was made between the 2 testes using a pair of fine dissecting scissors. The membrane covering the testis was cut,

and the lower end of the testis was pushed through the incision to reveal the epididymis and vas deferens. The vas deferens was isolated by gently tearing the membrane connecting it to the outer surface of the testis. Surgical silk (~6cm) was folded to produce a loop, placed between the testis and the vas deferens, and pulled through, leaving equal lengths of silk on either side. The loop was cut to give 2 separate strands; one was tied close to the epididymis using a tight reef knot, and the other was tied further up the vas deferens. Surgical dissection of a 0.5cm section of the vas deferens was performed between the 2 tightly-knotted ends. The procedure was then repeated for the other testis. Subsequently, the scrotal skin was sutured with at least 6 stitches. Following surgery, the rats were allowed to recuperate for 3 weeks and were then mated with a wild-type female to determine if the surgery was effective, before being introduced into the colony of vasectomised males.

4.2.2.1.3 Preparation of pseudopregnant female rats

On day -1 at 11:00h, (i.e. following mating of superovulated female SD rats with SD stud males; see 4.2.2.2), 15-25 mature SD rats (200-300g) were individually placed with a vasectomised male SD rat (4.2.2.1.2) overnight. The subsequent morning (day 0), the potential recipient/oviduct transfer (OVT) female rats were separated from the vasectomised males and examined for the presence of a copulatory plug (indicative of a successful mating). Rats showing evidence of copulation were considered pseudopregnant, and were set aside as recipient/OVT rats (4.2.2.5.2), whilst non-pseudopregnant females were returned to normal caging.

4.2.2.2 Superovulation of immature female rats and generation of fertilised one-cell eggs

Superovulation was initiated at 09:30h on day -3 by restraining an immature female SD rat (120-150g; 5-6 week old) in one hand, and administering an i.p. injection of 40IU Folligon (Pregnant Mare Serum Gonadotrophin, PMSG, Intervet; mimics the endogenous effects of FSH) with the other hand. Between 4-16 female rats were

treated in this way, per transgenesis session. On day -1 (i.e. two days following Folligon treatment), at 10:30h, the same female rats were treated with an i.p. injection of 30IU Chorulon (human Chorionic Gonadotrophin (hCG), Intervet; mimics the effects of LH), and then each placed with a (stud) male SD rat (3-9 month old) overnight to generate fertilised one-cell eggs. The subsequent morning (day 0; before 10:00h), the superovulated female rats were separated from the stud males and prepared for egg harvesting (4.2.2.3.3).

4.2.2.3 Harvesting of fertilised one-cell eggs from superovulated female rats

4.2.2.3.1 Preparation of transfer pipettes

To prepare pipettes for the transfer of eggs (for manipulation), the centre of a hard glass capillary (1.5mm; GC150-15, Harvard Apparatus Ltd.) was placed in a small bunsen burner flame to soften, then immediately removed and simultaneously pulled from either side to narrow the centre region (internal diameter 150-200μm). The narrow region was scored with a diamond cutter, cut into 2 halves, and then rounded at the wider end of the capillary using a small bunsen burner flame.

The pipette was assembled into a mouth-operated system, constructed from a mouthpiece (Gamidor), a rubber tube (Esco), and a pipette holder, and operated by gentle blowing and sucking of the mouthpiece.

4.2.2.3.2 Preparation of media and culture dishes

All media preparation was performed using aseptic techniques in a class 2 laminar flow cabinet. M2 media (Sigma; media used for manipulating eggs in culture) and M16 media (Sigma; media used for maintaining eggs in culture) were prepared, each containing additional reagents (streptomycin, penicillin, sodium lactate, and hyaluronidase; see appendix A). At 09:00h on day 0 (30 minutes - 1 hour prior to

harvesting fertilised one-cell eggs), a series of 35mm culture dishes (Corning) were prepared (see Table 4.1).

	Number of culture dishes	M2 media
i)	1	2-3ml
ii)	1	4x 50μl drops with hyaluronidase (10mg/ml) 1x 50μl drop
iii)	1	3 x 50μl drops
		M16 media
iv)	1	150μl drop covered with paraffin oil (Fluka)
v)	2	3 x 50μl drops covered with paraffin oil (Fluka) (drops labelled 'U', '√' and 'X', respectively)

Table 4.1 Preparation of culture dishes for various media for manipulating and maintaining eggs in culture. Dishes **i), ii)** and **iii)** were prepared per 4 superovulated female rats and kept at RT. Dishes **iv)** and **v)** were allowed to equilibrate for 1 hour at 37°C and 5% CO_2, in a humidified tissue culture incubator (Model 3164; Forma Scientific).

4.2.2.3.3 Isolation and collection of fertilised one-cell eggs

Between 09:30-10:00h on day 0, the superovulated female rats (4.2.2.2) were killed by concussion and cervical dislocation, in batches of four. Each rat was placed on its back, and checked for the presence of a copulatory plug, before soaking the abdomen with 70% ethanol. An incision was made in the skin and abdominal wall with a sharp pair of dissection scissors, to expose the abdominal cavity. One side of the reproductive tract was located, and the coiled oviduct was identified between the ovary and the uterus. The mesometrium membrane, which joins the uterine horn to the body wall, was removed using a pair of curved dissecting forceps, to allow an incision to be made in the bursa membrane between the ovary and the oviduct, using a

pair of fine dissecting scissors. Finally, the oviducts were excised from each uterine horn of the reproductive tract and immediately placed into the M2 manipulation media dish (see 4.2.2.3.2 **i**)).

The oviducts were viewed under a stereo dissection microscope (Nikon; x10-20 magnification), where the swollen ampulla was located and gripped with a pair of fine watchmakers forceps, and then incised using a pair of fine vannas spring scissors to release the cumulus mass (fertilised eggs surrounded by a multitude of cumulus cells). To remove the cumulus cells, the cumulus masses from each rat were transferred to a fresh microdrop of M2 media with hyaluronidase (see 4.2.2.3.2 **ii**)), for up to 5 minutes), using a transfer pipette (see 4.2.2.3.1), before being transferred to a microdrop of M2 media (same culture dish) to wash away any remaining traces of the enzyme. The eggs collected from each rat were pooled in this microdrop of M2 media (see 4.2.2.3.2 **ii**)), before being washed through a series of three microdrops of M2 media (see 4.2.2.3.2 **iii**)), then M16 media (see 4.2.2.3.2 **iv**)), to remove any traces of the M2 media, and finally incubated in a fresh microdrop of M16 media (see 4.2.2.3.2 **v**); labelled 'U' for 'uninjected'), at 37°C and 5 % CO_2, for at least 1 hour prior to micro-injection (4.2.2.4.5).

4.2.2.4 Pronuclear micro-injection of fertilised one-cell eggs

4.2.2.4.1 Preparation of holding pipettes

Glass capillaries (1mm; GC100-15; Harvard Apparatus Ltd.) were used to prepare holding pipettes (internal diameter ~100μm), using the same methodology applied for making transfer pipettes (see 4.2.2.3.1). The shaft length of a typical holding pipette measured 4-5cm from one end of the 8-10cm long capillary. The narrow end of the holding pipette was cut at the tip with a diamond cutter to obtain a straight cut, and then placed in a microforge and heated until the diameter of the hole was reduced to 10-15μm.

4.2.2.4.2 Preparation of micro-injection needles

Thin-walled boroscillicate glass capillaries (TW 100F-4, World Precision Instruments) were used to make the micro-injection needles. The needles were prepared using a mechanical puller (Model 773, Campden Instruments) with optimised settings (see Table 4.2).

Stage	Heat intensity	Heating time (seconds)	Pulling force	Pulling displacement
1	60	5	0	0
2	0	0	40	0.3
3	0	1	0	0
4	65	3	0	0
5	0	0	70	24

Table 4.2 Mechanical pipette puller (Model 773, Campden Instruments) parameters for preparing micro-injection needles.

The blunt end of each micro-injection needle was placed into the transgene DNA solution (on ice; see 4.2.1), where capillary action allowed the tip to fill with the solution. The needle was removed from the solution when a bubble was visible about 5mm away from the tip, ready for immediate use in micro-injection (4.2.2.4.5).

4.2.2.4.3 Inverted microscope fitted with micromanipulators – the automatic micro-injection system

An inverted microscope (Diaphot 200; Nikon) was used to provide the differential interference contrast optics required for observation of egg pronuclei during micro-injection. The microscope was positioned on a vibration-free base plate on an air table, with a micromanipulator (Leica) fitted on either side.

The left side manually-controlled micromanipulator provided attachment for the holding pipette (see 4.2.2.4.1) via a pneumatic screw and rubber tube (pneumatic manipulator, Microtec). Manipulation of the screw allowed eggs to be held in position during micro-injection, by controlling the displacement of air within the holding pipette. Anti-clockwise movements of the screw draws air/fluid into the holding pipette and clockwise movements expels air/fluid out from the holding pipette.

The right side micro-manipulator provided attachment for the micro-injection needle (see 4.2.2.4.2), and was used to inject transgene DNA (see 4.2.1) into the pronucleus of fertilised eggs, via an automated pico-injector system (IM300; Narishige) attached to a supply of compressed nitrogen (40-50 psi). Both micromanipulators were operated with the aid of joysticks, which enabled fine movement to be controlled in both horizontal planes, and a knob to adjust the manipulator in the vertical plane. Micro-injection was performed at a pressure of 7-15 psi, by switching on the 'vent mode,' and using a pre-programmed sequence controlled by operating a foot pedal or control pad. The 'clear' mode was operated via a button on the injection unit, and was used to remove debris from blocked needles at a pressure of 80-100psi.

4.2.2.4.4 Preparation of the micro-injection chamber

The micro-injection chamber was prepared by washing a depression slide with 70% ethanol, and then adding 50-100µl of M2 media covered with a layer of paraffin oil (Fluka) in the centre of the depression. The slide was placed centrally onto the microscope stage, with the M2 microdroplet positioned in the centre of the optical field (x40 magnification). Both the holding pipette and micro-injection needle were aligned centrally in the field of view, where the needle tip was broken, and the holding pipette was filled with M2 media. Immediately prior to micro-injection, the needle was primed by clearing the tip 2-3 times (4 seconds each), using the 'clear' mode on the automated injector unit.

4.2.2.4.5 Micro-injection procedure

Following incubation of the isolated fertilised eggs in the M16 medium for at least 1 hour (see 4.2.2.3.3), batches of 25-40 eggs were microinjected in a serial fashion. Each batch of eggs was removed from the microdrop of M16 media labelled 'U' ('uninjected'; see 4.2.2.3.3), washed in M2 media, and transferred to the M2 media droplet on the depression slide. The eggs were placed at the bottom of the optical field underneath the holding pipette (x40 magnification).

The holding pipette was used to immobilise each egg by altering the suction pressure in the holding pipette. The magnification was adjusted to x400 and the 2 pronuclei were brought into focus. The larger (male) pronucleus was then positioned centrally, since this was the target for micro-injection. The needle was placed in front of the zona pellucida and adjusted to bring the needle tip in focus with the large (male) pronucleus. Penetration of the egg was achieved by a sharp tapping action of the index finger on the joystick several times. Once the needle tip was inside the pronucleus, DNA micro-injection was activated by pushing on the foot pedal, which automatically injected the egg for a 4 second duration at a pressure of 7-15psi, and caused the pronucleus to swell. Micro-injection was conducted until the diameter of the pronucleus swelling increased to 25-50% (optimal; >50% increase in diameter killed most eggs). At this point, the needle was quickly withdrawn from the egg, maintaining the injection pressure to prevent needle blockage and extrusion of either pronuclear or plasma membrane. Injection parameters varied between eggs, and in some cases, the injector was activated several times. Successfully injected eggs were transferred to the right side of the visual field just above the midline under low magnification. Non-injectable eggs, such as eggs with no clear pronuclei (unfertilised), or a dense shrunken cell mass within the zona pellucida (dead), were transferred to an egg pool located to the left side of the visual field just above the midline. Eggs that died during microinjection were also transferred to this pool.

After the injection of each batch of eggs, the injected and non-injectable eggs were washed in the equilibrated M16 media (see 4.2.2.3.2 **iv**)), before being transferred to the M16 droplets labelled '√' and 'X', respectively (see 4.2.2.3.2 **v**)). Following completion of micro-injection, the eggs were incubated at 37°C and 5% CO_2 for at least 1 hour prior to commencing same day oviduct transfers (day 0 OVTs; 4.2.2.5.2), (or overnight for day 1 OVTs, depending on the availability of recipient females; the present study only involved day 0 OVTs since there were sufficient numbers of recipient females immediately available for OVT per transgenesis session).

4.2.2.5 Implantation of micro-injected eggs into the oviducts of recipient pseudopregnant female rats (oviduct transfer, OVT)

4.2.2.5.1 Preparation of oviduct transfer (OVT) pipettes

(OVT) Transfer pipettes (internal diameter 140-150μm) for implanting manipulated eggs into recipient female rat oviducts were prepared and operated, as described in 4.2.2.3.1.

During OVT (4.2.2.5.2), the required number of eggs were removed from the M16 media '√' microdrop (see 4.2.2.4.5) using a standard transfer pipette (see 4.2.2.3.1) containing M2 media, and were washed twice in fresh microdrops of M2 media, ready for OVT pipette loading (see below).

Under the stereo dissecting microscope (Nikon; x10-20 magnification), the OVT pipette was loaded with minimal M2 media (just past the shoulder of the pipette), immediately followed by 2 air bubbles, the micro-injected eggs (in M2 media; see above), a 3rd air bubble and finally a small volume of M2 media.

4.2.2.5.2 The oviduct transfer (OVT) Procedure

OVTs were conducted 1 hour subsequent to micro-injection (see 4.2.2.4.5) on day 0 (for same day OVTs; or between 0630-1000h the subsequent morning for next day (day 1) OVTs, depending on the availability of recipient females; the present study only involved day 0 OVTs since there were sufficient numbers of recipient females immediately available for OVT per transgenesis session). Up to 3 pseudopregnant rats were used as surrogate mothers per transgenesis session.

Between 20-50 manipulated eggs were implanted into one (unilateral) or both (bilateral) oviduct(s) of the pseudopregnant recipient female, dependant upon the number of eggs available for transfer, and the number of pseudopregnant females available.

Each pseudopregnant subject was anaesthetised (see 4.2.2.1.1) and laid on its abdomen on the surgical table, before shaving an area from the end of the rib cage towards the tail (~6x4 cm). Excess loose fur was was removed by swabbing with 70% alcohol containing hibitane. A ~3cm incision was made along the midline of the back, using a scalpel. The thumb and forefinger were used to grip, and gently lift up, the skin on one side. A Spencer Wells artery clamp was used to separate the skin from the body wall, by pushing it through the connective tissue, which was then clamped to one side using a pair of Drape forceps. The fat pad covering the ovary was located beneath the body wall using blunt forceps, and a site clear of blood vessels and nerves was found in which to make a ~1cm incision with a fine pair of dissecting scissors. The fat pad was carefully pulled through this incision, bringing with it the ovary, oviduct and uterine horn. A bulldog type clamp was attached to the fat pad to hold the ovary in a suitable position for the transfer procedure, so that the swollen ampulla was clearly visible. The exposed reproductive tract was kept moist by swabbing with saline using a cotton bud.

The ovary of the recipient female rat was viewed under the stereo dissecting microscope (Nikon; x10-20 magnification), where the coils of the oviduct were gently moved aside, using fine curved watchmakers forceps, to expose the infundibulum (swollen opening of the oviduct). At this point, the micro-injected eggs were loaded into the transfer pipette (see 4.2.2.5.1), ready for surgical transfer into the pseudopregnant recipient rat. The bursa membrane was gripped with fine curved watchmaker's forceps, and a small incision was made above the infundibulum with a fine pair of Vannas spring scissors. The infundibulum was carefully pulled out from below the bursa and held, whilst the tip of the OVT pipette was moved into the oviduct opening. The infundibulum was gripped around the OVT pipette, whereby the manipulated eggs were released into the oviduct. The OVT pipette was then gently removed on release of the 3rd bubble (see 4.2.2.5.1), the infundibulum was gradually pushed back into the bursa, and any further disruption of the oviduct was avoided. The ovary and fat pad were then placed back inside the body cavity, and the body wall was sutured with 4-5 stitches.

The procedure was repeated for the oviduct on the opposite side for bilateral OVTs. (The same procedure was used for both unilateral and bilateral OVTs. For unilateral OVTs, the eggs were transferred to the right infundibulum only (preferred site for this procedure).) Following the OVT procedure, the incision along the back of the subject was sutured with 12-15 stitches, and cleaned of blood using saline, before applying a topical antibiotic. After recovering from surgery (see 4.2.2.1.1), the subjects were individually caged, and regularly observed until complete healing of the incision was observed. For the remaining duration of the gestation period, which was usually prolonged in these animals (22-23 days), the subjects were disturbed as little as possible, except for a few days before the end of the gestation period, where extra bedding (shredded paper) was placed in the cage for nest building.

4.2.3 Genotyping of *mPer1*/d2EGFP transgenic rats

4.2.3.1 Screening of progeny for transgene incorporation

Genomic DNA extracted from tail and ear biopsies (see 4.2.3.1.1 and 2.2.1) of all progeny derived from OVT mothers (see 4.2.2.5.2) were screened for transgene incorporation using multiplex PCR and Southern analysis (see 4.2.3.1.1.1 and 2.2.2., and 4.2.3.1.1.2 and 2.2.5, respectively).

4.2.3.1.1 Identification marking by tail and ear biopsies

Rat pups were restrained in one hand, with the tail held between thumb and index finger. For identification purposes, the sex of each rat pup was first determined by observing the distance between the anus and external genitals, and for the presence of nipples. Different types of ear biopsies were then performed to ear-mark the rat pups:

i) cross-cut ('x') - a horizontal cut made across one ear

ii) incision ('i') - a v-shaped incision made in one ear

iii) hole punch ('o') – a hole in one ear made with an ear punch.

A tail biopsy was performed on those rat pups with either no ear mark, or marked with a hole (since an ear punch alone would yield an insufficient amount of genomic DNA). A combination of these markings in either one or both ears was sufficient to uniquely identify a rat pup in a litter.

For each biopsy, 2-3mm of tissue was cut from the tip of the rat's tail or ear, using sharp dissection scissors, and placed into a 1.5ml microcentrifuge tube. For tail biopsies, the tip of the tail was first anaesthetised with ethyl chloride (a local anaesthetic; Roche), and then, following tissue removal, cauterised by applying a silver nitrite pencil (Bay Health & Leisure) to the wound for 3-5 seconds. Biopsy samples were digested immediately (see 2.2.1), or stored at –70°C until ready for genomic DNA extraction.

4.2.3.1.1.1 Genomic screening by Multiplex Polymerase Chain Reaction (PCR) amplification

Transgenic offspring were identified by using a multiplex PCR strategy to screen genomic DNA using custom primers designed to amplify the β-actin gene and the EGFP transgene sequences. Following a successful PCR, amplified β-actin products served as a control to confirm the presence of genomic DNA, and amplified EGFP products represented the presence and integration of the transgene into the rat genome.

Standard PCR reactions (see 2.2.2) were performed. Each reaction contained 0.5-1.0μg of genomic DNA, 400nM (final concentration) of each EGFP-specific primer (forward (F1) and reverse (R); see Table 4.4), 200nM (final concentration) of each actin-specific primer (forward (F) and reverse (R); see Table 4.3). (All primers were manufactured by MWG Biotech). An aliquot of the micro-injection transgene fragment (*mPer1*/d2EGFP; 0.5ng) and a water sample were included as positive and blank (negative) controls, respectively. PCR was conducted using a pre-heated (95°C) PCR block (PCR Express, ThermoHybaid). Thermal cycling conditions used were: 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds and 62°C for 2 minutes; 72°C for 7 minutes. The products of the reactions were analysed by 1% 1x TAE agarose gel electrophoresis, as described in 2.2.3, with an aliquot of an appropriate marker added to the outer lane(s) (100bp ladder, New England Biolabs).

Primer	5' – Sequence - 3'
EGFP-F1	ATG GTG AGC AAG GGC GAG GAG C
EGFP-R	CTT GTA CAG CTC GTC CAT GCC G
Actin-F	TCA TGC CAT CCT GCG TCT GGA CCT
Actin-R	CCG GAC TCA TCG TAC TCC TGC TTG

Table 4.3 Oligonucleotide primer sequences used for genomic screening by PCR (see Figure 4.5; EGFP primers highlighted in green). The EGFP primers were designed to amplify a 716 bp PCR product from genomic DNA, and the β-actin primers were designed to amplify a 581 bp PCR product from genomic DNA.

4.2.3.1.1.2 Genomic screening by Southern Analysis

Southern analysis is used to detect a specific DNA sequence (target) within a mixture of DNA fragments, such as those obtained when genomic DNA is digested with a restriction endonuclease, and is essentially a technique based upon the complementarity between the target and a radioactively-labelled DNA fragment (probe). The procedure consists of 5 stages:-

i) sample, gel and Southern blot preparation;
ii) probe preparation;
iii) hybridisation of the probe to the Southern blot;
iv) stringency washes;
v) detection and visualisation of the hybridised probe.

Transgene integration was confirmed, and copy number estimated, by Southern analysis of genomic DNA extracted from founders, as described in 2.2.5.

4.2.3.1.1.2.1 Sample, gel and Southern blot preparation

Overnight *BamH*I restriction digests were performed on 10μg of genomic DNA (see 2.2.5.1), and the resulting restriction fragments were fractionated using 0.7% 1xTAE agarose gel electrophoresis (see 2.2.5.2). An aliquot of the full-length micro-injection transgene fragment (*mPer1*/d2EGFP; 0.5ng) was included as a positive control. An aliquot of an appropriate marker was also added to the outer lanes (1kb ladder, NEB).

Electrophoretic fractionation of the DNA fragments was then followed by partial acid depurination, alkaline denaturation, and capillary transfer in a high-salt buffer from the gel to a nylon membrane (Hybond-N) overnight (see 2.2.5.3). Acid depurination followed by alkaline hydrolysis increases the transfer efficiency of large molecular weight fragments from the agarose gel to the nylon membrane by reducing the length of the fragments. The alkaline solution cleaves the sugar-phosphate backbone at depurinated sites, denatures the DNA, and therefore makes the hydrogen bonding sites on the nitrogenous bases in the single-stranded genomic DNA available to hydrogen bond with the single-stranded probe DNA (4.2.3.1.1.2.2).

4.2.3.1.1.2.2 Probe preparation

An EGFP probe was prepared using a 736bp *BamH*I - *Hind*III fragment (nucleotide positions 79-815, see Figure 4.5) digested from the pd2EGFP-1 vector (Clontech – see appendix C; digest reaction conditions as described in 2.2.5.1), and contained the generic EGFP DNA sequence. Radioactively-labelled probes were synthesised from 20-50ng of DNA using the ^{32}P random primer labelling method (see 2.2.5.4.1), and purified by size exclusion chromatography (see 2.2.5.4.2).

4.2.3.1.1.2.3 Hybridisation of the probe to the Southern blot

Southern blot membranes (4.2.3.1.1.2.1) were treated and hybridised with the EGFP probe (4.2.3.1.1.2.2), as described in 2.2.5.5.

4.2.3.1.1.2.4 Stringency washes

Stringency washes were performed as described in 2.2.5.6.

4.2.3.1.1.2.5 Detection and visualisation of the hybridised probe

The decay of the radioisotope within the probe emits energy (mostly beta particles). The presence and size of a particular genomic DNA fragment (complementary to the probe) was therefore detected and visualised by exposing the membrane to a radiation sensitive material (X-ray film for 2-3 days, or, Phosphor screen for 1-2 days), as described in 2.2.5.7.

4.2.4 Establishment, breeding and maintenance of hemizygous transgenic lines

All *mPer1*/d2EGFP transgenic rat pups (founders) derived from OVT mothers (4.2.2.5.2), confirmed by multiplex PCR and Southern analysis (4.2.3.1.1.1 and 4.2.3.1.1.2, respectively), were weaned on post-natal day 21 into individual cages. In order to establish transgenic lines, transgenic founders were mated with a wild-type rat on reaching sexual maturity (6-9 week old) for 7-14 days. The 'fertilised' females were then individually caged and supplied with shredded paper 2 days prior to the expected date of birth.

All progeny derived from each founder were sexed and ear-marked between post-natal days 10-14, and genotyped, as described in 4.2.3. After weaning on post-natal day 21, the first generation (F1) of *mPer1*/d2EGFP transgenic rat pups were caged separately from non-transgenic rat pups for each transgenic line. The breeding process was repeated with either the founder or a transgenic descendant for each transgenic line, depending on experimental demand. Given that the transgene is in the founder germline, Mendelian inheritance predicts that 50% of offspring from hemizygous founders should be transgenic. Where possible, 2 hemizygous transgenic male rats were maintained for each transgenic line for breeding purposes, since males are able to mate with numerous females at one time, and do not require additional time to allow for births and weaning to occur.

4.2.5 Transgene expression analysis

Breeding lines of transgenic rats were established and maintained from founders, and subsequent generations were used for transgene expression analysis. (Non-transgenic litter-mates were used as experimental controls.)

The transgene used in the present study is convenient in that it contains the EGFP reporter gene. This allows transgene-specific (non-endogenous) expression to be analysed since EGFP does not exist in the mammalian genome. Transgene expression is manifested initially by transcript production within cells. RT-PCR can be used to identify whether these transcripts are present within a population of cells, using (trans)gene-specific oligonucleotide primers. The procedure consists of 4 stages:-

i) tissue sampling;

ii) total RNA extraction from rat tissues;

iii) first-strand cDNA synthesis from total RNA, using reverse transcriptase;

iv) PCR amplification of the cDNA template, using (trans)gene-specific oligonucleotide primers.

In order to establish that the transgene is expressed as a functional protein, (confocal) fluorescence microscopy can be used to directly detect transgene (EGFP) expression in fresh tissue sections. The procedure consists of 3 stages:-

i) tissue sampling;

ii) sectioning of tissue;

iii) detection of fluorescence by (confocal) flourescence microscopy.

All determinations of transgene expression were confirmed with duplicate experimental animals/groups.

4.2.5.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

4.2.5.1.1 Tissue sampling

Male and female adult SD rats (2-5 month old), were killed by stunning and decapitation according to UK Home Office Regulations (Schedule 1 humane killing methods).

Various tissues (see 4.2.5.1.1.1 and 4.2.5.1.1.2) were immediately dissected out (~mid-day), placed into a 1.5ml microcentrifuge tube and placed on dry ice for prompt freezing. The tissues were transferred on dry ice, and either placed in a -70°C freezer for temporary storage, or immediately used for total RNA extraction using a GTC-phenol-chloroform extraction method (see 2.3.1.1).

4.2.5.1.1.1 Retinal sampling

Whole eyes were excised from their respective sockets, placed on top of a culture dish (Corning) and bisected with a scalpel. The lens was removed, and the remainder of the eye was placed in a culture dish (Corning) containing saline, and viewed under the stereo dissecting microscope (Nikon; x10-20 magnification). The retina was then detached from the eye using fine curved watchmakers forceps.

4.2.5.1.1.2 Brain sampling

The roof of the rat skull was excised using a pair of sharp dissecting scissors, and the brain was removed using a pair of blunt curved forceps. The rat brain was laid ventral side up, and bisected in the coronal plane at both the rostral and caudal ends of the optic chiasm, using a scalpel (see appendix E). To obtain a sample of the basal hypothalamus containing the SCN, the resultant coronal brain slice was then laid flat and bisected ~1mm above the ventral brain surface. The ventral portion of the brain slice was laid ventral side up, and the central hypothalamic region was isolated with cuts on the outer margin of the optic chiasm on each side of the brain slice. A sample of the cortex was also taken using a scalpel.

4.2.5.1.2 RT-PCR

Total RNA extracted from rat tissues (see 4.2.5.1.1) was treated with DNase I, purified by P/C/IAA extraction, and used to synthesise first-strand cDNA with reverse transcriptase, as described in 2.3.2.

1µl of cDNA, 1.25 units of Taq polymerase (Promega), 400nM (final concentration) of each EGFP-specific primer (forward (F) and reverse (R); (MWG Biotech); see table 4.4 and Figure 4.5) and 200nM (final concentration) of each actin-specific primer (forward and reverse (MWG Biotech); see table 4.5) was used per PCR reaction (see 2.2.2.). Positive and negative controls were also included. PCR was conducted using a pre-heated (95°C) PCR block (PCR Express, ThermoHybaid). Thermal cycling conditions used were: 95°C for 2 minutes; 33 cycles of 95°C for 30 seconds, 61°C for 30 seconds, 72°C for 1 minute; 72°C for 7 minutes. The products of the reactions were analysed by 1% 1x TAE agarose gel electrophoresis, as described in 2.2.3, with an aliquot of an appropriate marker added to the outer lane(s) (100bp ladder, New England Biolabs).

Primer	5' – Sequence – 3'
EGFP-F2	CGG CAT CAA GGT GAA CTT CAA GAT CCG
EGFP-R	CTT GTA CAG CTC GTC CAT GCC G
Actin-F	TCA TGC CAT CCT GCG TCT GGA CCT
Actin-R	CCG GAC TCA TCG TAC TCC TGC TTG

Table 4.4 Oligonucleotide primer sequences used for screening by RT-PCR (see Figure 4.5; EGFP-F2 highlighted in red; EGFP-R highlighted in green). The EGFP primers were designed to amplify a 237bp PCR product from cDNA, and the β-actin primers were designed to amplify a 581bp PCR product from cDNA.

4.2.5.2 (Confocal) Fluorescence microscopy

4.2.5.2.1 Tissue preparation

Male and female SD rats (5-8 week old), were killed by stunning and decapitation according to UK Home Office Regulations (Schedule 1 humane killing methods). The brain was immediately dissected out and placed into a glass beaker of ice-cold artificial cerebral spinal fluid (ACSF) bubbled with oxygen. Immediately before transferring the brain to a vibrating blade microtome (vibratome; see 4.2.5.2.2), a coronal brain slice containing the SCN was obtained by laying the brain ventral side up, and bisecting in the coronal plane at both the rostral and caudal ends of the optic chiasm, using a scalpel (see appendix E).

4.2.5.2.2 Sectioning of tissue

A fresh blade was screwed onto the vibratome (Leica VT1000 S), before attaching the pre-frozen platform to the machine and surrounding it with ice and water. The cold brain slice (4.2.5.2.1) was mounted onto the chuck using 'superglue' (Loctite) and screwed down into the platform, which was then filled with ice-cold ACSF and bubbled with oxygen. Initially, the mounted tissue was approached with thick cuts (feed:thickness set at 300μm; speed 2) by moving the tissue towards the blade, using the start/stop switch of the vibratome. The thickness control was then adjusted to cut thin sections (feed:thickness set at 100-200μm; speed 1), prior to approaching the SCN region (identified using rat brain maps: Paxinos and Watson, 1986; Palkovits and Brownstein, 1988). Free-floating sections were transferred from the platform of the vibratome to a perspex bath filled with ice-cold ACSF (bubbled with oxygen), with a fine paintbrush, ready for detection of fluorescence by confocal microscopy (4.2.5.2.3).

4.2.5.2.3 Detection of fluorescence by (confocal) fluorescence microscopy

Brain sections were transferred from the perspex bath (4.2.5.2.2) to a petri dish (Corning) containing ice-cold ACSF, and held down by a harp (fine nylon wound round a piece of stainless steel). The sections were analysed using a confocal microscope (Leica DMIRBE), where the 488nm band of an argon laser was used to excite EGFP (emission detection at 507nm).

4.2.6 DNA sequencing

To verify that no mutations had occurred within the *mPer1* fragment, the P1PG #4 plasmid was sequenced. All DNA sequencing was performed by the Molecular Biology Support Unit, Cardiff University, using the Applied Biosystems Big Dye Terminator version 3.1 kit. Each sequencing reaction, containing 0.5-1.8μg/μl of the P1PG #4 plasmid and 1.6pmol/μl of each oligonucleotide primer (see Table 4.5), was loaded on an automated sequencer (ABI-PRISM 3100 capillary electrophoresis instrument, Applied Biosystems).

4.2.6.1 Oligonucleotide primer design and synthesis

The majority of oligonucleotide primers were designed by visual inspection using the following criteria (where possible): 50-60% G/C content, 20-30 bases, melting points (T_m) between 60-70°C, and avoidance of runs of identical nucleotides and palindromic sequences. Oligonucleotide primers were generated by MWG Biotech, where a high purity salt free (HPSF) grade was requested.

Primer	5' - Sequence - 3'
mPer1-F1	TAA CCG TAT TAC CGC CAT GC
mPer1-F2	CAG TCC TAC GGT GCT GGA AT
mPer1-F3	TGG CCA GAG AGC AAT CTC CA
mPer1-F4	CAG CAA CCG TGT ACA GTC TG
mPer1-F5	CAG CAG AGC CTG GTT ACT GT
mPer1-F6	CAG CTG ATT ATG TCA GCC GC
mPer1-F7	ACT TCC TGT GGC CCA GGT AT
mPer1-R1	TGG AGA TTG CTC TCT GGC CA
mPer1-R2	ACA GTA ACC AGG CTC TGC TG
mPer1-R3	CAG CGT CAG GAC TGA CAG TA
mPer1-R4	ATA CCT GGG CCA CAG GAA GT
mPer1-R5	CTG AAC TTG TGG CCG TTT AC

Table 4.5 Oligonucleotide primer sequences used for sequencing plasmid (P1PG #4) DNA containing the *mPer1*/d2EGFP transgene (see Figures 4.4 and 4.5).

4.2.6.2 Sequence analysis

Sequence files were analysed as chromatograms using the software package, Chromas (version 1.43). Subsequent alignment of these DNA sequences with the *mPer1* sequence (www.ncbi.nlm.nih.gov/entrez/ accession number AF223952; see Figure 4.4) were performed by a Gene Codes software package, 'Sequencher™'.

4.3 RESULTS

4.3.1 Preparation of transgene DNA for micro-injection

4.3.1.1 Extraction and purification of transgene DNA

1% 1xTAE agarose gel electrophoresis resolution of *Xho*I and *Afl*II restriction fragments of the P1PG #4 plasmid yielded a ~4.1 kb *mPer1*/d2EGFP transgene fragment (see Figure 4.6). The transgene DNA fragment was extracted from the gel using the QIAEX II gel extraction kit (Qiagen), purified through a Sephadex G-50 spin column, adjusted to 2-5ng/µl with MITE buffer, and filter-sterilised, ready for micro-injection (4.2.2.4.2).

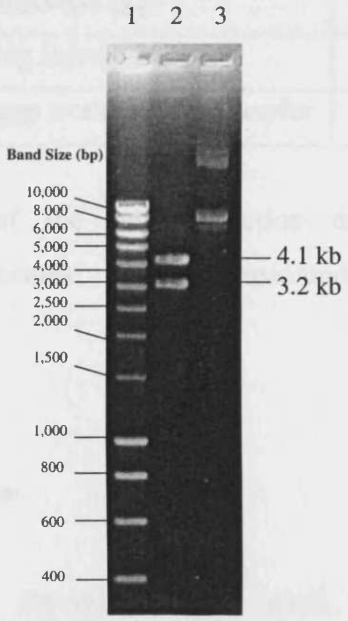


Figure 4.6 Gel electrophoresis analysis of the P1PG #4 plasmid (containing the *mPer1*/d2EGFP transgene). Lane 1 corresponds to the molecular weight marker (Hyperladder I, Bioline). Lane 2 corresponds to the P1PG #4 plasmid DNA digested with restriction enzymes *Xho*I and *Afl*II, yielding a ~4.1 kb band (*mPer1*/d2EGFP transgene fragment), and a ~3.2 kb band (pd2EGFP vector fragment). Lane 3 corresponds to non-digested P1PG #4 plasmid DNA.

4.3.2 Transgenesis

4.3.2.1 Micro-injection data

A total of 115 female rats were used for superovulation (~40 day old; ~130g), yielding 1614 eggs for micro-injection over 13 transgenesis sessions (raw data in appendix D3). 45% of harvested and micro-injected eggs were suitable for oviduct transfer (OVT), with the other eggs either non-injectable (35%; no clear pronucleus) or were lysed during the micro-injection procedure (11.5%; see table 4.6 and appendix D3).

	%
Non-injectable eggs	**35**
Micro-injected eggs	**62.5**
Surviving injected eggs	**51**
Total eggs available for transfer	**45**

Table 4.6 A summary of the micro-injection data accumulated over 13 transgenesis sessions. Data is presented in % values (raw data in appendix D3).

4.3.2.2 (OVT) Transfer data

Following micro-injection, surviving eggs were transferred into mature pseudopregnant female rats (n=22; weight 200-300g; 20-30 eggs per OVT; see table 4.7 and raw data in appendix D4). Following successful transfer, pups were born 22-23 days later (n=26; ~2 pups per litter; see table 4.7 and raw data in appendix D4 and D5). PCR and Southern blot analysis confirmed that 4 of these offspring were transgenic (see 4.3.3.1).

	Day 0 Bilateral OVT	Day 0 Unilateral OVT	Total
Total number of eggs used for OVT	324	349	**673**
Number of OVTs	(2x) 8	14	(8+) **22**
Average number of eggs per OVT	(2x) **20**	**25**	**23**
Number of OVTs with live births	3	6	**9**
Number of live pups	12	14	**26**
Average number of live pups per OVT	(2x) **2**	**2.3**	**2.2**
Number of pups surviving	11	7	**18**
Number of transgenics generated	**3**	**1**	**4**

Table 4.7 A summary of the OVT data accumulated over 13 transgenesis sessions. (Raw data and % values in appendix D4 and D5).

4.3.3 Genotyping of *mPer1*/d2EGFP transgenic rats

4.3.3.1 Screening of progeny for transgene incorporation

PCR and Southern blot analysis (see 4.3.3.1.1 and 4.3.3.1.2, respectively) confirmed that 4 of the offspring contained the exogenous EGFP sequence, thus indicating successful genomic integration of the transgene.

4.3.3.1.1 Genomic screening by PCR analysis

PCR amplification of genomic DNA derived from litters of OVT mothers, using primers specific for the EGFP and actin genes, was used to identify 4 transgenic founder rats, designated Y6, Y7, Y10 and Y16, respectively (Y refers to the *mPer1*/d2EGFP transgene, and the number refers to the pup number). Transgenic

lines were established from the respective founders (Y6, Y7 and Y16), and maintained as described in 4.2.5. No breeding line was established from the Y10 founder, however, due to contraction of an infection and its subsequent killing. All transgenic progeny from founders and transgenic descendants of each transgenic line were confirmed by Southern analysis (see 4.3.3.1.2). Genotyping of Y6 and Y7 lines of *mPer1*/d2EGFP transgenic rats are shown in Figures 4.8, and 4.7, 4.8 and 4.9, respectively.

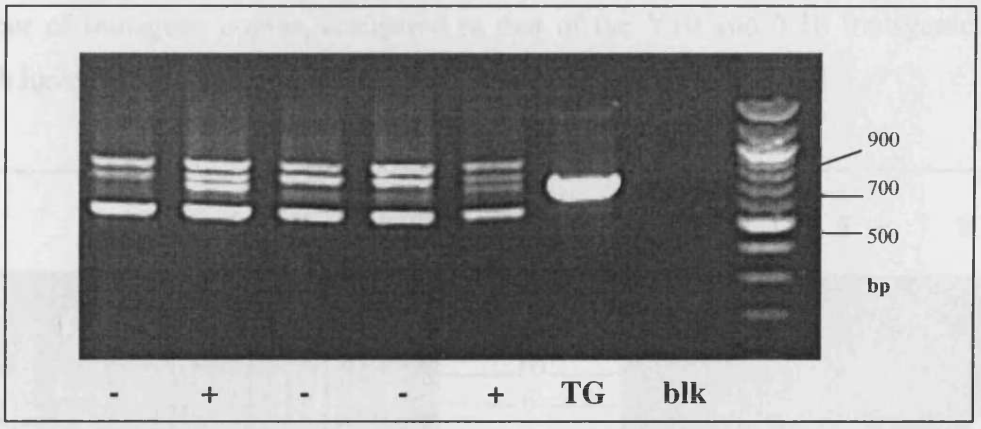


Figure 4.7 PCR amplification (using appropriate primers) of **Y7** transgenic (+) and non-transgenic (-) rat genomic DNA, extracted from tail or ear biopsies of a second generation Y7 litter, and resolved on an agarose gel. An EGFP-specific band (716 bp) is visible in transgenic samples, and an actin-specific band (581 bp) and 2 actin pseudogene bands are visible in all genomic DNA samples. The lanes marked **TG** and **blk** correspond to positive (transgene) and blank control samples, respectively; the right lane represents a 100 bp molecular weight marker (NEB).

4.3.3.1.2 Genomic screening by Southern analysis

Southern blot analysis of genomic DNA extracted from Y6, Y7, Y10 and Y16 founder rats revealed the presence of a major fragment on phosphor screen images (see Figure 4.8; Y10 and Y16 data not shown), which appears to migrate in parallel with the full length *mPer1*/d2EGFP transgene (4.1 kb), and would appear to constitute

a head to tail tandem array of transgenic integration (see appendix G). In addition, a smaller (2 kb) fragment is present in Y7 founder rat genomic DNA (see Figure 4.8), and may represent a tail to tail transgenic integration, and a larger (6 kb) fragment is present in Y6 founder rat genomic DNA (see Figure 4.8), and may represent a head to head transgenic integration. These observations were consistent in subsequent generations of the respective Y lines of transgenic rat (see Figure 4.9**B**, for example), and therefore confirm stable inheritance of the *mPer1*/d2EGFP transgene. Southern blot analysis indicated that the Y6 and Y7 transgenic lines have a relatively high number of transgene copies, compared to that of the Y10 and Y16 transgenic lines which have relatively low copy numbers (data not shown).

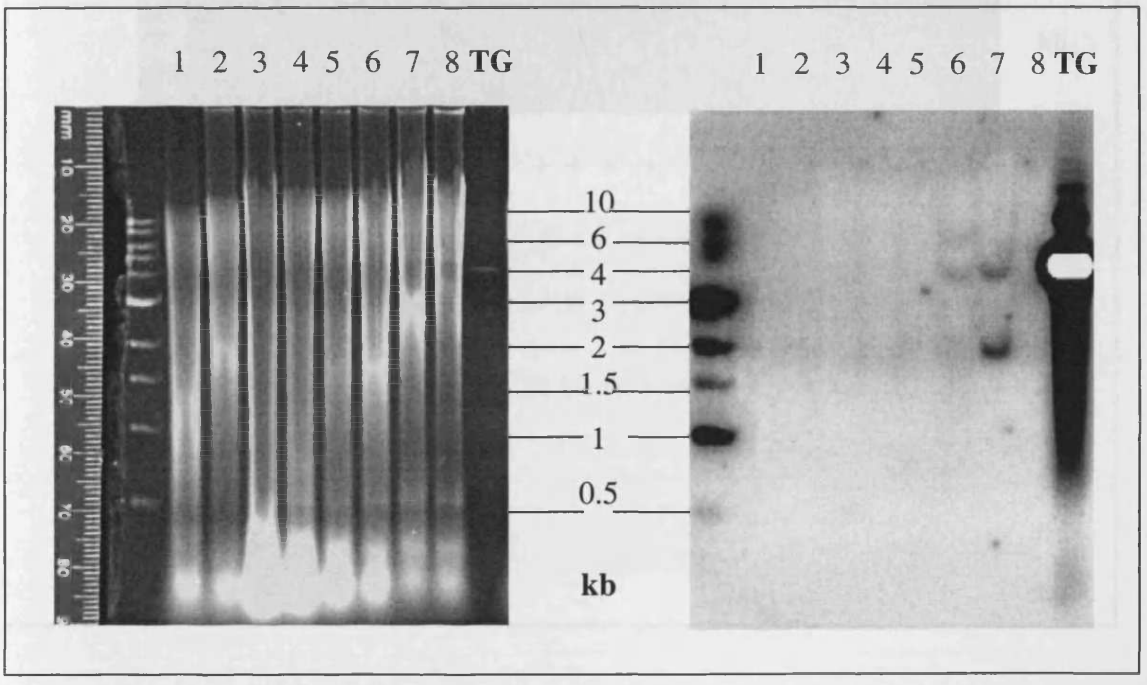


Figure 4.8 UV transilluminator image (left) of *BamH*I-restricted rat genomic DNA (10μg), extracted from tail or ear biopsies of OVT litter pups (numbered accordingly), and resolved on an agarose gel (before transfer to a nylon membrane). Phosphor screen image (right) of the membrane (from left) probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (OVT litter pup numbers 6 and 7; **Y6** and **Y7** transgenic founders, respectively). **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment. The left lane represents a 1 kb DNA ladder molecular weight marker (NEB).

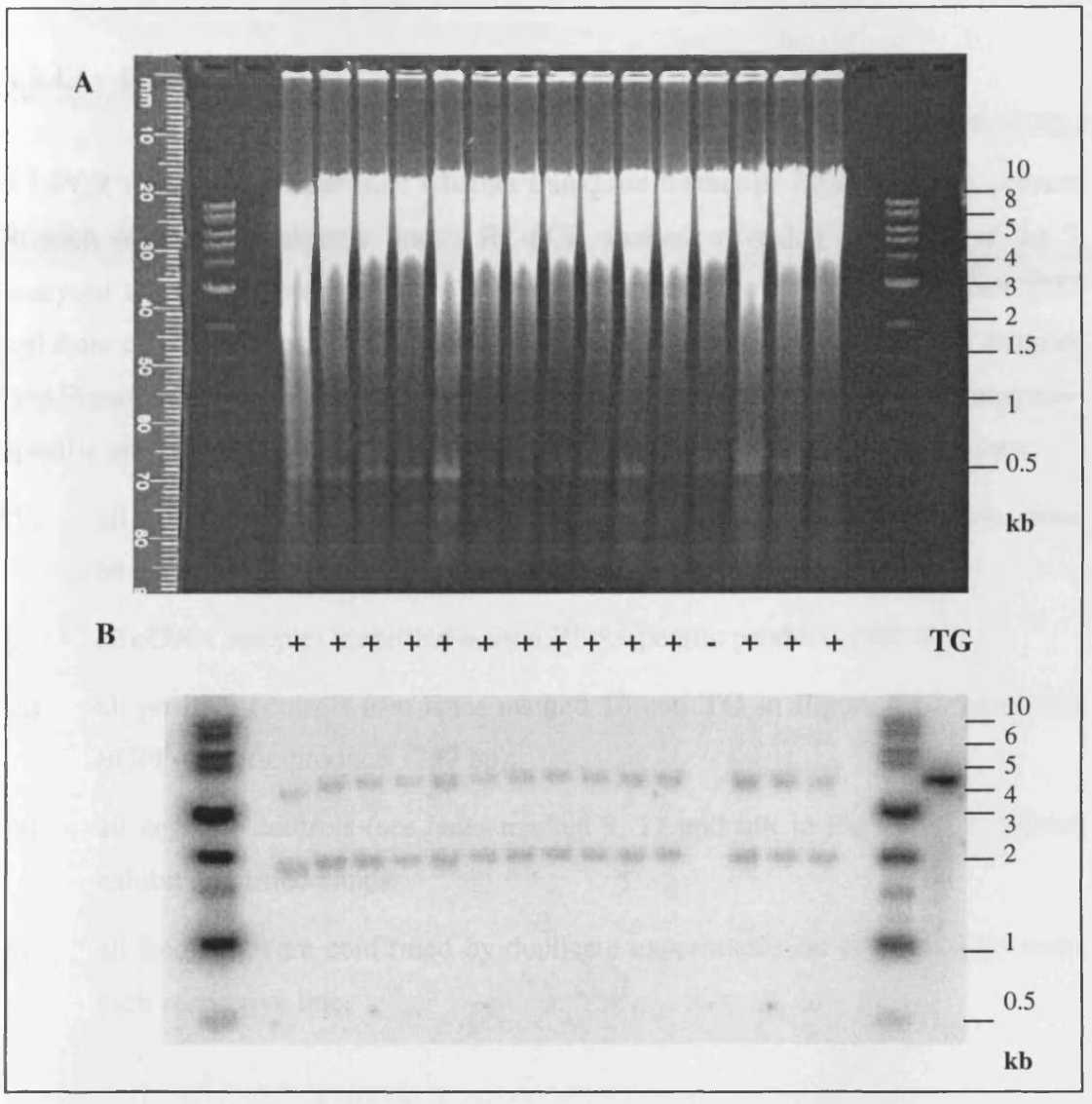


Figure 4.9 **A.** UV transilluminator image of *BamH*I-restricted **Y7** transgenic (+) and non-transgenic (-) rat genomic DNA (10μg), extracted from tail or ear biopsies of a third generation Y7 rat litter, and resolved on an agarose gel (before transfer to a nylon membrane). **B.** Phosphor screen image of the membrane (from **A**) probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples. **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment. The lanes on either side of the genomic DNA samples represent a 1 kb DNA ladder molecular weight marker (NEB).

4.3.4 Transgene expression analysis

4.3.4.1 RT-PCR

RT-PCR was used to determine whether transgene transcript expression was present in each of the Y transgenic lines. RT-PCR analysis revealed that none of the 3 analysed transgenic lines generated a transgene specific amplification product (237 bp) from cDNA synthesised from total RNA derived from the various tissues samples (see Figure 4.10 for Y6 and Y16 samples; Y7 data not shown). The lack of transgene-specific amplification products were considered genuine for the following reasons:

i) all cDNAs were free from significant levels of genomic contamination, since all non-RT controls generated no amplification products;

ii) all cDNA samples amplified actin mRNA-specific products (581 bp);

iii) all positive controls (see lanes marked 10 and **TG** in Figure 4.10) amplified EGFP-specific products (237 bp);

iv) all negative controls (see lanes marked 9, 11 and **blk** in Figure 4.10) did not exhibit amplified bands;

v) all findings were confirmed by duplicate experiments on other animals from each respective line.

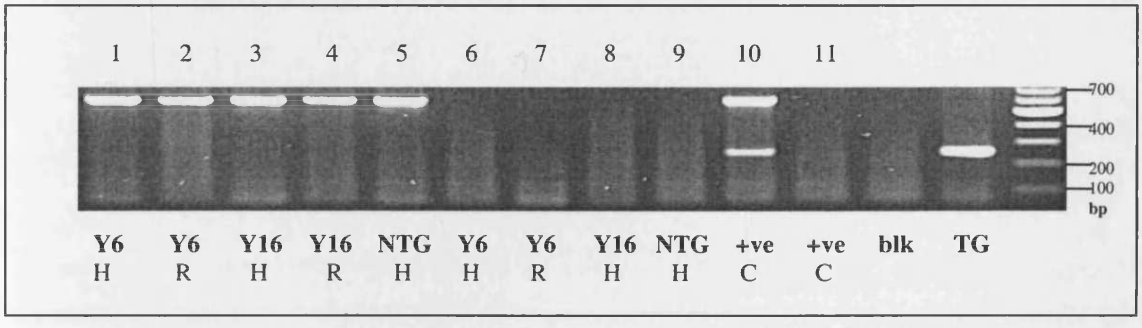


Figure 4.10 PCR amplification (using specific primers) of rat cDNA template (lanes 1-5 and 10), and non-RT controls (lanes 6-9 and 11), derived from transgenic (**Y6** and **Y16**) and non-transgenic (**NTG**) samples of third generation litters. The

lanes marked **+ve** correspond to samples derived from a different transgenic rat model (positive control for RT-PCR and EGFP; see 6.3.3.1). H, R and C represent hypothalamic, retinal and cortical samples, respectively. The lanes marked **blk** and **TG** correspond to blank and positive (transgene) RT-PCR and EGFP control samples, respectively; the right lane represents a 100 bp DNA ladder molecular weight marker (NEB). EGFP-specific bands (237 bp) are visible in the **+ve** cDNA and **TG** samples. Actin-specific bands (581 bp) are visible in all cDNA samples.

4.3.4.1 Detection of fluorescence by (confocal) fluorescence microscopy

Fluorescence microscopy was used to determine whether potential transgene transcripts were translated into mature functional proteins. In each of the Y transgenic lines, no fluorescence was detected by (confocal) fluorescence microscopy, thus revealing that none of the 3 transgenic lines (Y6, Y7 and Y16) expressed the transgene as a functional protein, at least at a level that was readily detectable.

4.3.5 DNA sequencing

Thorough sequencing of the P1PG #4 plasmid containing the transgene was performed to confirm that no mutations had occurred within the *mPer1* promoter fragment of the transgene. Chromas (version 1.43) was used to analyse DNA sequence files (obtained from the Molecular Biology Support Unit (see 4.2.6)), in the form of chromatograms, an example of which is shown in Figure 4.11. All chromatogram files (labelled with the respective oligonucleotide primer used for the sequencing reaction) are located in Appendix D1.

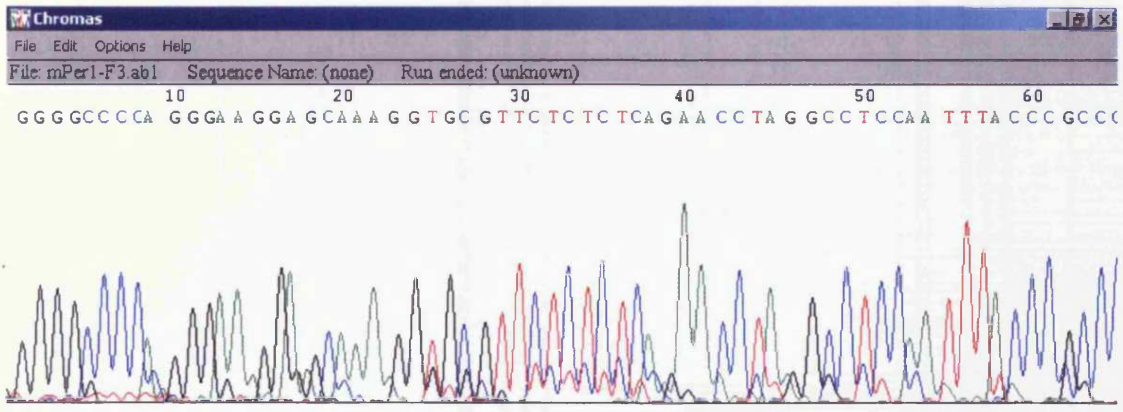


Figure 4.11 An example of a chromatogram file, acquired from the Chromas DNA analysis software package.

4.3.5.1 Sequence analysis

Sequencher™ DNA analysis software package was used to align Chromas sequence files (4.3.5; see appendix D1) with the *Xho*I-*Bgl*I *mPer1* promoter sequence (nucleotides 2580-5562, accession number AF223952; see Figure 4.4), as demonstrated by Figure 4.12.

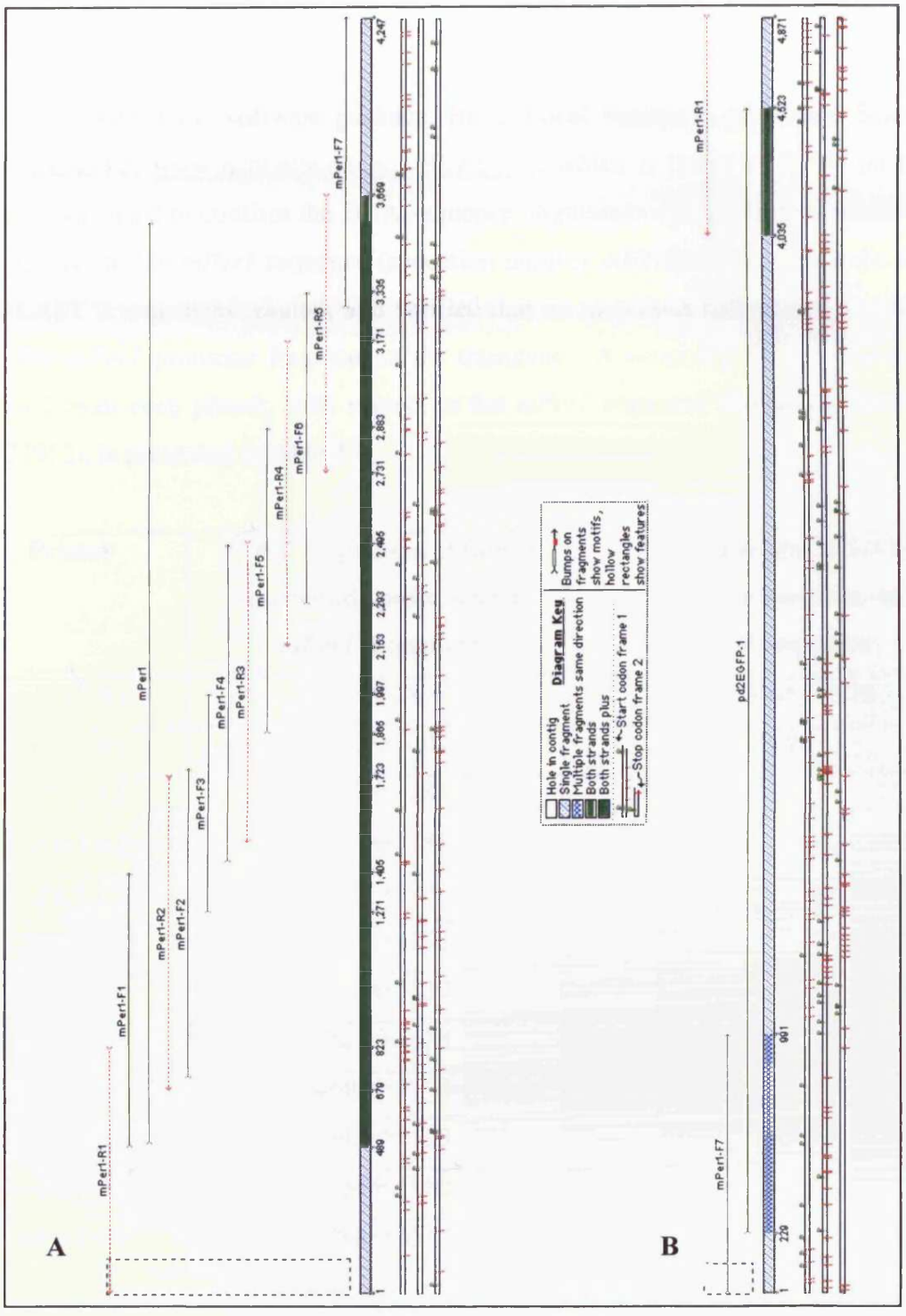


Figure 4.12 The display above represents the alignment of DNA sequences (obtained from sequencing reactions with the respective primers), with **A**, the *Xho*I-*Bgl*I *mPer1* promoter sequence (nucleotides 2580-5562, accession number AF223952; see Figure 4.4), and **B**, the pd2EGFP-1 vector sequence (Clontech; see Figure 4.5), acquired from the Sequencher™ DNA analysis software package.

Another bioinformatic software package, Basic Local Sequence Alignment Search Tool (BLAST2; www.ncbi.nlm.nih.gov/BLAST/), which is freely available on the internet, was used to confirm the DNA sequence alignments and nucleotide positions with respect to the *mPer1* sequence (accession number AF223952; see appendix D2 for BLAST 2 sequences results), and verified that no mutations had occurred within the ~3kb *mPer1* promoter fragment of the transgene. A summary of the sequences obtained from each primer, with respect to the *mPer1* sequence (accession number AF223952), is presented in table 4.8.

Primer	**5'→3' Sequences obtained (nucleotide positions on *mPer1* sequence)**	**5'→3' Sequences obtained (nucleotide positions on d2EGFP sequence)**
mPer1-R1	2578←2890	3991←4280; 1←36
mPer1-F1	2581→3402	47→529
mPer1-F2	2802→3281	
mPer1-R2	3214←3781	
mPer1-F3	3347→3933	
mPer1-F4	3511→4250	
mPer1-R3	3782←4539	
mPer1-F5	3924→4678	
mPer1-R4	4440←5172	
mPer1-F6	4345→5123	
mPer1-R5	5134←5560	
mPer1-F7	5288→5558	

Table 4.8 A summary of the DNA sequences obtained from the respective oligonucleotide primer used for each sequencing reaction, with respect to the *mPer1* sequence (accession number AF223952; see Figure 4.4 and appendix D1 and D2), and the pd2EGFP-1 vector sequence (Clontech; see Figure 4.5 and appendix C and D1 and D2).

4.4 DISCUSSION

The transgenesis protocol used in the present study was successful in generating 4 transgenic lines, each demonstrating integration of the *mPer1*/d2EGFP transgene.

4.4.1 Optimisation of transgenesis

Increasing the efficiency of transgenesis procedures is paramount, since each transgenesis session is expensive and time-consuming. To optimise the overall efficiency of transgenesis, various practices were adhered to in each transgenesis session, for example, keeping detailed and accurate records of all aspects of the sessions, including superovulation, transgene DNA preparation and concentration, and micro-injection and surgical records.

The majority of rat transgenesis publications fail to specify the technical variables used (for example data selection and technical experience), and/or the efficiencies obtained for the procedures involved, and therefore restrict the extent to which transgenesis efficiencies may be compared between different laboratories. In comparison with published data, the egg survival rate following micro-injection (82%; see appendix D3) was over twice as efficient in the present study (compared to 36.5-39.4%, Hochi *et al.* 1990, Swanson *et al.* 1992, Charreau *et al.* 1996b). Numerous factors may have contributed to the marked increase in egg survival, for example, the small batches of eggs used for micro-injection (~30 eggs), and the shape of the micro-injection needles used. The latter was paramount since poor quality needles critically reduce egg viability, thus only needles with long narrow shafts and fine tips, which would allow injection of eggs at an optimal pressure of 7-15psi, were used for micro-injection. Any needles requiring pressures outside this range were immediately discarded as they tended to rupture either the nuclear- or plasma-membrane. Fresh aliquots of transgene preparations (microcentrifuged at 14,000rpm

for 3 minutes before use) were also used each session, thus reducing the possibility of micro-injection needles becoming blocked (as well as decreasing potential transgene DNA degradation). In addition, the size and concentration of the transgene may influence the integration efficiency of transgenic DNA into the host genome, since the absolute number of transgene DNA molecules micro-injected in a set volume will be greater for a smaller transgene. Brinster *et al.*, (1985) demonstrated that micro-injection of transgene concentrations between 3-5 ng/μl, into the mouse genome, were optimal for egg survival, and concentrations greater than 10 ng/μl reduced egg viability. This was taken into account in the present study, and therefore transgene DNA concentrations between 2-5 ng/μl were used.

In the present study, all OVTs were performed on the same day (day 0) as micro-injection of the fertilised one-cell eggs. Consequently, there was no data to statistically compare efficiencies between day 0 and day 1 OVTs, respectively. It can be argued, however, that day 0 unilateral OVTs are more efficient since the procedure limits the amount of time the eggs are exposed to conditions outside of their natural environment, and consequently allows the eggs to continue to develop naturally (i.e. to the 2-cell stage) *in vivo* within 1-5 hours subsequent to micro-injection. (Eggs used for day 1 OVTs typically only progress to the 2-cell stage in M16 media overnight, this may have detrimental effects on the developing embryo since any further progress is blocked under cell culture conditions, unlike mouse embryos; the cause for this remains unknown). In addition, day 0 OVTs allow the subsequent developing embryos and surrogate (pseudopregnant recipient) mothers to be at complementary stages of development. It can also be argued that unilateral OVTs are more efficient than bilateral OVTs, since less invasive surgery is performed, the amount of time the pseudopregnant recipient mothers are subjected to anaesthesia is restricted, and therefore enables improved post-surgical recovery and entry into a normal gestation period. Again, there was insufficient data to statistically compare efficiencies between unilateral and bilateral OVTs, respectively.

In summary, transgenesis was optimised in the rat, and subsequently resulted in the successful generation of 4 transgenic lines (which, over 13 sessions, gives an overall trangenesis efficiency of 31%; see appendix D5).

4.4.2 Conclusions

The *mPer1*/d2EGFP transgene was successfully integrated into the rat genome, as shown by PCR and Southern analysis (see 4.3.3) of genomic DNA derived from founder (or descendant) transgenic rats. The 3 transgenic lines studied (Y6, Y7 and Y16), however, were unable to express reporter gene (EGFP) transcripts, and also therefore protein, as shown by RT-PCR analysis and fluorescence microscopy, respectively (see 4.3.4). Thus, the sequences required for conferring appropriate *mPer1* expression in the rat could not be defined by the present transgenic rat model.

The reason for the lack of transgene expression in the respective lines is unknown, but it appears that some aspect of gene expression had failed. Given that the respective lines showed no distinct phenotype, it can be assumed that the integration event had not disrupted the genome, but may have integrated into a transcriptionally silent region of the chromosome (an example of a position effect, see 3.4.2). Another possibility is that the transgene could have been silenced by DNA methylation or post-injection disruption of the transgene. Thorough sequencing of the P1PG #4 plasmid containing the transgene also confirmed that no mutations had occurred within the *mPer1* promoter fragment of the transgene (see 4.3.5). Furthermore, concomitant experiments (see 6.3.3) verified that the d2EGFP fragment of the transgene was intact.

There is no evidence to suggest that the *mPer1* promoter fragment, in the *mPer1*/d2EGFP transgene used in the present study, would not function in a similar fashion in the rat as in the mouse, since it contains all the characteristics of a basic mammalian promoter, and there are many examples of promoters from one species successfully driving transgene expression in another (host) species (reviewed in Wells and Carter, 2001). Yamazaki *et al.* (2000) generated an appropriate example of this, whereby 6.75 kb of the *mPer1* promoter, 5' sequence and the *luc* reporter gene were incorporated into rat hosts (see Figure 4.2**c**). This particular transgenic rat model demonstrated that the 6.75 kb *mPer1* promoter fragment was sufficient to confer appropriate transgene expression (i.e. both circadian and photic regulation *in vivo*).

4.4.3 Future Directions

The sequences of the 3 kb *mPer1* promoter fragment used in the *mPer1*/d2EGFP transgene of the present study have already demonstrated appropriate expression in murine hosts (Kuhlman *et al.*, 2003; Kriegsfeld *et al.*, 2003; LeSauter *et al.*, 2003; Witkovsky *et al.*, 2003; see 4.1.4). It can be speculated that these sequences lack the elements required for conferring appropriate *mPer1* expression in rat hosts. Therefore, additional sequences (up to 6.75 kb of the *mPer1* promoter, as established by Yamazaki *et al.*, 2000) may be required in future studies.

Since there were no distinct phenotypic abnormalities (such as in physical appearance and overt behaviour) observed in the respective *mPer1*/d2EGFP transgenic lines (Y6, Y7 and Y16), and there was no evidence of any transgene expression, additional experiments were neither required nor justified. Therefore, further study of these transgenic animals was ceased, and all colonies were subsequently culled.

CHAPTER 5

A TRANSGENIC RAT MODEL FOR STUDYING THE REGULATION OF EGR-1 GENE EXPRESSION I

5.1 OVERVIEW AND AIMS OF STUDY

The aim of the present study was to investigate *egr-1*/d4EGFP transgene expression and its physiological regulation in the rat brain and anterior pituitary gland.

5.1.1 Egr-1

Early growth response gene-1 (*egr-1*; also termed *NGFI-A*, *zif268*, *krox-24*) encodes a zinc-finger transcription factor (Egr-1); it was originally isolated in molecular screens for growth factor- inducible genes (Milbrandt, 1987; Gashler and Sukhatme, 1995). Egr-1 is one member of a transcription factor family that includes three related factors (Egr-2,3 and 4; O'Donovan *et al.*, 1999), each of which exhibits a characteristic CyS_2-His_2 zinc-finger DNA binding domain, and interact with a common response element (consensus: GCGGGGGCG) to regulate the transcription of target genes (Gashler and Sukhatme, 1995; O'Donovan *et al.*, 1999).

5.1.2 The functional roles of Egr-1

Extensive analysis of the roles of the transcription factor Egr-1 has revealed important, and unique, roles in fertility (Lee *et al.*, 1996; Topilko *et al.*, 1998), memory (Jones *et al.*, 2001), and the ischaemic stress response (Yan *et al.*, 2000b). In particular, null mutation studies in mice have established that fertility is Egr-1-dependent since Egr-1 acts as a transcriptional regulator of the pituitary hormone gene that codes for the LH β-subunit, and thus maintains appropriate expression of the LH

β-subunit (Lee *et al.*, 1996; Topilko *et al.*, 1998; see also Dorn *et al.*, 1999; Tremblay and Drouin, 1999; Wolfe and Call, 1999; Sevetson *et al.*, 2000; Duan *et al.*, 2002). Recent studies have shown that Egr-1 may also affect fertility through regulation of LH receptor gene expression (Topilko *et al.*, 1998; Yoshino *et al.*, 2002).

The functional association between Egr-1 and the reproductive axis has been explored mainly at the developmental level using null-mutant mice (Lee *et al.*, 1996; Topilko *et al.*, 1998), but recent studies have provided evidence of an additional association, at a physiological level. It has been described that cyclical changes in anterior pituitary gland Egr-1 activity correlate with cyclical changes in the reproductive axis in adult female rats (Slade and Carter, 2000). Another study (Knight *et al.*, 2000) has shown that the nuclear 75kDa form of Egr-1 protein is primarily co-localised with LH β-subunit expressing cells in the adult rat anterior pituitary gland. Taken together, these findings indicate that Egr-1 might play a role in mediating changes in gonadotroph function during the rat oestrous cycle, possibly contributing to the cyclical changes in LH release (Butcher *et al.*, 1974; Fink, 1979). This potential role is obscure, however, because gonadotroph function is regulated at multiple levels, and many different molecular factors have now been shown to be associated with this axis (see Brown and McNeilly, 1999).

Currently, there is no direct evidence to link physiological changes in Egr-1 expression (Slade and Carter, 2000) with changes in gonadotroph gene (LH β-subunit, or otherwise) expression because the null mutant genetic models (Lee *et al.*, 1996; Topilko *et al.*, 1998) lack Egr-1 prior to the establishment of reproductive cycles, and adult-onset genetic models have yet to be developed. Consequently, further studies are required to address the physiological role of Egr-1.

In addition to the essential physiological role of Egr-1 in maintaining fertility, this factor is also implicated in reprogramming gene expression in various pathological states including atherosclerosis (McCaffrey *et al.*, 2000), hypoxia (Yan *et al.*, 1999b) and ischaemia (Yan *et al.*, 2000).

5.1.3 Induction of the *egr-1* gene

The expression of *egr-1* is rapidly and transiently induced in response to a variety of stimuli (see Beckmann and Wilce, 1997). For example, early studies demonstrated that *egr-1* expression is activated in various cell types by growth and differentiation stimuli such as nerve growth factor (NGF; Gashler and Sukhatme, 1995; Milbrandt, 1987), serum growth factors (Christy *et al.*, 1988), and mitogens (Lau and Nathans, 1987; Sukhatme *et al.*, 1987, 1988). Other studies have shown that electrically- and drug-induced seizure activity also induces *egr-1* expression in the rat hippocampus (Saffen *et al.*, 1988; Cole *et al.*, 1990; Mack *et al.*, 1990; Wisden *et al.*, 1990; O'Donovan *et al.*, 1998; see Figure 5.1).

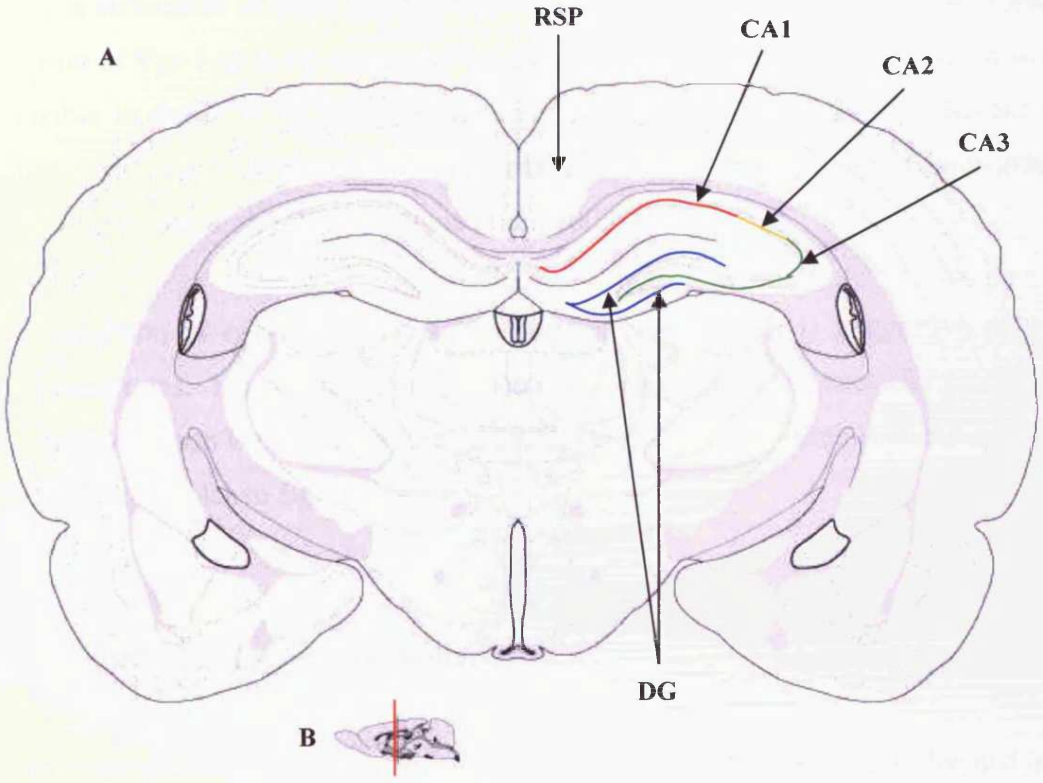


Figure 5.1 Schematic representation of *egr-1* induction in the brain (following seizure activity; Saffen *et al.*, 1988), in different regions of the hippocampus (unilaterally shown here in coronal section (**A**)). Coloured lines (where appropriate) and arrows indicate the retrosplenial (RSP) cortex, dentate gyrus (DG; blue), and cornu ammonis 1, 2 and 3 regions (CA1, 2, and 3; red, orange and green, respectively). The position of the coronal section in the rat brain is indicated (red line) in the parasagittal view (**B**). Modified from Swanson (1998/1999).

Extensive studies have shown that exposure to light during the subjective night (but not during the subjective day) induces *egr-1* expression in the rat SCN (Rusak *et al.*, 1990, 1992; Sutin and Kilduff, 1992; Tanaka *et al.*, 1997; Guido *et al.*, 1999; see Figure 3.2 for schematic representation of SCN), and therefore suggests that transcriptional activation of *egr-1* may be an essential event linking light and circadian entrainment in the SCN. However, initial studies of the SCN in *egr-1* knock-out mice did not detect any abnormalities in entrainment or phase shifting (Kilduff *et al.*, 1998), thus, failed to determine whether Egr-1 plays a role in the signalling and transcriptional events that underlie light entrainment of the clock since Egr-1 is redundant in these mice. It has been proposed that the absence of apparent function of Egr-1 in these mice may be due to compensation by Egr-3, which is also inducible in the SCN by photic stimulation (O'Donovan *et al.*, 1999). Recent data indicates that the MAPK pathway functions as an upstream regulator of light-induced *egr-1* expression in the SCN (Dziema *et al.*, 2003).

The induction of *egr-1* in response to these various stimuli is suggestive of many functional roles. However, the concomitant induction of other genes (for example, c-*fos* and *jun*-B; see Herdegen and Leah, 1998) means that it is often difficult to ascribe further unique roles to Egr-1.

5.1.4 Studies of the *egr-1* promoter

The transcription of a gene is regulated by *cis*-acting elements generally located in the 5' upstream promoter region, or alternatively, within introns. Early studies of the *egr-1* promoter established that the proximal 5' flanking region contains several different consensus response elements and the region from -532 to +100 relative to the transcription start site is sufficient for conferring NGF responsiveness (Changelian *et al.*, 1989). More recent studies have focused on the role of multiple serum response elements (SREs) within the 5' flanking region in mediating transcriptional responses to a variety of stimuli including NGF (DeFranco *et al.*, 1993), depolarization and glucose (Bernal-Mizrachi *et al.*, 2000), and calmodulin antagonists (Shin *et al.*, 2001).

These *in vitro* studies have begun to elucidate the role of specific *egr-1* promoter elements in considerable detail, but a complete functional description of *egr-1* regulatory sequences will require an *in vivo* (transgenic) analysis in which transgene constructs are confronted by the full complement of cell-specific and physiologically regulated trans-acting factors.

5.1.5 The *egr-1*/d4EGFP transgenic rat model

The *egr-1*/d4EGFP transgenic rat model was generated by our laboratory (see Slade *et al.*, 2002 and Figure 5.2) using similar regulatory sequences as in the original *egr-1* promoter analysis (Changelian *et al.*, 1989). In addition to 1.58 kb of 5'-flanking *egr-1* sequences, the rat *egr-1* intron (0.9 kb) was also included within the transgene construct because it is possible that intronic sequences may be important in conferring appropriate *in vivo* expression, as shown, for example, in the rat arylalkamine N-acetyltransferase (AA-NAT) gene (Burke *et al.*, 1999). In order to visualize the dynamics of *egr-1* induction in the brain, a destabilised and enhanced version of green fluorescent protein (1.1 kb of d4EGFP-N1; Clontech) was used as the reporter gene in this transgene construct.

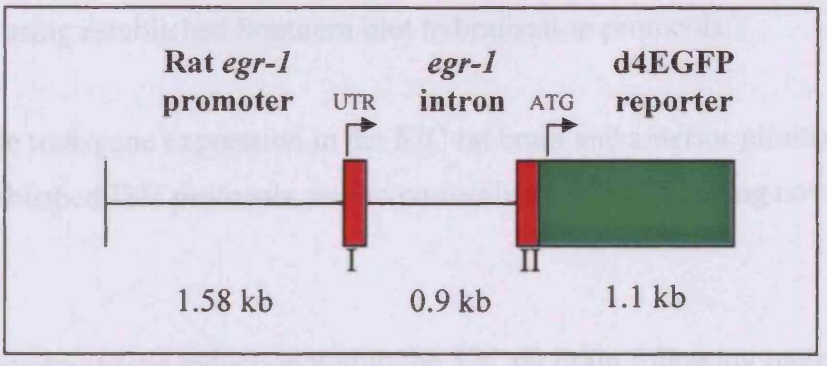


Figure 5.2 Schematic representation of the *egr-1*/d4EGFP transgene (not to scale). Red boxes represent the *egr-1* exons. The construct contains 1.58 kb of rat *egr-1* 5' flanking sequence, and 160 bp of untranslated (UTR) exon 1 sequence (red box I) linked via the full-length rat *egr-1* intron to 194 bp of exon 2 sequence (red box II), prior to the EGFP coding sequence. Left arrow indicates the transcription initiation site. Right arrow indicates the translation initiation site (ATG).

The present study involves a line of hemizygous *egr-1*/d4EGFP transgenic rats (designated 57C), and investigates transgene expression in the brain and anterior pituitary gland, with the primary aim of defining the extent to which the transgene recapitulates expression of the endogenous *egr-1* gene. The particular functional interest of the present study is in the induction, and role, of Egr-1 within the brain and neuroendocrine system (Carter, 1996; Slade and Carter, 2000; Knight *et al.*, 2000; Slade *et al.*, 2001), where Egr-1 is implicated in neuronal plasticity. The physiological factors that determine both endogenous *egr-1* and transgene expression in the 57C transgenic anterior pituitary gland are also investigated.

The rat has been used as an experimental model because this species has been used in previous studies of *egr-1* induction in the brain (Tanaka *et al.*, 1999; Slade *et al.*, 2001).

5.1.6 Experimental aims

The aims of the present study are:

1. To confirm stable inheritance of the *egr-1*/d4EGFP transgene in third generation 57C rats, using established Southern blot hybridisation protocols.

2. To localise transgene expression in the 57C rat brain and anterior pituitary gland, using established ISH protocols, and to co-localise expression using novel protocols.

3. To compare transgene induction within the 57C rat brain following metrazole treatment, and nocturnal photic stimulation.

4. To investigate the physiological factors that determine both endogenous *egr-1* and transgene expression in the 57C rat anterior pituitary gland.

5. To define the sequences required for conferring physiologically-regulated *egr-1* gene expression in the rat brain and anterior pituitary gland.

5.2 METHODS

The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

5.2.1 Animal procedures

All animal procedures were conducted according to UK Home Office regulations (Schedule 1 humane killing methods), and local ethical review. SD rats were maintained in approved laboratory conditions on a 14 hour light - 10 hour dark cycle, with food and water available *ad libitum*. Unless otherwise stated, all determinations of gene expression were confirmed with duplicate experimental animals/groups.

5.2.2 Genomic screening by PCR amplification and Southern analysis

Genomic DNA from third generation rats of the 57C line (Slade *et al.*, 2002) was extracted as described in 4.2.3.1.1 and 2.2.1. Transgenic rats were identified by PCR amplification with the appropriate specific primers (see 4.2.3.4.1.1.1), and confirmed by Southern analysis (see 4.2.3.1.1.2) of *Bgl*II-cut genomic DNA using an EGFP-sequence-specific probe (774bp *Hind*III fragment (nucleotide positions 623-1397) of pd4EGFP-N1 (Clontech - see appendix C)).

5.2.3 Transgene expression analysis

5.2.3.1 Fresh frozen sampling of 57C rat brains (following experimental stimuli)

For transgene expression analysis, animals (6-8 week old) were killed by decapitation at appropriate times relative to experimental stimuli: 1 hour after an injection of either pentylenetetrazole (metrazole; 50mg/kg, i.p.; Sigma,-Aldrich, Poole, Dorset, UK) or

sterile water, or 1 hour after exposure to light at night (24:00-01:00 hour). Brains were rapidly dissected and rinsed in cold saline before snap-freezing in isopentane cooled with dry ice pellets, prior to *in situ* hybridization analysis (ISH; see 3.2).

5.2.3.1.1 Transgene expression analysis in the 57C rat brain by ISH

ISH analysis of transcript expression was performed essentially as described (see 2.3.4). Frozen control and experimentally-stimulated 57C brains (5.2.3.1) were mounted in embedding medium (Bright Cryo-M-Bed), with the third ventricle positioned vertically, and the ventral surface of the brain facing down, as described in 2.3.4.1.2. 12 μm sections of the hippocampal region (for metrazole-treated brains and the respective control brains), and of the SCN region (for nocturnal light-stimulated brains and the respective control brains) were collected (identified using brain maps:- Paxinos and Watson, 1986; Palkovits and Brownstein, 1988), dried and fixed as described in 2.3.4.1.2 and 2.3.4.3.1, and hybridised with the appropriate probe.

In the present EGFP riboprobe study, the plasmid, pd4EGFP-GEM11Z, is a 426 bp fragment of pd4EGFP (Clontech) corresponding to nucleotides 971-1396, cloned into pGEM-11Z (Promega; vector map in appendix C), orientation T7...5'-3'...SP6. For the anti-sense probe, the plasmid was linearised with *Sal*I (and transcribed with SP6 RNA polymerase), and for the sense probe, the plasmid was linearised with *Hind*III (and transcribed with T7 RNA polymerase). In the present *egr-1* riboprobe study, the plasmid, p726*Egr-1*, is a 726bp fragment of NGFI-A cDNA (Milbrandt, 1987) corresponding to nucleotides 228-953, cloned into pBS-KS (Stratagene; vector map in appendix C), orientation T7...5'-3'...T3. For the anti-sense probe, the plasmid was linearised with *Hind*III (and transcribed with T7 RNA polymerase), and for the sense probe, the plasmid was linearised with *Xba*I (and transcribed with T3 RNA polymerase). Following purification of the linearised plasmid templates (see 2.3.4.2.2.2), ^{35}S-labelled RNA probes were generated using the Riboprobe™ *In vitro* Transcription System (Promega) as described in 2.3.4.2.2.3 and 3.2.2.2.

Probe hybridisation ($1x10^7$ cpm/ml) was performed overnight at 56°C (see 2.3.4.3.3), prior to washing (see 2.3.4.3.4.2) and exposure to a storage phosphor screen (Kodak – K; see 2.3.4.3.5.2) for 3 days, and subsequent coating in photographic emulsion (LM-1, Amersham Pharmacia Biotech; see 2.3.4.3.5.3). After 3 weeks, coated slides were developed, counter-stained (Mayer's haematoxylin; see 2.3.4.3.5.3.1), and viewed with a Leica DM-RD microscope. Images were captured as described (2.3.4.3.5.3.2).

5.2.3.1.2 Transgene expression analysis in the 57C rat brain (following experimental stimuli) by dual ISH (using ^{35}S- and digoxigenin-labelled riboprobes) : co-localisation of *egr-1* and transgene transcripts

ISH analysis of *egr-1* and transgene transcript expression was performed essentially as described (see 2.3.4). Control and experimentally-stimulated 57C brains (5.2.3.1) were collected as described in 5.2.3.1.1, and hybridised with the appropriate probe.

Riboprobes (see 3.2.2.2) were prepared from *egr-1* and EGFP plasmid templates as described in 5.2.3.1.1. ^{35}S-labelled RNA probes were generated from the linearised EGFP plasmid templates using the Riboprobe™ *In vitro* Transcription System (Promega), as described in 2.3.4.2.2. Digoxigenin-labelled RNA probes were generated from the linearised *egr-1* plasmid templates using the RNA labelling kit (Roche; see below and appendix B), which labels RNA with DIG-11-UTP during *in vitro* transcription (5.2.3.1.2.1).

5.2.3.1.2.1 Digoxigenin labelling system

20µl reactions were set up on ice for each of the *egr-1* probes (anti-sense and sense), using components from the Digoxigenin RNA labelling kit (Roche), and comprised:

DEPC-treated water, 1x (final concentration) transcription buffer, 10mM DTT (final concentration), 1-2 units (final concentration) of RNase Inhibitor, 1x (final concentration) RNA labelling mix, 1μg linearised template DNA and 1 unit (final concentration) of either T7 or T3, as appropriate. The reagents were mixed gently, centrifuged briefly, and incubated at 37°C for 2 hours.

To remove the template DNA, each probe reaction mixture was treated with: 1μg (final concentration) tRNA (Sigma), 1 unit (final concentration) RNase-free DNase 1 (Promega) and 1-2 units (final concentration) RNase inhibitor (Promega). The reagents were mixed gently, incubated at 65°C for 5 minutes, and quenched on ice for 2 minutes. The probes were then purified by P/C/IAA extraction (5.2.3.1.2.1.1).

5.2.3.1.2.1.1 Digoxigenin riboprobe purification

For each Digoxigenin-labelled riboprobe sample (5.2.3.1.2.1), 280μl TE and 300μl P/C/IAA for 5 minutes at RT. The resultant upper aqueous phase was transferred to a fresh 1.5ml microfuge tube containing 300μl chloroform/isoamylalcohol (C/IAA; 49:1), and tubes were vortexed briefly, and centrifuged at 13,500rpm for 1 minute at RT.

The resultant upper aqueous phase was again transferred to a fresh 1.5ml microfuge tube, and 0.1 volume 3M NaAc (pH 5.8) and 2.5 volumes 100% ethanol were added. Tubes were inverted several times, incubated at -70 °C for 30 minutes to allow the RNA pellet to precipitate, and centrifuged at 14,000rpm for 20 minutes at 4°C. The supernatant was carefully removed by pipette, and the pellet was then washed in 50μl (pre-chilled) 70% ethanol. The tubes were centrifuged at 14,000rpm for 5 minutes at 4°C, and the resultant supernatant pipetted off. The RNA pellet was briefly air-dried and resuspended in 50μl sterile DEPC-treated water.

5.2.3.1.2.2 Pre-treatment – fixation of tissue sections

Tissue sections (see 5.2.3.1.1) were directly immersed into DEPC-treated plastic jars containing PAF, and incubated at room temperature for 5 minutes. The PAF was poured off, and the slides rinsed by filling the jars with DEPC-treated PBS (~35 ml), twice, for 5 minutes each. The PBS was then replaced with a fresh mix of DEPC-treated 0.25% acetic anhydride / 0.1M triethanolamine for 10 minutes.

To dehydrate the sections, the slides were transferred through the following ascending concentrations of fresh ethanol (used to reduce osmotic shock that may loosen thick sections):- 70% and 80% ethanol, for 1 minute each; 95% ethanol, for 2 minutes, and 100% ethanol for 1 minute. To delipidate the sections, the slides were incubated in chloroform for 5 minutes. The sections were then transferred through 100% and 95% ethanol for 1 minute each. The ethanol was poured off, and sections were allowed to air-dry at RT.

5.2.3.1.2.3 Hybridisation of Digoxigenin-labelled *egr-1* riboprobes and ^{35}S-labelled EGFP riboprobes to tissue

The hybridisation solution comprised: 50μl Digoxigenin-labelled *egr-1* riboprobe (approximately 1 μg; see 5.2.3.1.2.1 and 5.2.3.1.2.1.1), 1x10^7 cpm/ml ^{35}S-labelled riboprobe (see 5.2.3.1.2), 30μl sterile DEPC-treated water and 40μl nucleic acid solution. The reagents were mixed gently, incubated at 65°C for 5 minutes, and quenched on ice for 2 minutes, before adding: 840μl *in situ* hybridisation buffer I, 20μl 5M DTT, 10μl 10% sodium thiosulphate and 10μl 10% SDS (total volume = 1ml). 45μl of the hybridisation solution was applied to each slide (5.2.3.1.2.2) and covered with a Nescofilm coverslip. The slides were incubated at 56°C overnight, in a sealed sandwich box containing paper towels dampened with 4x SSPE/50% formamide, prior to washing (5.2.3.1.2.4).

5.2.3.1.2.4 Washing of double riboprobe-probed sections

Nescofilm coverslips were removed from slides (5.2.3.1.2.3) by submersion in small plastic beakers of 1x SSPE/1mM DTT. The slides were then rinsed in small glass staining dishes (washed as described in 2.3.4.3.4.1) containing 100ml 4x SSPE/1mM DTT. Following this, the slides were incubated in 4x SSPE/1mM DTT, at RT, 4 times, for 5 minutes each, and then transferred to the pre-prepared RNase A solution at 37°C, for 30 minutes. The slides were then washed in 0.1x SSPE/1mM DTT, at RT, on a rotary shaker (Stuart Scientific rocking platform; 35 rev/min), twice, for 5 minutes each. The slides were subsequently washed in 0.1x SSPE/1mM DTT at 65°C, on a shaker, twice, for 30 minutes each, and then rinsed with 0.1x SSPE/1mM DTT at RT, twice for 5 minutes each.

5.2.3.1.2.5 Detection and visualisation of probes

For the detection and visualisation of the Digoxigenin-labelled *egr-1* riboprobe, the slides (5.2.3.1.2.4) were rinsed in Buffer 1, twice, for 5 minutes each, incubated in approximately 50μl of Buffer 1 / 5% NGS / 0.6% Triton X-100, for 30 minutes (with Nescofilm coverslip), and then in Buffer 1 / 5% NGS / 0.6% Triton X-100 / 1:500 anti-digoxigenin-AP (Roche 1500mU/ml), at RT, for 2 hours (with Nescofilm coverslip). The slides were rinsed in Buffer 1, twice, for 10 minutes each, and then in Buffer 2, for 5 minutes, before an overnight incubation in 0.4mg/ml nitro blue tetrazolium chloride (NBT; Roche) / 0.19mg/ml 5-bromo-4-chloro-3-indolyl-phosphate p-toluidinium salt (BCIP; Roche) / 1mM Levamisole (Sigma), at RT, in the dark (with Nescofilm coverslip). The colour reaction was stopped the following day by incubation with Buffer 3.

For immediate analysis of *egr-1* riboprobe hybridisation, the slides were briefly dipped in distilled water and mounted in aqueous mounting solution (BDH).

For analysis proceeding detection of the EGFP riboprobe hybridisation, the slides were washed in 1x SSPE, at RT, 4 times, for 30 minutes each (to eliminate any residual NBT / BCIP which may interact non-specifically with nuclear emulsion), briefly dipped in distilled water, and allowed to air-dry. For the detection and visualisation of the ^{35}S-labelled EGFP riboprobe see 2.3.4.5 and 3.2.5. Note: no counterstaining was required, thus slides were mounted in a drop of DPX (BDH), and glass coverslipped, directly following development of the nuclear emulsion.

5.2.3.2 Fresh frozen sampling of proestrus 57C rat pituitary glands

In further studies, proestrous female transgenic rats (determined by microscopic examination of vaginal washings) were killed at 12:00, and whole pituitary glands were removed and snap-frozen as described above, prior to ISH / immuno-histochemical (IHC) analyses.

5.2.3.1.3 Transgene expression analysis in the proestrus 57C rat pituitary gland by dual ISH / IHC

Dual ISH / IHC was performed using a previously described protocol (Allen *et al.*, 1997; Morgan *et al.*, 2000) adapted for gonadotrophs. All solutions used in the initial immunohistochemical step were prepared in diethylpyrocarbonate (DEPC)-treated water, and also contained RNasin ribonuclease inhibitor (Promega; 0.1U/μl).

Sections (8μm) of 57C rat pituitary gland were post-fixed in 4% paraformaldehyde in phosphate buffered saline (5 min), and permeabilised in methanol (-20°C, 2 min). LH β-subunit immunoreactivity was detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol, using DAB as chromogen.

The primary antisera to LH β-subunit (NIDDK-anti-rbetaLH-IC-2; AFP22238790-GPOLHB) was diluted 1:1000. Following development of the chromogen, slides were washed, dried and stored at -70°C prior to ISH analysis (see 5.2.3.1.1). Following development of the photographic emulsion, sections were counterstained with Mayer's haematoxylin.

5.2.3.1.4 Transgene expression analysis in the 57C rat pituitary gland, following ovariectomy and oestrogen replacement, as determined by northern blot analysis

In this experiment, transgenic rats (8 week old) were either ovariectomised (see below) and injected with vehicle (s.c., sesame oil, daily on days 8-10 after surgery), ovariectomised and injected with 17β–estradiol (s.c. 20μg/100g body weight, Sigma) or sham-ovariectomised.

Each ovariectomy subject was anaesthetised (see 4.2.2.1.1) and laid on its abdomen on the surgical table, before shaving an area from the end of the rib cage towards the tail (~6x4 cm). Excess loose fur was was removed by swabbing with 70% alcohol containing hibitane. A ~3cm incision was made along the midline of the back, using a scalpel. The thumb and forefinger were used to grip, and gently lift up, the skin on one side. A Spencer Wells artery clamp was used to separate the skin from the body wall, by pushing it through the connective tissue, which was then clamped to one side using a pair of Drape forceps. The fat pad covering the ovary was located beneath the body wall using blunt forceps, and a site clear of blood vessels and nerves was found in which to make a ~1cm incision with a fine pair of dissecting scissors. The fat pad was carefully pulled through this incision, bringing with it the ovary, oviduct and uterine horn. A bulldog type clamp was attached to the fat pad to hold the ovary in a suitable position for manipulation. The exposed reproductive tract was kept moist by swabbing with saline using a cotton bud.

The ovary was viewed under the stereo dissecting microscope (Nikon; x10-20 magnification), and surgical silk (~3cm) was tied to the oviduct, close to the ovary, using a tight reef knot. Surgical dissection (and removal) of the ovary was then performed, prior to placing the uterine horn and restricted oviduct back inside the body cavity, and suturing the body wall with 4-5 stitches. The procedure was repeated for the other ovary. Following the bilateral ovariectomy procedure, the incision along the back of the subject was sutured with 12-15 stitches, and cleaned of blood using saline, before applying a topical antibiotic. Following surgery, rats were maintained for 10 days prior to killing.

Subsequent total RNA extraction and northern analysis of transgene and *egr-1* transcript expression was performed by Dr. D. Carter, as described (Slade *et al.*, 2002; see 2.3.3). Blots were sequentially probed with ^{32}P-labelled cDNA probes specific for transgene (*Not*I - *Sal*I fragment of pEGFP-N1), *egr-1* (*EcoR*I - *Dra*I fragment of the rat NGFI-A cDNA (Milbrandt, 1987), and 18S ribosomal RNA (DecaTemplate, Ambion, Austin, Texas), washed, and exposed to a Phosphor screen (Kodak-K). Densitometric analysis of mRNA levels between groups was performed using Imagequant™ software (Amersham Pharmacia Biotech, Uppsala, Sweden).

5.3 RESULTS

5.3.1 Genotyping of 57C transgenic rats

5.3.1.1 PCR amplification of genomic DNA

PCR amplification of third generation 57C rat genomic DNA, using primers specific for the EGFP and actin genes, was used to identify 57C transgenic rats (see Figure 5.3). All 57C transgenic rats, and selected non-transgenic litter mates, were confirmed by Southern blot (see 5.3.1.2).

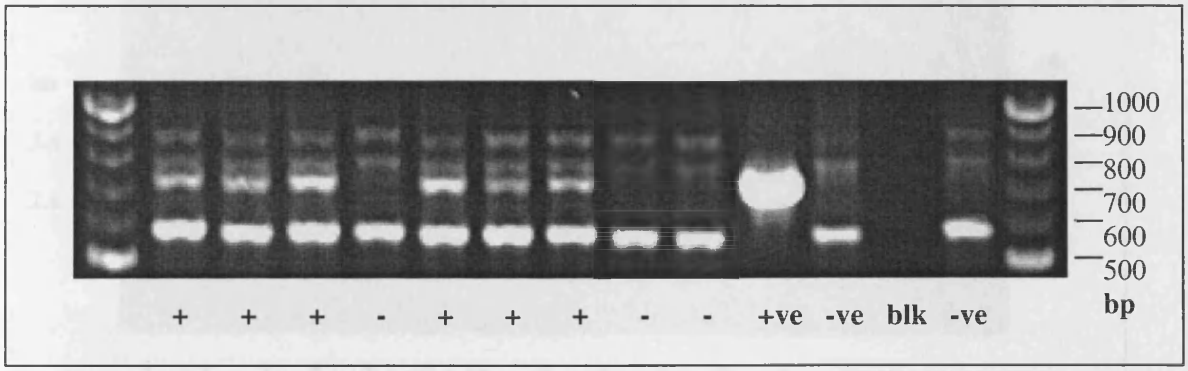


Figure 5.3 PCR amplification (using specific primers) of **57C** transgenic (**+**) and non-transgenic (**-**) rat genomic DNA, extracted from tail or ear biopsies of a third generation 57C rat litter, and resolved on an agarose gel. An EGFP-specific band (716 bp) is visible in transgenic samples, and an actin-specific band (581 bp) and 2 actin pseudogene bands are visible in all genomic DNA samples. The lanes marked **+ve**, **-ve** and **blk** correspond to positive (transgene), negative and blank control samples, respectively; the outer lanes represent a 100 bp DNA ladder (NEB).

5.3.1.2 Southern blot analysis of genomic DNA

Southern blot analysis of third generation 57C rat genomic DNA revealed the presence of a major 3.6 kb band on autoradiographs (see Figure 5.4), which appears to represent the integration of two full length copies of the transgene. A smaller 2.8 kb fragment is also present, and would appear to represent either the integration of an additional truncated transgene fragment, or a junction fragment. Taken together, these findings are consistent with those of previous generations of the 57C transgenic rat (Slade, 2001), and therefore confirm stable inheritance of the transgene (see Figure 5.4), and also indicate that the fragments are integrated at a single chromosomal site.

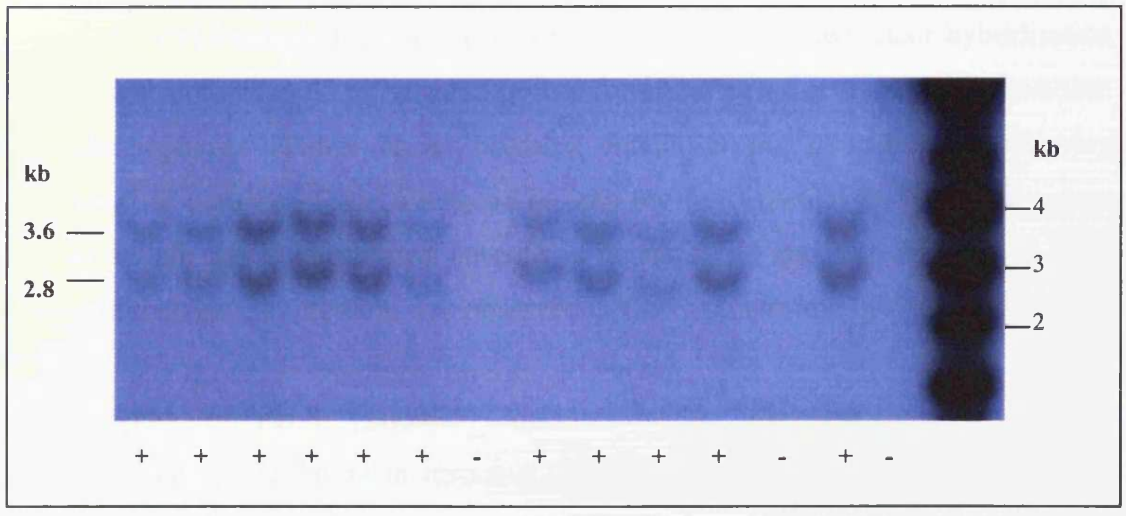


Figure 5.4 Film autoradiograph of *Bgl*II-restricted **57C** transgenic (+) and non-transgenic (-) rat genomic DNA (10µg), extracted from tail or ear biopsies of a third generation 57C rat litter, and probed with an EGFP-specific radiolabelled probe. EGFP-hybridising bands are visible in transgenic samples. The right lane represents a 1 kb DNA ladder (Promega).

5.3.2 ISH analysis of *egr-1* gene and transgene (EGFP) expression in 57C rat brains (subjected to pharmacological stimulus) using specific ^{35}S-labelled riboprobes

Control and metrazole-induced *egr-1* and EGFP (transgene) expression were localised in the brain by ISH. All ISHs were performed by myself, and the images generated from these have been included in both Slade *et al.* (2002) and Slade (2001), and therefore have not been shown in the present results (see Figure 5.1 for schematic representation, and appendix H for published data).

ISH analysis of the basal pattern of transgene (EGFP) expression in control 57C brains were comparable with that of *egr-1* mRNA. Storage phosphor images of transgenic brain sections hybridised with either anti-sense probes revealed a diffuse pattern of hybridisation in dorsal areas of the cortex. In contrast, clear hybridisation signals were not observed using sense probes for either *egr-1* or transgene. Emulsion autoradiographs of control brains revealed details of the hybridisation, showing expression of both *egr-1* and transgene in both the retrosplenial and parietal cortex, with an abundance of hybridising neurons in layers II, IV and VI. In addition, both *egr-1* and transgene expression was observed in the CA1 subfield of the hippocampus.

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to a (generalised, pharmacological) neural stimulus was investigated using the convulsant metrazole, which has been used in previous studies of endogenous *egr-1* induction (Saffen *et al.*, 1988; Mack *et al.*, 1990; Morita *et al.*, 1996). Following metrazole treatment, dramatic increases in both *egr-1* and transgene expression, compared with control animals, were observed. Of particular prominence was an increase in the dentate gyrus, but increased expression was also detected in CA2 and CA3 subfields of the hippocampus, in retrosplenial, parietal and perirhinal cortex. In addition, induction of both *egr-1* and transgene was observed in the amygdaloid complex, with prominent expression in the anterodorsal medial amygdaloid nucleus. Transgene induction was also observed in the ventro-medial nucleus (VMN) of the hypothalamus, predominantly in the DM region.

In accord with previous studies at both protein (Mack *et al.*, 1990) and mRNA (Herdegan *et al.*, 1995; Cullinan *et al.*, 1995) levels, hybridisation of *egr-1* and transgene probes appeared to be primarily, if not exclusively, associated with neurons rather than other cell types in the brain. In general, ISH analysis of transgene expression revealed localised, high-level expression in specific groups of neurons with an absence of expression in many brain areas. Similar observations were made for *egr-1* mRNA.

5.3.3 ISH analysis of *egr-1* gene and transgene (EGFP) expression in 57C rat brains (subjected to physiological stimulus) using specific ^{35}S-labelled riboprobes

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to a physiological stimulus was investigated using an established circadian, light-pulse paradigm. Following this stimulus, a discrete induction of transgene was observed throughout the SCN (see Figures 5.5**D** and 5.6), and in a subset of neurons within the PeN (see Figure 5.7). Both of these observations mirrored the light-induced expression of *egr-1* (SCN; see Figure 5.5**B**; PeN not shown). The light stimulus was not associated with transgene induction in other areas of the neural axis including the hippocampus and cortex (not shown), and SCN / PeN induction was not observed in non-stimulated animals sampled at 01:00h (SCN; see Figures 5.5**A** and **C**). The apparent induction of *egr-1* mRNA in cortical layers Figure 5.5**D**) was not consistently observed. In addition, no hybridisation signals were visible, in animals subjected to a nocturnal light pulse, following ISH with the respective sense probes (not shown).

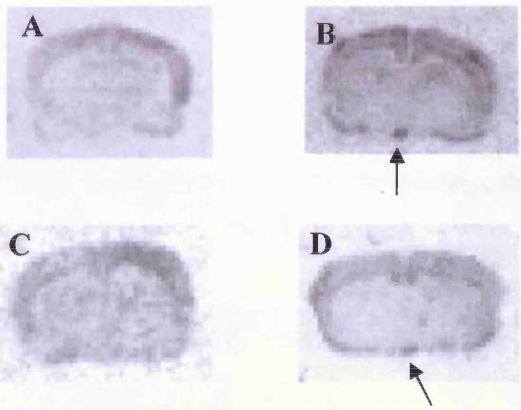


Figure 5.5 Phosphor screen images of *egr-1* mRNA and transgene mRNA in coronal section (12 μm) of **57C** rat hypothalamus, following ISH with specific ^{35}S-labelled anti-sense riboprobes: (**A**) *Egr-1* mRNA in control 57C rat (non-stimulated); (**B**) *Egr-1* mRNA in 57C rat subjected to a nocturnal light pulse; (**C**) transgene mRNA in control 57C rat (non-stimulated); (**D**) transgene mRNA in 57C rat subjected to a nocturnal light pulse. Arrows indicate light-induced expression in the SCN (only visible in 57C rats subjected to a nocturnal light pulse). Magnification x1.

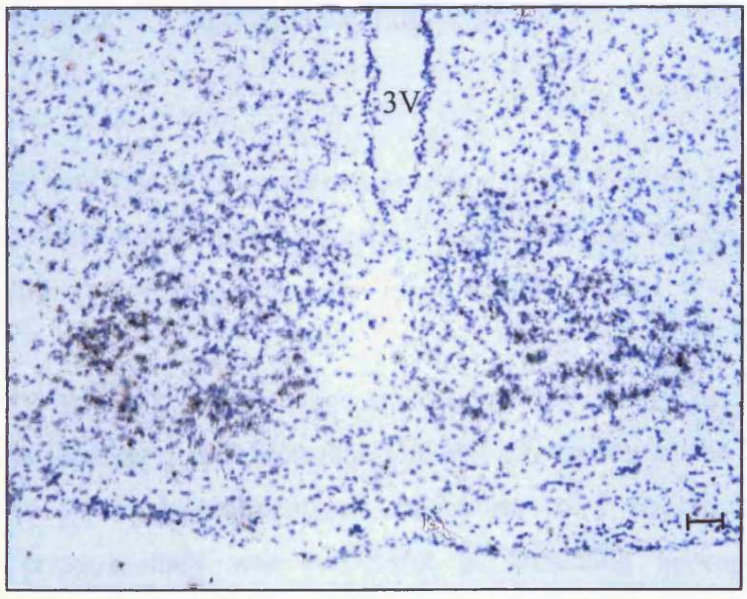


Figure 5.6 Emulsion autoradiograph of transgene mRNA in coronal section (12μm) of **57C** rat hypothalamus subjected to a nocturnal light pulse, following ISH with an EGFP anti-sense riboprobe. Clusters of silver grains (representing hybrid-disation signal) are visible throughout the SCN. Scale bar = 200μm. 3V represents the third ventricle.

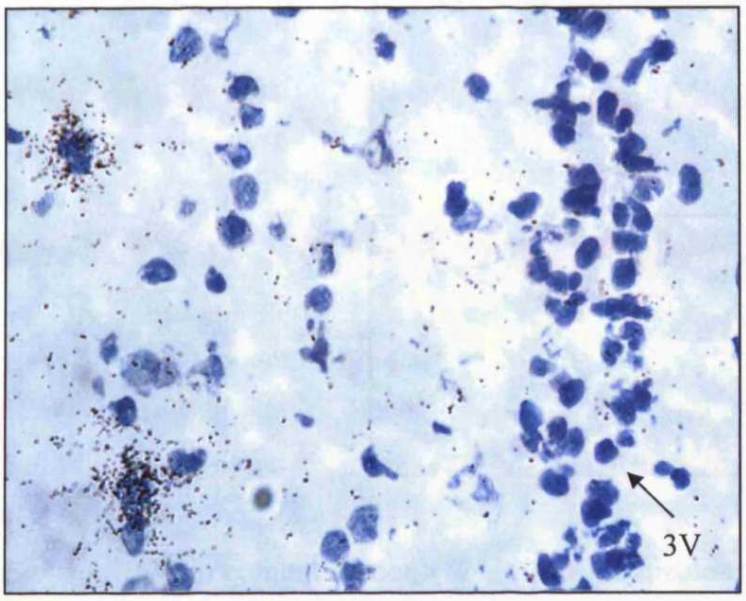


Figure 5.7 Emulsion autoradiograph of transgene mRNA in coronal section (12μm) of **57C** rat hypothalamus subjected to a nocturnal light pulse, following ISH with an EGFP anti-sense riboprobe. Clusters of silver grains are visible in individual periventricular neurons. 3V represents the third ventricle.

5.3.4 Dual ISH analysis of transgene expression in the 57C rat brain (following experimental stimuli) using specific ^{35}S-labelled and Digoxigenin-labelled riboprobes : co-localisation of *egr-1* and EGFP

Dual ISH, involving Digoxigenin-labelled and ^{35}S-labelled riboprobes, was used in an attempt to co-localise *egr-1* and transgene (EGFP) expression in the 57C rat brain, following either metrazole-treatment, or subjection to a nocturnal light pulse. Although the present study was successful in localising appropriate induced expression of *egr-1* (using a specific Digoxigenin-labelled anti-sense probe; see Figure 5.8 **C** + **D** and 5.9 **A** + **B**), and of EGFP (using a specific ^{35}S-labelled anti-sense probe; see 5.3.2 and Figures 5.5**D** and 5.6) individually, hence confirming previous findings (see 5.3.2 and 5.3.3), the present technique failed to co-localise the respective transcripts; there was no simultaneous visualisation of the signals.

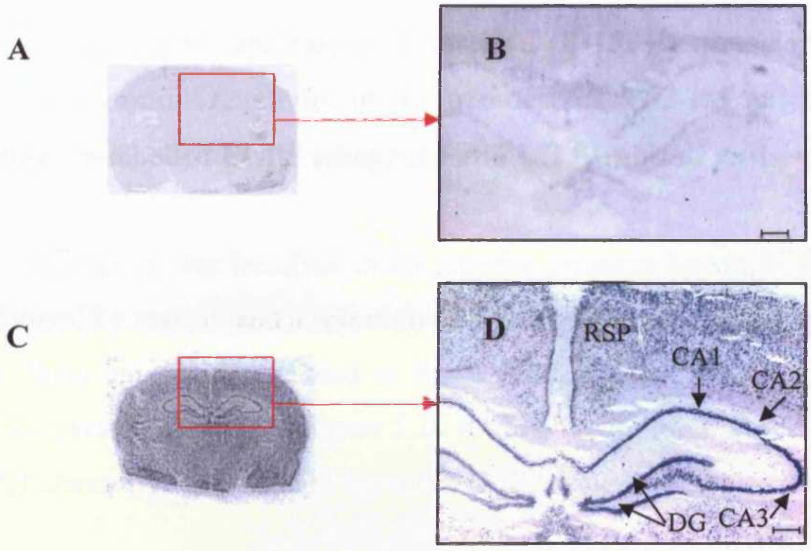


Figure 5.8 *Egr-1* mRNA in coronal section (12 µm) of metrazole-treated **57C** rat hypothalamus, following ISH with specific Digoxigenin-labelled riboprobes: (**A** + **B**) No specific hybridisation signals are visible in *egr-1* sense-probed sections; (**A**) magnification approximately x1; image rendered greyscale; (**B**) scale bar 400µm; (**C** + **D**) Hybridisation signals are visible in the hippocampus (retrosplenial (RSP) cortex, dentate gyrus (DG), and cornu ammonis 1, 2 and 3 regions (CA1, 2, and 3, respectively) of *egr-1* anti-sense-probed sections; (**C**) magnification approximately x1; image rendered greyscale; (**D**) scale bar 400µm.

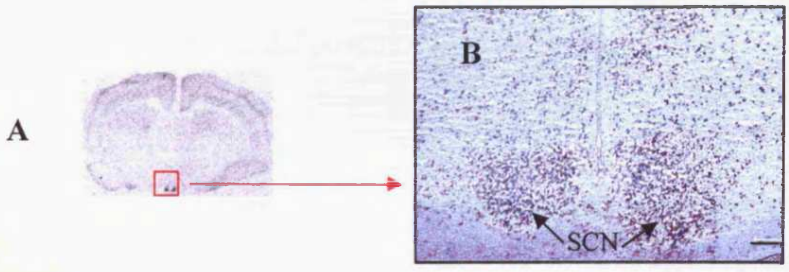


Figure 5.9 *Egr-1* mRNA in coronal section (12 µm) of **57C** rat subjected to a nocturnal light pulse, following ISH with specific Digoxigenin-labelled riboprobes: (**A** + **B**) Hybridisation signals are visible in the SCN of *egr-1* anti-sense-probed sections; (**A**) magnification approximately x1; image rendered greyscale; (**B**) scale bar 100µm. No hybridisation signals are visible in *egr-1* sense-probed sections (not shown).

5.3.5 Dual ISH / IHC analysis of transgene (EGFP) expression and LH β-subunit immunoreactivity in the pro-oestrus 57C rat anterior pituitary using ^{35}S-labelled EGFP riboprobe and LH β-subunit antiserum

Transgene expression was localised in the anterior pituitary by single ISH. All ISHs were performed by myself, and a selection of higher resolution images generated from the single ISHs have been included in Slade (2001), and therefore have not been shown in the present thesis (see Figure 5.10 for low resolution images, Figure 5.11**A**, **B** and **C** for alternative images, and appendix H for published data).

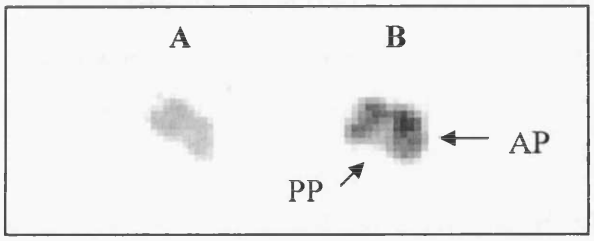


Figure 5.10 Low resolution phosphor screen images of transgene mRNA in proestrous **57C** rat pituitary sections (12 μm), following ISH with specific riboprobes: (**A**) No hybridisation signals are visible in EGFP sense-probed section; (**B**) Hybridisation signals are visible in the anterior pituitary (AP) lobe of EGFP anti-sense-probed sections, and not in the posterior pituitary (PP).

Proestrus 57C rat pituitaries were used in the present study since it was determined that, in a similar manner to the regulation of the endogenous *egr-1* gene (Slade and Carter, 2000), there is a clear up-regulation of transgene mRNA levels on pro-oestrous (Man and Carter, 2003).

ISH analysis of transgene expression in the pro-oestrus pituitary gland revealed that expression was restricted to the anterior lobe (see Figure 5.11), and, within this lobe, to a sub-population (approximately 10-15%) of cells (see Figure 5.11**A**). The cells exhibiting hybridisation were often associated into small groups (Figure 5.11**A**). The

aggregations of silver grains that define transgene mRNA expression were restricted to the anterior lobe of the pituitary gland (see Figure 5.11**C**). In control experiments, using a sense transgene probe, there was no evidence of hybridisation above background either in the anterior pituitary (Figure 5.11**B**), or in other lobes of the gland (not shown).

IHC analysis of transgenic rat pituitary glands using a previously characterised (Knight *et al*, 2000) LH β-subunit antiserum revealed a sub-population of β-subunit expressing cells in the anterior pituitary gland (Figure 5.11**D**). Dual ISH / IHC (Allen *et al.*, 1997; Morgan *et al.*, 2000) using the LH β-subunit antiserum in combination with the anti-sense transgene probe revealed co-localisation of LH β-subunit immunoreactivity with transgene mRNA (Figure 5.11**D**).

The distribution and abundance of silver grain aggregations in the dual analysis sections (Figure 5.11**D**) was similar to that observed for single ISH analysis (see Figures 5.11**A**, 5.11**B** and 5.11**C**; Slade, 2001), indicating that the specificity of transgene probe hybridisation was not compromised in the dual analysis procedure. Co-localisation was observed in the majority of LH β-subunit-expressing cells; however a sub-population (approximately 10%) of β-subunit-positive/ transgene mRNA-negative cells was also observed. Conversely, a similar sub-population of transgene mRNA-positive/ β-subunit-negative cells was also found, indicating, firstly, that the aggregations of silver grains are not non-specifically associated with immuno-positive cells, and secondly, that the transgene is also expressed a small population of cells other than gonadotrophs.

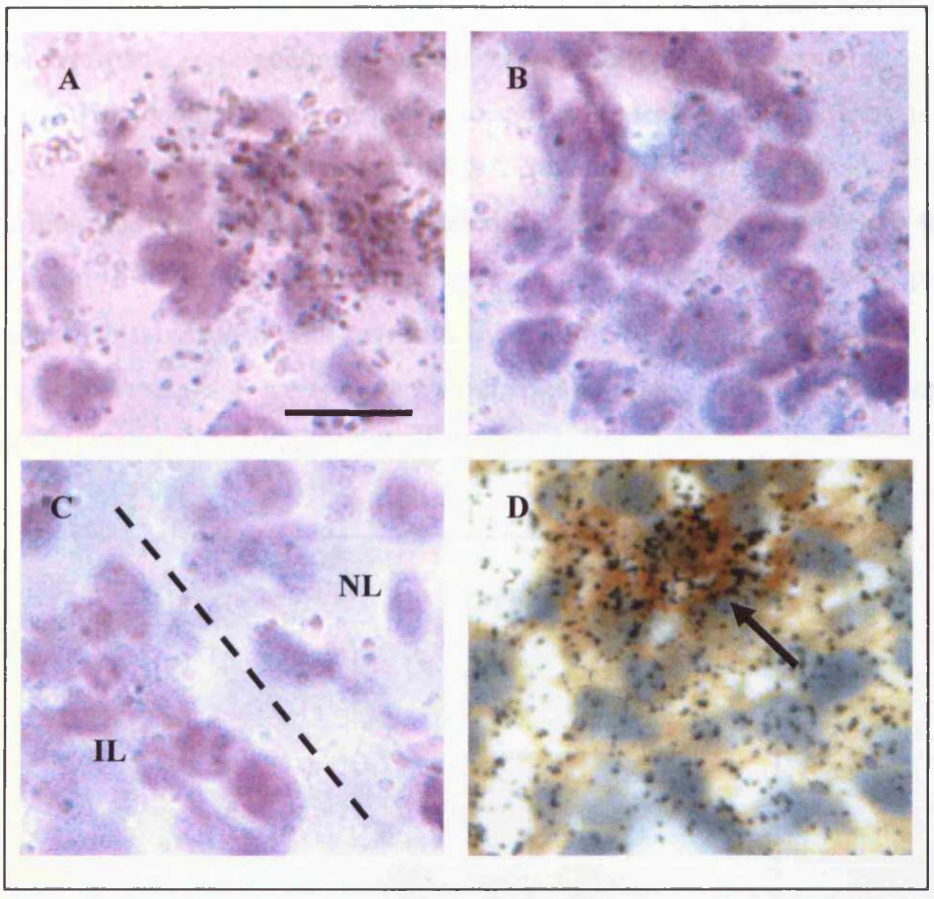


Figure 5.11 High resolution emulsion autoradiograms of transgene (EGFP) mRNA in pro-oestrus **57C** female rat pituitary sections (12μm), following ISH with a ^{35}S-labelled RNA probe specific for transgene (EGFP) mRNA. (**A**) Representative image of anterior lobe cells probed with the anti-sense transgene probe. Note the presence of a group of cells associated with aggregated silver grains, and other cells associated with a background distribution of silver grains. (**B**) Representative image of anterior lobe cells probed with the sense transgene probe. (**C**) Representative image of the neural (NL) – intermediate (IL) lobe boundary (dashed line) probed with the anti-sense probe. (**D**) Representative image of anterior lobe cells probed with both a LH β-subunit antisera, and the anti-sense transgene probe. Arrow indicates the association of an aggregation of silver grains with cells exhibiting the orange-brown staining that is representative of LH β-subunit immunoreactivity. Scale bar = 15μm.

5.3.6 Northern blot analysis of 57C anterior pituitary gland mRNA following ovariectomy and oestrogen replacement

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to physiological (endocrine) stimuli was investigated by observing transgene mRNA levels in ovariectomised 57C rats. Northern blot analysis (Figure 5.12) revealed that following ovariectomy, a marked and significant ($P<0.05$) elevation of transgene mRNA levels in the anterior pituitary was detected; this response was reversed by treatment with 17β–estradiol. These responses of the transgene were shown to recapitulate regulation of the endogenous *egr-1* mRNA (Slade and Carter, 2000).

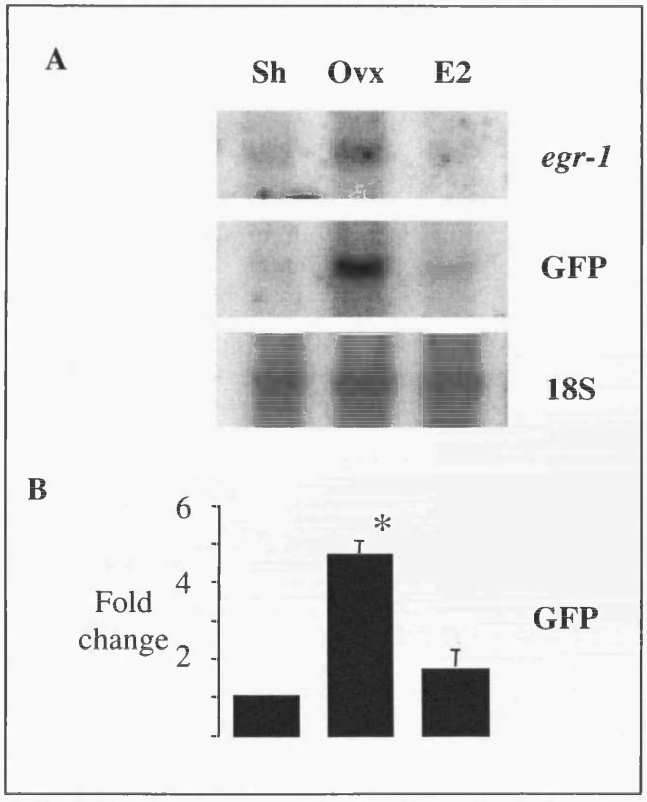


Figure 5.12. Phosphor screen images of transgene, and *egr-1* transcript expression in 57C rat anterior pituitary gland following ovariectomy, and oestrogen replacement. (**A**) Representative northern blot analysis of transgene mRNA in sham-operated (**Sh**), ovariectomised (**Ovx**) and ovariectomised, 17β-estradiol (**E2**) -treated rats. Immobilised total cellular RNA extracted from individual glands (12μg/lane) was probed sequentially with ^{32}P- labelled cDNA probes specific for transgene, *egr-1* and

18S ribosomal RNA. Blots were exposed to a Phosphor screen (Kodak-K) for 3 days (GFP and *egr-1*) and 10 minutes (18S). (**B**) Histogram showing densitometric analysis of multiple similar northern blots, as described in (**A**). Levels of transgene mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level for sham-operated animals (Mean ± S.E.M.). *$P<0.05$ compared with other groups. (Analysis of variance followed by Duncan's multiple range test.) n=4 (Sh & Ovx) and 3 (E2) pituitary glands in each group.

5.4 DISCUSSION

The present transgenic rat model study demonstrates that the *egr-1*/d4EGFP transgene (comprising proximal 5'-flanking, and intronic regions of the *egr-1* gene) confers both tissue-specific constitutive expression and inducible expression in the brain. These findings paralleled the results obtained with a recently generated transgenic mouse model (Tsai *et al.*, 2000) in which 5'-flanking sequences of the murine *egr-1* gene were shown to direct both constitutive expression in brain, heart and liver, and also hepatectomy-associated gene induction in liver.

More importantly, the transcriptional up-regulation that is inferred from the present observations has been shown to be stimulus-specific; discrete induction in the SCN and PeN by a nocturnal light pulse (Figures 5.5**D**, 5.6, and 5.7) compared to the induction of expression in the cortex, forebrain and VMN following a pharmacological stimulus (Slade *et al.*, 2002). In further studies, it will be interesting to observe whether transgene expression in the caudal brain areas can also be selectively induced by different specific stimuli. For example, the novel pattern of VMN induction observed here, which is not mirrored by *egr-1* during stress (Cullinan *et al.*, 1995), may be selectively regulated during mating stimuli (Polston and Erskine, 1995). Amygdala-specific induction during fear-memory retrieval (involving the conditioning of subjects to associate an experimental context with a footshock) is another possibility (Hall *et al.*, 2001). In addition to providing correlative evidence of Egr-1 function within specific areas, the demarcation of regulation within discrete cell groups also provides for conditional transgenesis models in which transgene promoter-linked open reading frames (ORFs; e.g. an *Egr-1* dominant-negative (Levkovitz *et al.*, 2001)) may be expressed in a stimulus-specific manner. In the paradigm demonstrated here, a non-invasive light stimulus is shown to have the potential for driving functional transgene expression in the SCN and PeN. The regulation and role of Egr-1 in PeN somatostatin neurons has been documented (Tanaka *et al.*, 1999; Slade *et al.*, 2001; Davies *et al.*, 2004).

In the brain, the fidelity of transgene-regulated constitutive expression is particularly well documented in the hippocampal formation in which restriction of a high level of basal expression of transgene transcripts to the CA1 subfield reflects a similar pattern

of constitutive *egr-1* expression (Cullinan *et al.*, 1995; Slade 2001; Slade *et al.*, 2002). Furthermore, pharmacological induction of transgene expression is observed in the CA2 and CA3 subfields, but predominantly in the dentate gyrus, as documented both here and previously for *egr-1* (Saffen *et al.*, 1988). A similar pattern of *egr-1* induction has also been observed following an alternative stimulus (swim stress; Cullinan *et al.*, 1995).

In addition, the findings of the present study indicate that sequences within the rat *egr-1*/d4EGFP transgene confer region-specific and physiologically-regulated expression in the rat pituitary gland. Each of these aspects of transgene expression mirror expression of the endogenous *egr-1* gene (see Slade and Carter, 2000; Tsai *et al.*, 2000; Slade *et al.*, 2002). The body of data that is emerging from these transgenic studies therefore argues that proximal sequences within the *egr-1* gene are sufficient to, at least broadly, recapitulate expression of the endogenous *egr-1* gene in rodents. Taken together with extensive *in vitro* evidence of the regulatory capacity of the *egr-1* proximal 5'-flanking sequence (Changelian *et al.*, 1989; Sakamoto *et al.*, 1991; DeFranco *et al.*, 1993; Cohen *et al.*, 1996; Yan *et al.*, 1999b; Bernal-Mizrachi *et al.*, 2000), it can be further argued that this region alone is sufficient to confer an appropriate pattern of expression.

The restriction of *egr-1*/d4EGFP transgene expression to cells of the anterior lobe of the pituitary, demonstrated in the present study (see Figures 5.11 and 5.12), indicates that the transgene contains sufficient regulatory information to discriminate the embryologically distinct cells of the neural and intermediate pituitary lobes. Within the anterior lobe, transgene transcripts are further restricted to a sub-population of cells indicating that the transgene is also able to mediate cell-type specific transcriptional control. Thus, using a previously validated (Morgan *et al.*, 2000) dual ISH / IHC technique, co-localisation of the transgene transcript with LH β-subunit immunoreactivity has been demonstrated. This finding reflects the demonstration of Egr-1 protein in rat gonadotrophs (Knight *et al.*, 2000), and indicates a remarkable fidelity of cell-type specific expression that is conferred by *egr-1* sequences in the transgene. (It should be noted that cell-type specific expression of the *egr-1*/d4EGFP transgene in brain (Slade *et al.*, 2002) has also been observed.) The full extent of the

cellular specificity of transgene expression in the anterior pituitary will, however, require considerable further analysis in which, firstly, the small population of transgene-positive / LH β-subunit-negative cell type(s) are characterised, and secondly the co-localisation of transgene and *egr-1* transcripts is quantitated. It is apparent from the present results, however, that the former population of cells is limited in number and would not appear to constitute a major anterior pituitary cell type. A similar distribution of expression is observed for Egr-1 protein (see Knight *et al.*, 2000).

Furthermore, the present study revealed that transgene mRNA levels are up-regulated (5-fold) following ovariectomy, in an oestrogen-dependent manner (see Figure 13). This is most likely explained by increased hypothalamic drive to the pituitary following the removal of the negative feedback effects of oestrogen. In this respect, luteinising hormone-releasing hormone (LH-RH) stimulated *egr-1* expression has been well documented (Tremblay and Drouin, 1999; Wolfe and Call, 1999; Duan *et al.*, 2002).

5.4.1 Conclusions

In conclusion, the present study used a transgenic approach to demonstrate that proximal regions of the *egr-1* gene can direct cell-specific and physiologically-regulated expression in the brain and anterior pituitary gland. Expression of the transgene, both anatomical and regulated, recapitulates expression of the endogenous gene, a finding that reflects observations in an *egr-1* transgenic mouse model (Tsai *et al.*, 2000). Therefore, only a single transgenic line (57C) was used for these studies.

The demonstration of stimulus-specific neuronal induction of an *egr-1* transgene in the present study is timely with respect to the recent definition of a role for *egr-1* in neuronal plasticity (Jones *et al.*, 2001). The present findings facilitate the development of functional transgenes that can be used to address the role of specific substrates of adaptive plasticity in different neurophysiological and behavioural paradigms.

The present study also provides further evidence of endocrine regulation of *egr-1* in the rat, and demonstrates that proximal sequences of the *egr-1* gene mediate endocrine-regulated expression.

5.4.2 Future directions

The present transgene construct could be used in future studies to direct expression of functional cDNAs to pituitary cells. The novel evidence of a direct effect of oestrogen on transgene (and *egr-1*) expression at the level of the pituitary (see Figure 5.12), together with previous evidence of *egr-1* gene regulation by LH-RH (see 5.4), suggests that the molecular response to these two physiological factors may be integrated at the *egr-1* promoter.

The presence of consensus, albeit functionally unproven, *cis*-acting element sequences within the *egr-1* intron (incorporated in the rat, but not the mouse transgene) that include a core SRE at base 1444, and an AP-1-like element at base 1682 of the rat *egr-1* gene (Acc. No. J04154; Changelian *et al.*, 1989) suggests that a more extensive analysis of the contribution of the *egr-1* intron sequences should be conducted. In this context, the transgenic studies of the melatonin-regulating AA-NAT gene (Burke *et al.*, 1999) have demonstrated an important role of intronic sequences for appropriate expression of this gene in the pineal gland. The relative importance of intronic *egr-1* sequences (absent in the mouse transgene; Tsai *et al.*, 2000) in conferring both basal and regulated transgene expression (Burke *et al.*, 1999) must await further analysis.

Similarly, the absence of detectable GFP fluorescence here (see 6.1.1), despite robust transcription of the transgene, requires additional investigation, including a full analysis of transgene activity *in vitro*, before this deficit can be explained.

CHAPTER 6

A TRANSGENIC RAT MODEL FOR STUDYING THE REGULATION OF EGR-1 GENE EXPRESSION II

A. GENERATION AND TRANSCRIPT ANALYSIS

6.1 OVERVIEW AND AIMS OF STUDY

The aim of the present study was to generate an *egr1*/d2EGFP promoter/reporter transgenic rat model, and characterise *egr1*/d2EGFP transgene expression.

6.1.1 Destabilised (d2) and enhanced green fluorescent protein (d2EGFP)

To date, no published data has demonstrated expression of the d4EGFP protein in an *in vivo* system. Thus, the absence of detectable GFP fluorescence in the previous *egr1*/d4EGFP transgenic model, despite robust transcription of the transgene (see Chapter 5), could possibly be due to the d4EGFP sequence itself. In contrast, translation of an alternative destabilised and enhanced green fluorescent protein, d2EGFP (see 4.1.6), has been successfully demonstrated in conjunction with the *mPer1* promoter (Kuhlman *et al.*, 2000; see 4.1.4). d2EGFP is derived from EGFP (GFPmut1; Cormack *et al.*,1996), and with its half-life of approximately two hours, is suitable as an *in vivo* reporter of dynamic changes in gene expression (Chalfie *et al.*, 1994), for a given functional promoter of interest. To construct d2EGFP, residues 422-461 of mouse ornithine decarboxylase (MODC) were fused to the C terminus of EGFP (Li *et al.*, 1998). This region of MODC contains a PEST (proline - P, glutamic acid - E, serine - S, and threonine - T) amino acid sequence that targets the protein for degradation and results in rapid protein turnover.

The d4EGFP variant is derived from d2EGFP by making an amino acid substitution in the MODC region (Thr-436 to Ala; Li *et al.*, 1998).

6.1.2 Generation of an *egr-1*/d2EGFP promoter/reporter transgenic rat model

The present study aims to generate an *egr-1*/d2EGFP transgenic rat model using similar sequences of the rat *egr-1* promoter as in the *egr-1*/d4EGFP transgene (~1.58 kb of *egr-1* gene promoter fragment (see Figure 6.1), derived from the *pJDM290* plasmid (Slade *et al.*, 2002)). However, the *egr-1* intron was replaced with a chimaeric intron derived from the *pSTEC-1* vector (Stec *et al.*, 2001), and d4EGFP was substituted by ~0.9 kb of d2EGFP coding sequence (see Figures 6.1 and 6.3), derived from the *mPer1*/d2EGFP transgene (see 4.1.7).

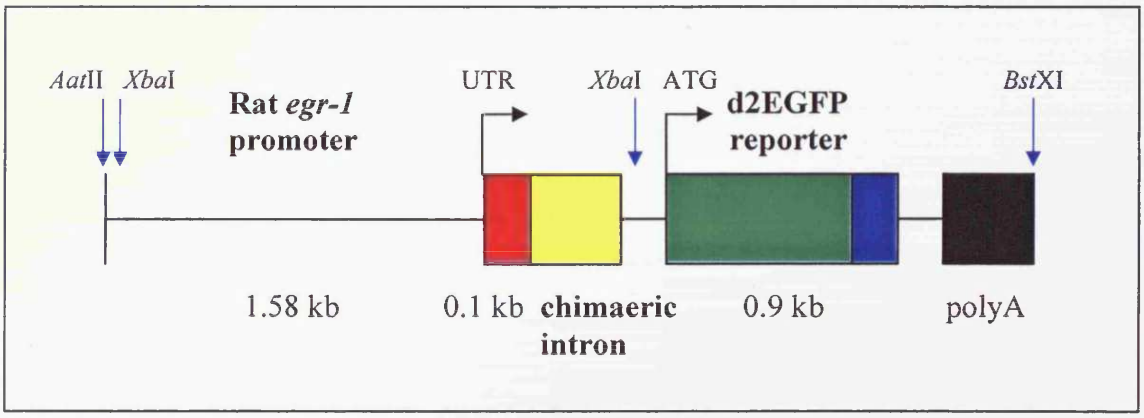


Figure 6.1 Schematic representation of the (3 kb) *egr-1*/d2EGFP transgene (not to scale). The construct contains 1.58 kb of rat *egr-1* 5' flanking sequence and 0.1 kb of untranslated (UTR) exon 1 sequence (red box) linked via a chimaeric intron to 0.9 kb of d2EGFP coding sequence, and was generated as described in 6.2.1. Blue box represents PEST sequences that target the protein for degradation and results in rapid protein turnover. Blue arrows indicate cleavage sites. Left black arrow indicates the transcription initiation site. Right black arrow indicates the translation initiation site (ATG).

6.1.3 Experimental aims

The aims of this study addressed in the present chapter are:

1. To generate an *egr1*/d2EGFP transgenic rat model using established transgenesis techniques.

2. To confirm transgene integration (and stable inheritance of the *egr1*/ d2EGFP transgene in further generations) using established Southern blot hybridisation protocols.

3. To investigate transgene transcript expression in the *egr1*/d2EGFP transgenic rat using reverse-transcriptase polymerase chain reactions (RT-PCRs) and Northern blot hybridisation protocols.

6.2 METHODS

6.2.1 Transgene construction

The cloning strategy used to generate the *egr-1*/d2EGFP transgene (see Figure 6.1) involved ligating the *egr-1* promoter and d2EGFP into *pSTEC-1* (Stec *et al.*, 2001) and *pGEM-7Z* (Promega; vector map in appendix C), to provide suitable flanking sequences for transgene insertion. The cloning steps involved (see below) were performed by Dr. D. Carter, and has been summarised here by Figure 6.2.

The plasmid *p-int1* was generated by ligating an *Xba*I - *Xho*I fragment (~1.5 kb) of *pJDM290* (see Slade *et al.*, 2002) into the *Xba*I - *Xho*I cut *pGEM-7Z* vector (Figure 6.2, step **1A**; restriction endonuclease digestion reaction conditions: Promega buffer D, 37°C, 1 hour). The plasmid *p-int2* was generated by ligating a *Hind*III - *BamH*I fragment (~0.5 kb) of *pSTEC1* (see Stec *et al.*, 2001) into the *Hind*III - *BamH*I cut *pGEM-7Z* vector (Figure 6.2, step **1B**; restriction endonuclease digestion reaction conditions: Promega buffer E, 37°C, 1 hour). The plasmid *p-int3* was generated by ligating a *Hind*III - *BstX*I fragment (~0.5 kb) of the *p-int2* plasmid into the *Hind*III - *BstX*I cut *p-int1* plasmid (Figure 6.2, step **2**; restriction endonuclease digestion reaction conditions: Promega buffer E, 37°C, 1 hour).

The d2EGFP fragment (~0.9 kb) of the transgene was derived from the *mPer1/ d2EGFP* plasmid (P1PG #4 plasmid; see 4.2.1) by digestion with *Sal*I and *Not*I (Figure 6.2, step **3A**; restriction endonuclease digestion reaction conditions: Promega buffer D, 37°C, 1 hour). The *egr-1* fragment of the transgene was derived from the *p-int3* plasmid by partial digestion with *Sal*I (Figure 6.2, step **3B**; restriction endonuclease digestion reaction conditions: enzyme added at approximately 1:50 dilution, Promega buffer D, 37°C, separate 5 and 10 minute reactions), followed by gel purification and further digestion with *Not*I (Figure 6.2, step **3B**; restriction endonuclease digestion reaction conditions: Promega buffer D, 37°C, 90 minutes), and gel extraction of the resultant larger band. The *egr-1*/d2EGFP plasmid was finally generated by ligating the *Sal*I - *Not*I fragment (~0.9 kb, see Figure 6.3) of the *mPer1/ d2EGFP* transgene into the *Sal*I - *Not*I cut *p-int3* plasmid (Figure 6.2, step **4**).

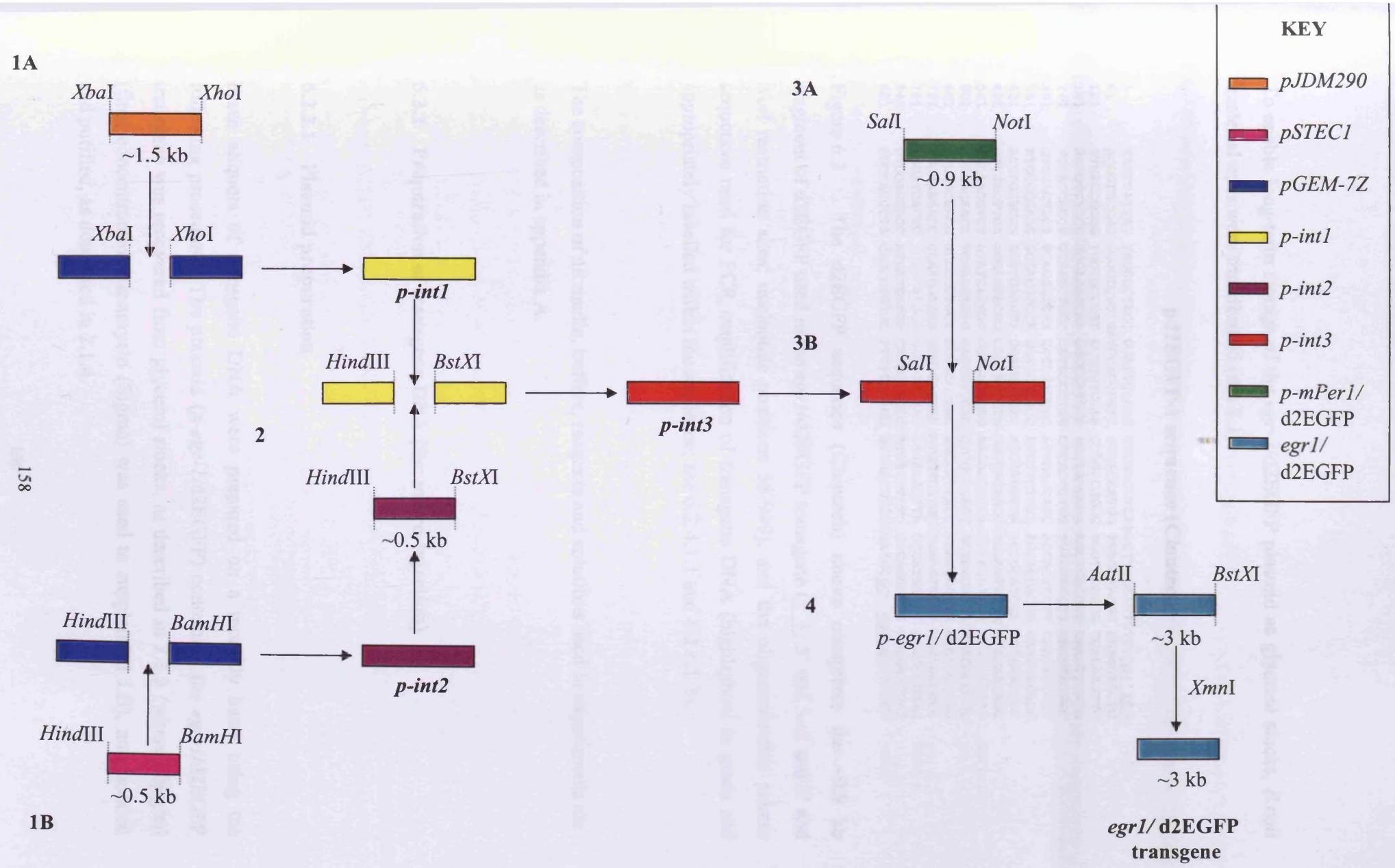


Figure 6.2 The cloning strategy used for generating the *egr1/* d2EGFP transgene construct. Numbers in bold indicate the various cloning steps involved (see text).

To enable long-term storage of the *egr-1*/d2EGFP plasmid as glycerol stocks, *E.coli* bacterial cells were transformed (see 2.1).

pd2EGFP-1 sequence (Clontech)

```
1     TAGTTATTAC TAGCGCTACC GGACTCAGAT CTCGAGCTCAAGCTTCGAATTCTGCAG{'TCG   SalI
61    ACGGTACCGC GGGCCCGG'GATCCACCGGTC GCCACCATGG TGAGCAAGGG CGAGGAGCTG
121   TTCACCGGGG TGGTGCCCAT CCTGGTCGAG CTGGACGGCG ACGTAAACGG CCACAAGTTC
181   AGCGTGTCCG GCGAGGGCGA GGGCGATGCC ACCTACGGCA AGCTGACCCT GAAGTTCATC
241   TGCACCACCG GCAAGCTGCC CGTGCCCTGG CCCACCCTCG TGACCACCCT GACCTACGGC
301   GTGCAGTGCT TCAGCCGCTA CCCCGACCAC ATGAAGCAGC ACGACTTCTT CAAGTCCGCC
361   ATGCCCGAAG GCTACGTCCA GGAGCGCACC ATCTTCTTCA AGGACGACGG CAACTACAAG
421   ACCCGCGCCG AGGTGAAGTT CGAGGGCGAC ACCCTGGTGA ACCGCATCGA GCTGAAGGGC
481   ATCGACTTCA AGGAGGACGG CAACATCCTG GGGCACAAGC TGGAGTACAA CTACAACAGC
541   CACAACGTCT ATATCATGGC CGACAAGCAG AAGAACGGCA TCAAGGTGAA CTTCAAGATC   EGFP-F2
601   CGCCACAACA TCGAGGACGG CAGCGTGCAG CTCGCCGACC ACTACCAGCA GAACACCCCC
661   ATCGGCGACG GCCCCGTGCT GCTGCCCGAC AACCACTACC TGAGCACCCA GTCCGCCCTG
721   AGCAAAGACC CCAACGAGAA GCGCGATCAC ATGGTCCTGC TGGAGTTCGT GACCGCCGCC
781   GGGATCACTC TCGGCATGGA CGAGCTGTAC AAGAAGCTTA GCCATGGCTT CCCGCCGGAG   EGFP-R
841   GTGGAGGAGC AGGATGATGG CACGCTGCCC ATGTCTTGTG CCCAGGAGAG CGGGATGGAC
901   CGTCACCCTG CAGCCTGTGC TTCTGCTAGG ATCAATGTGTAGATGCGC'}GGCCGCGACTCT   NotI
```

Figure 6.3 The d2EGFP sequence (Clontech) above comprises the ~0.9 kb fragment of d2EGFP used in the *egr1*/d2EGFP transgene ({ }; 5' end *Sal*I and 3' end *Not*I restriction sites; nucleotide positions 58-949), and the oligonucleotide primer sequences used for PCR amplification of transgene DNA (highlighted in green and appropriately labelled within the sequence; see 6.2.4.1.1 and 6.2.6.1.2).

The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

6.2.2 Preparation of transgene DNA (for micro-injection)

6.2.2.1 Plasmid preparation

Fresh aliquots of transgene DNA were prepared on a monthly basis using the following procedure. The plasmid (*p-egr-1*/d2EGFP) containing the *egr-1*/d2EGFP transgene was recovered from glycerol stocks, as described in 2.1.3 (where 50µg/ml (final concentration) kanamycin (Sigma) was used to supplement LB), and isolated and purified, as described in 2.1.4.

6.2.2.2 Extraction and purification of transgene DNA

The transgene plasmid (6.2.2.1) was subjected to *Aat*II - *Bst*XI restriction digestion (50µl DNA, 30µl sterile water, 10µl 10x buffer 4 (New England Biolabs, NEB; final concentration 1x), 5µl *Aat*II (NEB; final concentration 0.5 unit), and 5µl *Bst*XI (NEB; final concentration 0.5 unit)), at 37°C, for 1 hour, and at 55°C, for 1 hour. Subsequent restriction fragments were resolved by 1% 1xTAE agarose gel electrophoresis (see 2.2.3), to give multiple band fragments. A 3 kb band (transgene DNA and vector fragment), was briefly visualised under UV, excised using a scalpel (see 6.3.1.1, Figure 6.5**A**), and then extracted from the gel using the QIAEX II gel extraction kit (Qiagen, see appendix B).

The resultant pellet was eluted in 50µl MITE buffer, and then finally subjected to *Xmn*I restriction digestion (55µl DNA, 29µl sterile water, 10µl 10x buffer 2 (NEB; final concentration 1x), 1µl 100x BSA (final concentration 1x), and 5µl *Xmn*I (NEB; final concentration 0.5 unit)), at 37°C, for 90 minutes. Subsequent restriction fragments were resolved by 1% 1xTAE agarose gel electrophoresis (see 2.2.3), to give a 3 kb band (transgene DNA fragment), and 2 and 1 kb bands (vector fragments), respectively. The transgene DNA fragment (3 kb) was briefly visualised under UV, excised using a scalpel (see 6.3.1.1, Figure 6.5**B**), and then extracted from the gel using the QIAEX II gel extraction kit (Qiagen, see appendix B). The resultant pellet (transgene DNA) was eluted in 50µl MITE buffer, and then purified through a Sephadex G-50 spin column (prepared as described in 2.2.5.4.2; equilibrated with 100µl of MITE buffer, centrifuged at 3000g for 3 minutes, 5 times, prior to the application of the transgene DNA to the surface of the Sephadex), centrifuged at 3000g for 4 minutes. The transgene DNA was then quantitated and further purified as described (see 4.2.1.3).

6.2.3 Transgenesis

Transgenic rats were generated by pronuclear injection of transgene (*egr-1*/d2EGFP) DNA into fertilised oocytes (obtained from superovulated donor female rats mated with male studs). The cultured oocytes were then surgically transferred into recipient pseudopregnant mothers (oviduct transfer (OVT) mothers mated with vasectomised males), and resulting offspring were screened for transgene incorporation. Rat transgenesis has been described previously (see 4.2.2), and has been summarised here by Figure 6.4.

6.2.4 Genotyping of *egr1*/d2EGFP transgenic rats

6.2.4.1 Screening of progeny for transgene incorporation

Genomic DNA extracted from tail and ear biopsies (see 4.2.3.1.1) of all progeny derived from OVT mothers (see 4.2.2.5.2) were screened for transgene incorporation using multiplex PCR and Southern analysis (see 6.2.4.1.1 and 2.2.2., and 6.2.4.1.2 and 2.2.5, respectively).

6.2.4.1.1 Genomic screening by Multiplex Polymerase Chain Reaction (PCR) amplification

Transgenic offspring were identified by using a multiplex PCR strategy (see 4.2.3.1.1.1). Standard PCR reactions (see 2.2.2) were performed, each containing 0.5-1.0μg of genomic DNA, 400nM (final concentration) of each EGFP-specific primer (forward (F2) and reverse (R); see Table 6.1 and Figure 6.3), 200nM (final concentration) of each actin-specific primer (forward (F) and reverse (R); see Table 6.1). (All primers were manufactured by MWG Biotech). An aliquot of the micro-injection transgene fragment (*egr1*/d2EGFP; 0.5ng) and a water sample were included as positive and blank (negative) controls, respectively. PCR was conducted using a

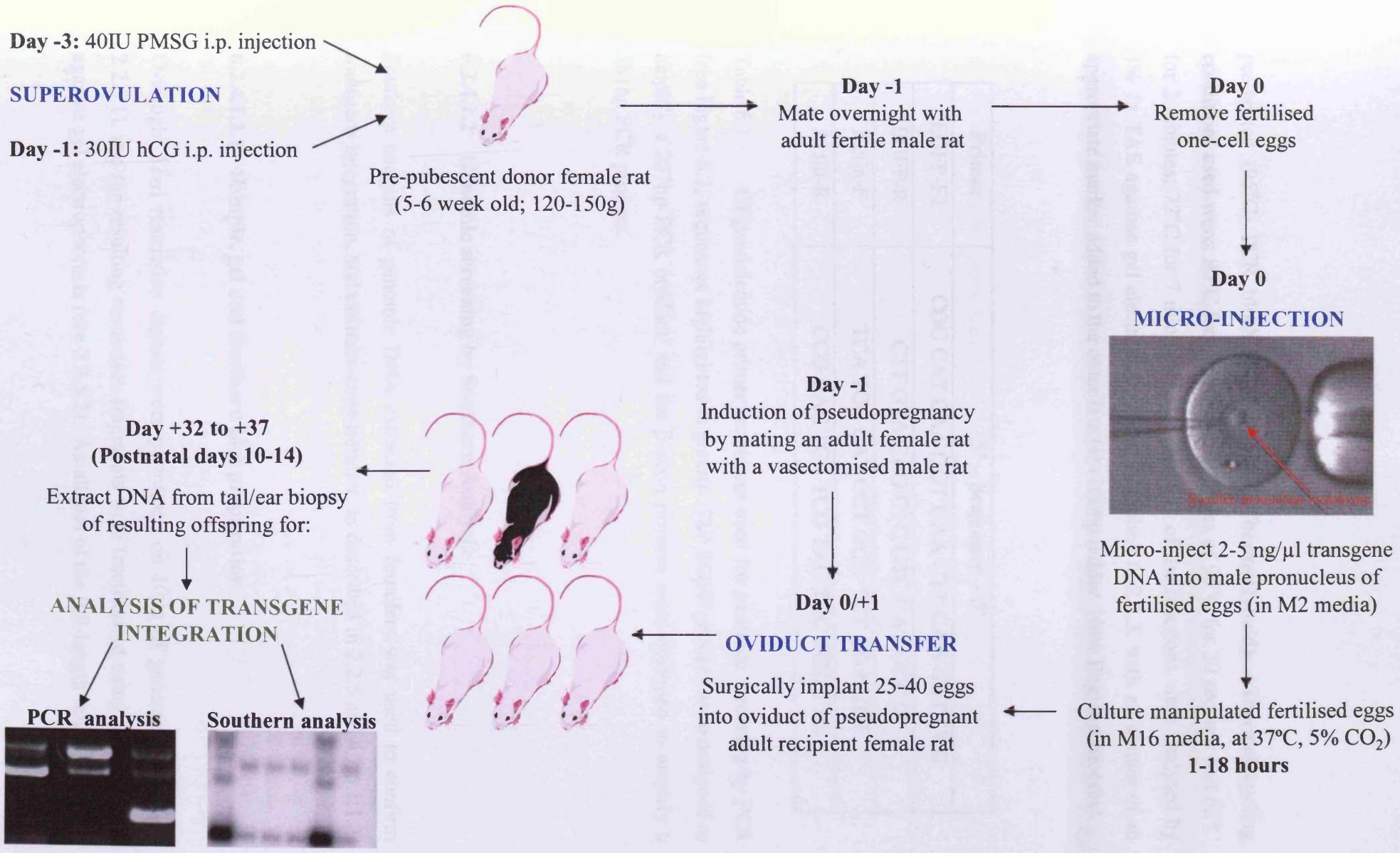


Figure 6.4 Schematic representation of the main processes involved in the generation of transgenic rats.

pre-heated (95°C) PCR block (PCR Express, ThermoHybaid). Thermal cycling conditions used were: 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds and 62°C for 2 minutes; 72°C for 7 minutes. The products of the reactions were analysed by 1% 1x TAE agarose gel electrophoresis, as described in 2.2.3, with an aliquot of an appropriate marker added to the outer lane(s) (100bp ladder, New England Biolabs).

Primer	5' – Sequence – 3'
EGFP-F2	CGG CAT CAA GGT GAA CTT CAA GAT CCG
EGFP-R	CTT GTA CAG CTC GTC CAT GCC G
Actin-F	TCA TGC CAT CCT GCG TCT GGA CCT
Actin-R	CCG GAC TCA TCG TAC TCC TGC TTG

Table 6.1 Oligonucleotide primer sequences used for genomic screening by PCR (see Figure 6.2; sequences highlighted in green). The EGFP primers were designed to amplify a 237bp PCR product, and the β-actin primers were designed to amplify a 581bp PCR product.

6.2.4.1.2 Genomic screening by Southern Analysis

Southern analysis of genomic DNA extracted from founders was used to confirm transgene integration, and estimate copy number, as described in 2.2.5 and 4.2.3.1.1.2.

6.2.4.1.2.1 Sample, gel and Southern blot preparation

Overnight *Xba*I restriction digests were performed on 10μg of genomic DNA (see 2.2.5.1), and the resulting restriction fragments were fractionated using 0.7% 1xTAE agarose gel electrophoresis (see 2.2.5.2). An aliquot of the full-length micro-injection

transgene fragment (*egr1*/d2EGFP; 0.5ng) was included as a positive control. An aliquot of an appropriate marker was also added to the outer lanes (1kb ladder, NEB). Electrophoretic fractionation of the DNA fragments was then followed by partial acid depurination, alkaline denaturation, and capillary transfer in a high-salt buffer from the gel to a nylon membrane (Hybond-N) overnight (see 2.2.5.3).

6.2.4.1.2.2 Probe preparation

An EGFP probe was prepared as described in 4.2.3.1.1.2.2 (see 6.3.2.1.2.1).

6.2.4.1.2.3 Hybridisation of the probe to the Southern blot

Southern blot membranes (6.2.4.1.2.1) were treated and hybridised with the EGFP probe (6.2.4.1.2.2), as described in 2.2.5.5.

6.2.4.1.2.4 Stringency washes

Stringency washes were performed as described in 2.2.5.6.

6.2.4.1.2.5 Detection and visualisation of the hybridised probe

The presence and size of a particular genomic DNA fragment (complementary to the probe) was detected and visualised by exposing the membrane to a radiation sensitive material (X-ray film for 2-3 days, or, Phosphor screen for 1-2 days), as described in 2.2.5.7.

6.2.5 Establishment, breeding and maintenance of hemizygous transgenic lines

All *egr1*/d2EGFP transgenic rat pups (founders) derived from OVT mothers (4.2.2.5.2), confirmed by multiplex PCR and Southern analysis (6.2.4.1.1 and 6.2.4.1.2, respectively), were weaned on post-natal day 21 into individual cages. In order to establish transgenic lines, transgenic founders were mated with a wild-type rat on reaching sexual maturity (6-9 week old) for 7-14 days. The 'fertilised' females were then individually caged and supplied with shredded paper 2 days prior to the expected date of birth.

All progeny derived from each founder were sexed and ear-marked between post-natal days 10-14, and genotyped, as described in 6.2.4. After weaning on post-natal day 21, the first generation (F1) of *egr1*/d2EGFP transgenic rat pups were caged separately from non-transgenic rat pups for each transgenic line. The breeding process was repeated with either the founder or a transgenic descendant for each transgenic line, depending on experimental demand. Given that the transgene is in the founder germline, Mendelian inheritance predicts that 50% of offspring from hemizygous founders should be transgenic. Where possible, 2 hemizygous transgenic male rats were maintained for each transgenic line for breeding purposes, since males are able to mate with numerous females at one time, and do not require additional time to allow for births and weaning to occur.

6.2.6 Transgene expression analysis

Breeding lines of transgenic rats were established and maintained from founders, and subsequent generations were used for transgene expression analysis. (Non-transgenic litter-mates were used as experimental controls.)

The transgene used in the present study is convenient in that it contains the EGFP reporter gene. This allows transgene-specific (non-endogenous) expression to be analysed since EGFP does not exist in the mammalian genome. Transgene expression is manifested initially by transcript production within cells. RT-PCR (see 4.2.5),

using (trans)gene-specific oligonucleotide primers, and Northern analysis (see below), using a transgene-specific probe, can be used to identify whether these transcripts are present within a population of cells.

Northern analysis is used to detect the presence of a specific sequence (target) within RNA samples extracted from a population of cells, and is essentially a technique based upon the complementarity between the target and a radioactively-labelled fragment (probe). In this respect, the procedure is similar to that of Southern analysis. The procedure consists of 7 stages:-

i) tissue sampling;
ii) total RNA extraction from rat tissues;
iii) sample, gel and northern blot preparation;
iv) probe preparation;
v) hybridisation of the probe to the northern blot;
vi) stringency washes;
vii) detection and visualisation of the hybridised probe.

All determinations of transgene expression were confirmed with duplicate experimental animals/groups.

6.2.6.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

6.2.6.1.1 Tissue sampling

Male and female adult SD rats (2-5 month old) were killed by stunning and decapitation, according to UK Home Office Regulations (Schedule 1 humane killing methods). Various tissues (see 6.2.6.1.1.1) were immediately dissected out (~mid-day), placed into a 1.5ml microcentrifuge tube and placed on dry ice for prompt freezing. The tissues were transferred on dry ice, and either placed in a -70°C freezer for temporary storage, or immediately used for total RNA extraction using a GTC-phenol-chloroform extraction method (see 2.3.1.1).

6.2.6.1.1.1 Brain and pituitary sampling

The roof of the rat skull was excised using a pair of sharp dissecting scissors, and the brain was removed using a pair of blunt curved forceps. The rat brain was laid ventral side up, and bisected in the coronal plane at both the rostral and caudal ends of the optic chiasm (see appendix E), and a sample of the cortex was taken using a scalpel. Whole pituitary glands were immediately dissected out, subsequent to brain excision, using a pair of fine curved watchmakers forceps.

6.2.6.1.2 RT-PCR

Total RNA extracted from rat tissues (see 6.2.6.1.1) was treated with DNase I, purified by P/C/IAA extraction, and used to synthesise first-strand cDNA with reverse transcriptase, as described in 2.3.2. The subsequent PCR reaction was performed as described in 4.2.5.1.2.

6.2.6.2 Northern analysis

6.2.6.2.1 Tissue sampling (following experimental stimuli) and total RNA extraction

Male and female adult SD rats (2-4 month old) were killed by stunning and decapitation, according to UK Home Office Regulations (Schedule 1 humane killing methods; 1 hour after an injection of either pentylenetetrazole (metrazole; 50mg/kg, i.p.; Sigma,-Aldrich, Poole, Dorset, UK) or sterile water, where applicable). Various tissues (see 6.2.6.2.1.1 and 6.2.6.1.2) were immediately dissected out, placed into a 1.5ml microcentrifuge tube and placed on dry ice for prompt freezing. The tissues were transferred on dry ice, and either placed in a -70°C freezer for temporary storage, or immediately used for total RNA extraction using a GTC-phenol-chloroform extraction method (see 2.3.1.1).

6.2.6.2.1.1 Brain and pituitary sampling

The roof of the rat skull was excised using a pair of sharp dissecting scissors, and the brain was removed using a pair of blunt curved forceps. The rat brain was laid ventral side up, and bisected in the coronal plane at both the rostral and caudal ends of the optic chiasm, using a scalpel (see appendix E). The resultant coronal brain slice was then laid flat (caudal end facing down), and a sample of the hippocampus was obtained using a scalpel and a pair of fine curved watchmakers forceps. A sample of the cortex was taken using a scalpel, and whole pituitary glands were immediately dissected out, subsequent to brain excision, using a pair of fine curved watchmakers forceps.

6.2.6.2.1.2 Liver, kidney and heart sampling

Each rat was placed on its back, and an incision was made in the skin and abdominal wall with a sharp pair of dissection scissors, to expose the abdominal cavity. Samples of the liver, kidney and heart were obtained, using a pair of curved dissecting forceps to grip the respective tissues, and a sharp pair of dissection scissors to excise a portion of the respective tissues.

6.2.6.2.2 Sample, gel and Northern blot preparation

RNA samples were prepared, fractionated by gel electrophoresis, and transferred to a nylon membrane, as described in 2.3.3.

6.2.6.2.3 Probe preparation

An EGFP probe was prepared as described in 4.2.3.1.1.2.2. An 18S (Decatemplate, Ambion) probe was used as an internal standard for the northern blot.

6.2.6.2.4 Hybridisation of the probe to the Northern blot

Northern blot membranes (6.2.6.2.2) were treated and hybridised with the EGFP and 18S probes (6.2.6.2.3), as described in 2.2.5.5.

6.2.6.2.5 Stringency washes

Stringency washes were performed as described in 2.2.5.6.

6.2.4.1.2.5 Detection and visualisation of the hybridised probe

The hybridised probe was detected and visualised by exposing the membrane to a radiation sensitive material (X-ray film for 2-3 days, or, Phosphor screen for 1-2 days), as described in 2.2.5.7.

6.3 RESULTS

6.3.1 Transgenesis

6.3.1.1 Preparation of transgene DNA for micro-injection

1% 1xTAE agarose gel electrophoresis resolution of *Aat*I and *Bst*II restriction fragments of the *p-egr-1*/d2EGFP plasmid yielded a 3 kb fragment (mixture of transgene and vector). The 3 kb fragment was extracted from the gel (see Figure 6.5**A**), using the QIAEX II gel extraction kit (Qiagen), and subjected to *Xmn*I restriction digestion, to cleave the vector from the transgene. Subsequent 1% 1xTAE agarose gel electrophoresis resolution of restriction fragments yielded a 3 kb *egr-1*/d2EGFP transgene fragment, and 2kb and 1kb vector fragments, respectively. The transgene fragment (see Figure 6.5**B**) was extracted from the gel, using the QIAEX II gel extraction kit (Qiagen), purified through a Sephadex G-50 spin column, adjusted to 2-5ng/µl with MITE buffer, and filter-sterilised, ready for micro-injection.

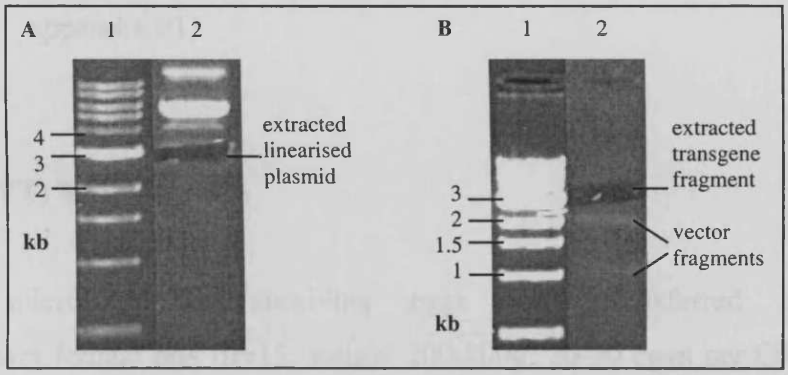


Figure 6.5 Gel electrophoresis resolution of the *p-egr-1*/d2EGFP plasmid. **A** Lane 1 corresponds to the molecular weight marker (1kb ladder, NEB). Lane 2 corresponds to the plasmid DNA linearised with restriction enzymes *Aat*I and *Bst*II, yielding a 3 kb band (transgene and vector fragment). **B** Lane 1 corresponds to the molecular weight marker (1kb ladder, NEB). Lane 2 corresponds to the 3 kb *egr-1*/d2EGFP transgene fragment, following cleavage from the vector (2kb and 1kb bands, respectively) by *Xmn*I restriction digestion.

6.3.1.2 Microinjection data

A total of 63 female rats were used for superovulation (~40 day old; ~130g), yielding 778 eggs for micro-injection over 9 transgenesis sessions (raw data in appendix F1). 52% of harvested and micro-injected eggs were suitable for oviduct transfer (OVT), with the other eggs either non-injectable (34%; no clear pronucleus) or were lysed during the micro-injection procedure (12%; see table 6.2 and appendix F1).

	%
Non-injectable eggs	**34**
Micro-injected eggs	**66**
Surviving injected eggs	**53**
Total eggs available for transfer	**52**

Table 6.2 A summary of the micro-injection data accumulated over 9 transgenesis sessions. Data is presented in % values (raw data in appendix F1).

6.3.1.3 (OVT) Transfer data

Following micro-injection, surviving eggs were transferred into mature pseudopregnant female rats (n=15; weight 200-300g; 20-30 eggs per OVT; see table 6.3 and raw data in appendix F2). Following successful transfer, pups were born 22-23 days later (n=56; ~4 pups per litter; see table 6.3 and raw data in appendix F2 and F3). PCR and Southern blot analysis confirmed that 6 of these offspring were transgenic (see 6.3.2).

	Day 0 Bilateral	Day 0 Unilateral	Total
Total number of eggs used for OVT	70	338	**408**
Number of OVT's	(2x) 1	14	(1+) **15**
Average number of eggs / OVT	(2x) **35**	**24**	**27**
Number of OVT's with live births	0	10	**10**
Number of live pups	0	56	**56**
Average number of pups / OVT	**0**	**5.6**	**5.6**
Number of pups surviving	N/A	56	**56**
Number of transgenics generated	**0**	**6**	**6**

Table 6.3 A summary of the OVT data accumulated over 9 transgenesis sessions. (Raw data and % values in appendix F2 and F3).

6.3.2 Genotyping of *egr1*/d2EGFP transgenic rats

6.3.2.1 Screening of progeny for transgene incorporation

PCR and Southern blot analysis (see 6.3.2.1.1 and 6.3.2.1.2, respectively) confirmed that 6 of the offspring contained the exogenous EGFP sequence, thus indicating successful genomic integration of the transgene.

6.3.2.1.1 Genomic screening by PCR analysis

PCR amplification of genomic DNA derived from litters of OVT mothers, using primers specific for the EGFP and actin genes, was used to identify 6 transgenic founder rats, designated Z13, Z14, Z16, Z22, Z25 and Z27, respectively (see Figure 6.6 for example; Z refers to the *egr1*/d2EGFP transgene, and the number refers to the pup number). Transgenic lines were established from the respective founders (Z13, Z14, Z16, Z25 and Z27), and maintained as described in 6.2.5. All transgenic progeny from founders and transgenic descendants of each transgenic line were confirmed by Southern analysis (see Figures 6.8, 6.9, 6.10 and 6.11, respectively). No breeding line was established from the Z22 founder, however, since the transgene failed to transmit to the F1 generation (see Figure 6.9), therefore the Z22 founder was subsequently killed.

Genotyping of the various Z lines of *egr1*/d2EGFP transgenic rats are shown in Figures 6.6, 6.8, 6.9, 6.10, and 6.11, respectively.

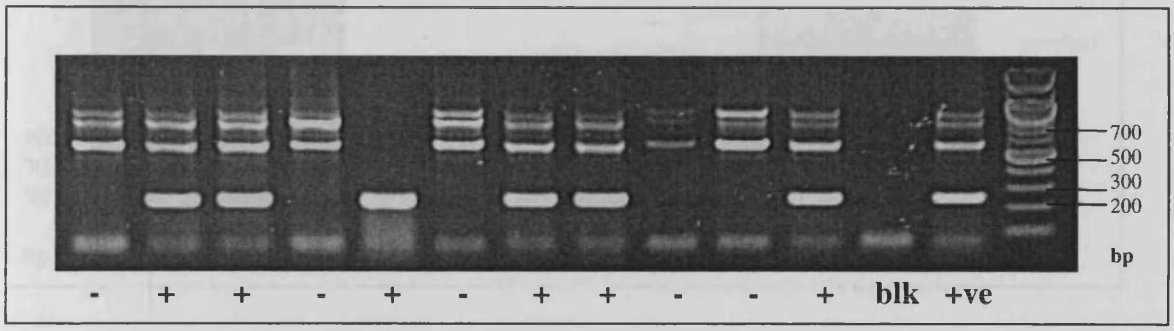


Figure 6.6 PCR amplification (using appropriate primers) of **Z16** transgenic (**+**) and non-transgenic (**-**) rat genomic DNA, extracted from tail or ear biopsies of a third generation Z16 litter, and resolved on an agarose gel. An EGFP-specific band (237 bp) is visible in transgenic samples, and an actin-specific band (581 bp) and 2 actin pseudogene bands are visible in all genomic DNA samples. The lanes marked **blk** and **+ve** correspond to blank and positive control samples, respectively; the right lane represents a 100 bp molecular weight marker (NEB).

6.3.2.1.2 Genomic screening by Southern analysis

6.3.2.1.2.1 Probe preparation

1% 1xTAE agarose gel electrophoresis resolution of *BamH*I and *Hind*III restriction fragments of the *p-egr-1*/d2EGFP plasmid yielded a 736bp EGFP fragment (probe). The 736 bp EGFP probe was extracted from the gel (see Figure 6.7**A**), using the QIAEX II gel extraction kit (Qiagen), and the resultant pellet was resuspended in 35 μl of TE. An aliquot of the probe was quantitated using the Hyperladder I molecular weight marker (Bioline; see Figure 6.7**B**). Radioactively-labelled probes were synthesised from 20-50ng of DNA using the ^{32}P random primer labelling method (see 2.2.5.4.1), and purified by size exclusion chromatography (see 2.2.5.4.2).

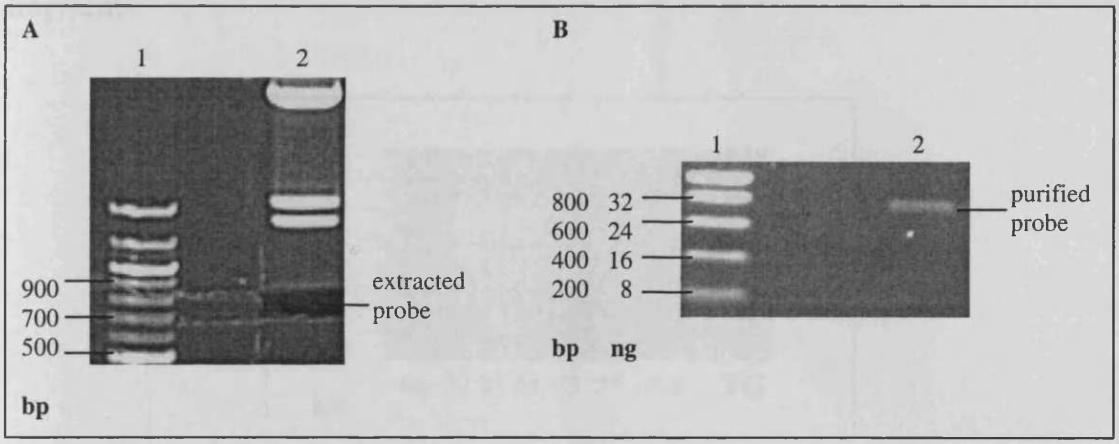


Figure 6.7 Gel electrophoresis resolution of the 736 bp EGFP probe. **A** Lane 1 corresponds to the molecular weight marker (1kb ladder, NEB). Lane 2 corresponds to the *p-egr-1*/d2EGFP plasmid linearised with restriction enzymes *BamH*I and *Hind*III, yielding a 736 bp band (EGFP probe). **B** Lane 1 corresponds to the molecular weight marker (Hyperladder I (Bioline), 8 μl of a 1:4 dilution). Lane 2 corresponds to an aliquot (2 μl) of the 736 bp EGFP probe, following purification using the QIAEX II gel extraction kit (quantitated here as 3.5 ng/μl).

6.3.2.1.2.2 Southern blot analysis

6.3.2.1.2.2.1 Southern blot analysis of founder transgenic rats

Southern blot analysis of *Xba*I-restricted genomic DNA extracted from Z13, Z14, Z16, Z22, Z25 and Z27 founder rats revealed the presence of a major band (~1.2 kb) on phosphor screen images (see Figure 6.8 for Z22, Z25 and Z27 data; see Figure 6.9 for other Z13, Z14 and Z16 data), which appears to represent hybridisation of the EGFP-specific probe to *Xba*I-flanked d2EGFP coding sequences (see Figure 6.1) that would be formed in a head to tail tandem array of transgene integration. Southern blot analysis of genomic DNA extracted from Z13, Z14, Z16, Z25 and Z27 founder rats also revealed the presence of other bands (see Figure 6.8 for Z25 and Z27 data; see Figure 6.9 for other Z13, Z14 and Z16 data), and may represent various junction fragments.

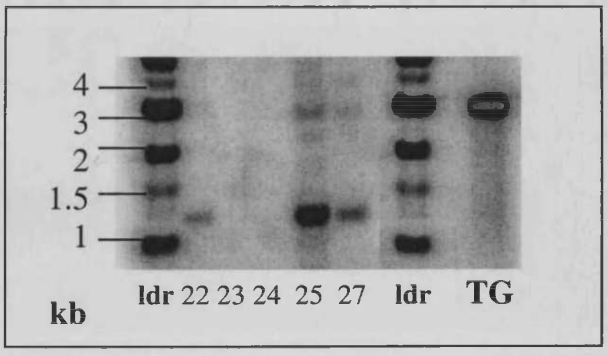


Figure 6.8 Phosphor screen image of *Xba*I-restricted rat genomic DNA (10μg), extracted from tail or ear biopsies of OVT litter pups (numbered accordingly), and resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (OVT litter pup numbers 22, 25 and 27; **Z22**, **Z25** and **Z27** transgenic founders, respectively). **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment; **ldr** represents a 1 kb DNA ladder molecular weight marker (NEB).

6.3.2.1.2.2.2 Southern blot analysis of first generation (F1) Z13, Z14, Z16 and Z22 transgenic rats

Southern blot analysis of *Xba*I-restricted genomic DNA extracted from Z13, Z14 and Z16 first generation (F1) transgenic rats revealed the presence of a major band (~1.2 kb) and other bands (junction fragments) on phosphor screen images (see Figure 6.9). These observations were consistent with the respective Z line founder transgenic rat (data not shown), and therefore confirm stable inheritance of the *egr1*/d2EGFP transgene. Southern blot analysis indicated that the Z13, Z14 and Z16 transgenic lines have a relatively low number of transgene copies, compared to that of the Z25 and Z27 transgenic lines which have relatively high copy numbers, respectively (see Figure 6.8), and that the transgene failed to transmit to the F1 generation of the Z22 line (see Figure 6.9).

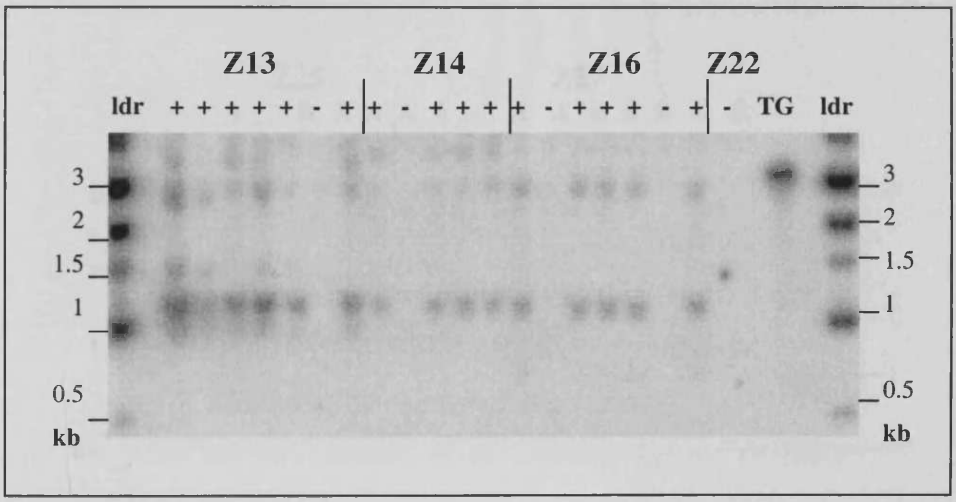


Figure 6.9 Phosphor screen image of *Xba*I-restricted rat genomic DNA (10μg), extracted from tail or ear biopsies of F1 litter pups from the respective **Z13**, **Z14**, **Z16** and **Z22** founder rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (+), and absent in non-transgenic samples (-). **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment; **ldr** represents a 1 kb DNA ladder molecular weight marker (NEB).

6.3.2.1.2.2.3 Southern blot analysis of first generation (F1) Z25 and Z27 transgenic rats

Southern blot analysis of genomic DNA extracted from Z25 and Z27 F1 generation transgenic rats revealed the presence of two distinct subtypes within each of these lines (see Figure 6.10). Southern blot analysis indicated one subtype (designated 'A') comprising a major band (~1.2 kb) and multiple junction fragments on phosphor screen images (see Figures 6.10 and 6.11), and another subtype (designated 'B') comprising a major band (~1.2 kb) and a minor band (~2.9 kb) on phosphor screen images (see Figures 6.10 and 6.11). Southern blot analysis revealed that the 'A' subtypes of the Z25 and Z27 transgenic lines have a relatively high number of transgene copies, compared to the 'B' subtypes of the Z25 and Z27 transgenic lines which have relatively low copy numbers (see Figures 6.10 and 6.11).

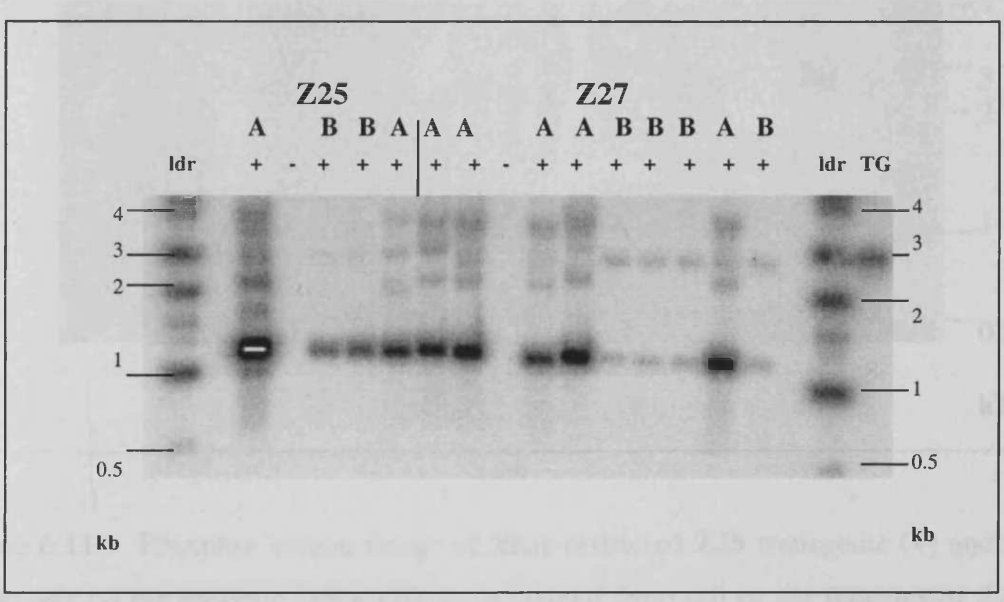


Figure 6.10 Phosphor screen image of *Xba*I-restricted rat genomic DNA (10μg), extracted from tail or ear biopsies of F1 litter pups from the respective **Z25** and **Z27** founder rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (+), and absent in non-transgenic samples (-). **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment; **ldr** represents a 1 kb DNA ladder molecular weight marker (NEB).

The breeding process was repeated with each subtype from the Z25 and Z27 lines (see Figure 6.10), respectively, and the subsequent observations (see Figure 6.11 for Z25-A and Z25-B data; Z27-A and Z27-B data not shown) were consistent with the respective parent (F1) transgenic rat. In addition to confirming stable inheritance of the *egr1*/d2EGFP transgene, these findings demonstrate that the original Z25 and Z27 founders were chimaeric. This would explain the different subtypes (A or B) inherited by the F1 progeny of the Z25 and Z27 founders (see Figure 6.10), and the subsequent lines generated from them (Z25-A and Z25-B, and Z27-A and Z27-B lines, respectively; see Figure 6.11).

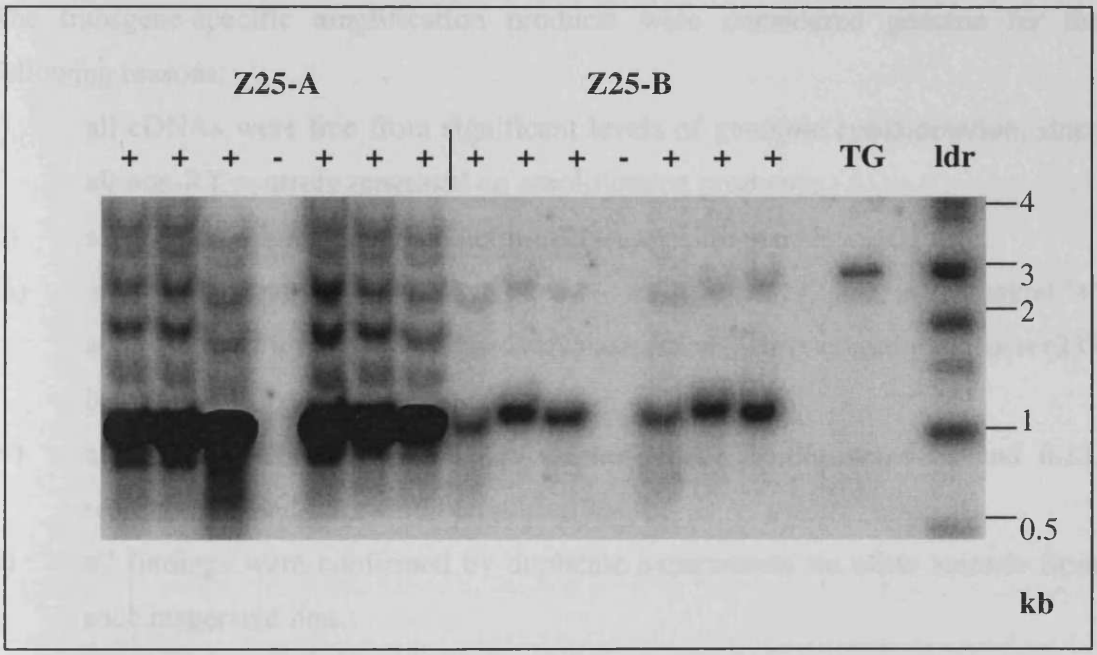


Figure 6.11 Phosphor screen image of *Xba*I-restricted **Z25** transgenic (+) and non-transgenic (-) rat genomic DNA (10μg), extracted from tail or ear biopsies of **Z25-A** and **Z25-B** litter pups from the respective parent (F1) transgenic rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (+), and absent in non-transgenic samples (-). **TG** corresponds to an aliquot of the full-length micro-injection transgene fragment; **ldr** represents a 1 kb DNA ladder molecular weight marker (NEB).

6.3.3 Transgene expression analysis

6.3.3.1 RT-PCR

RT-PCR was used to determine whether transgene transcript expression was present in the Z25 and Z27 transgenic lines. RT-PCR analysis revealed that both subtypes (A and B, respectively) of the Z25 and Z27 transgenic lines generated a transgene specific amplification product (237 bp) from cDNA synthesised from total RNA derived from pituitary and cortical samples (see Figures 6.12 and 6.13, respectively). The transgene-specific amplification products were considered genuine for the following reasons:

i) all cDNAs were free from significant levels of genomic contamination, since all non-RT controls generated no amplification products;

ii) all cDNA samples amplified actin-mRNA specific products (581 bp);

iii) all positive controls (see lane marked '**+**' in Figure 6.12, and lanes marked '**+**' and '**+ve**' in Figure 6.13, respectively) amplified EGFP-specific products (237 bp);

iv) all negative controls (see lanes marked '**blk**' in Figures 6.12 and 6.13, respectively) did not exhibit amplified bands;

v) all findings were confirmed by duplicate experiments on other animals from each respective line.

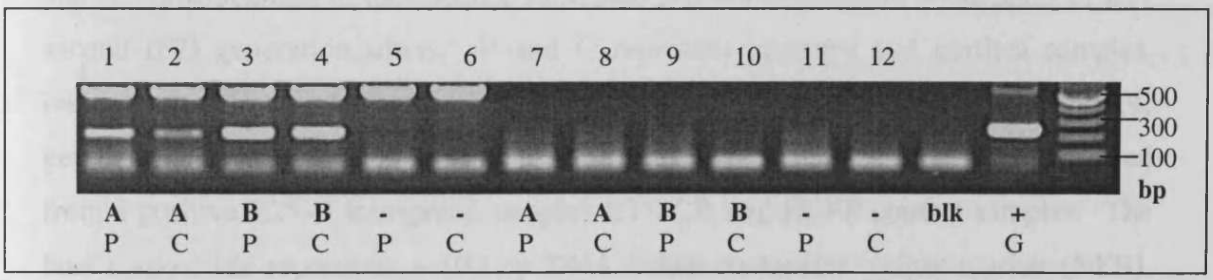


Figure 6.12 PCR amplification (using specific primers) of **Z25** rat cDNA template (lanes 1-6), and non-RT controls (lanes 7-12), derived from transgenic (**A** and **B**;

corresponding to the specific subtypes) and non-transgenic (-) samples of Z25 second (F2) generation litters. P and C represent pituitary and cortical samples, respectively. The lanes marked **blk** and (+) correspond to blank and positive (genomic DNA (G) derived from a positive transgenic sample) RT-PCR and EGFP control samples. The right lane represents a 100 bp DNA ladder molecular weight marker (NEB). EGFP-specific bands (237 bp) are visible in the **+ve** cDNA samples, and actin-specific bands (581 bp) are visible in all cDNA samples.

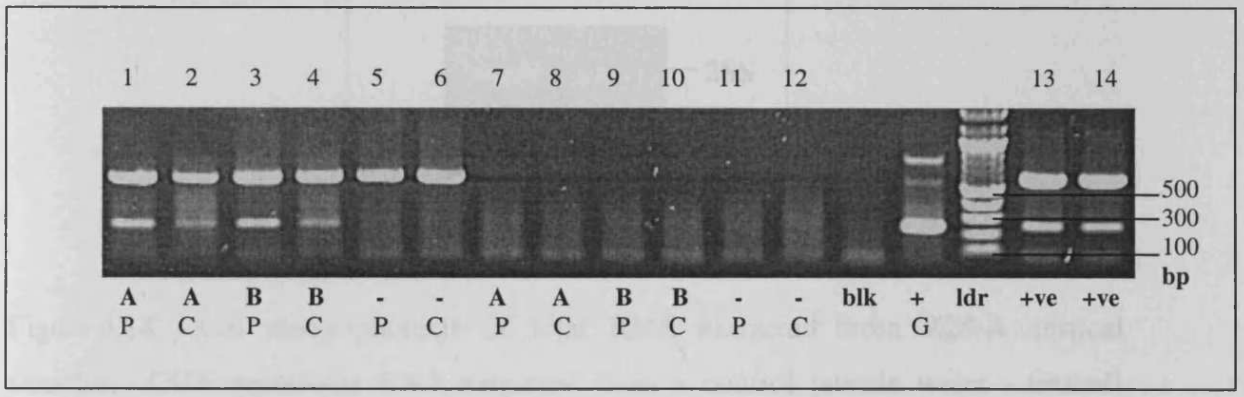


Figure 6.13 PCR amplification (using specific primers) of **Z27** rat cDNA template (lanes 1-6, and 13-14), and non-RT controls (lanes 7-12), derived from transgenic (**A** and **B**; corresponding to the specific subtypes) and non-transgenic (-) samples of Z27 second (F2) generation litters. P and C represent pituitary and cortical samples, respectively. The lanes marked **blk**, **+** and **+ve** correspond to blank and positive (**+**: genomic DNA (G) derived from a positive transgenic sample; **+ve**: cDNA derived from a positive Z25-B transgenic sample) RT-PCR and EGFP control samples. The lane marked **ldr** represents a 100 bp DNA ladder molecular weight marker (NEB). EGFP-specific bands (237 bp) are visible in the **+ve** cDNA samples, and actin-specific bands (581 bp) are visible in all cDNA samples.

6.3.3.2 Northern blot analysis

Northern blot analysis was used to detect levels of transgene transcript expression in various tissues of the Z13, Z25-A and Z27 (A and B) lines.

6.3.3.2.1 RNA integrity

The integrity of the RNA samples was checked prior to use in northern blot analysis (see Figure 6.14 for example).

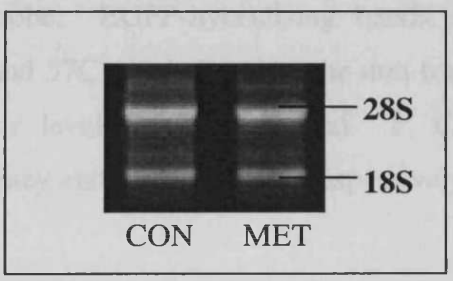


Figure 6.14 Gel electrophoresis of total RNA extracted from **Z25-A** cortical samples. CON represents RNA extracted from a control (sterile water - treated) animal, and MET represents RNA extracted from a metrazole-treated animal. The 28S and 18S RNA bands are indicated (~5kb and ~2kb, respectively; molecular weight marker not shown).

6.3.3.2.2 Northern blot analysis of basal transgene expression

Northern blot analysis of the basal pattern of EGFP (transgene) expression in the Z27 transgenic line revealed highest levels of hybridisation in the pituitary and cortex, followed by the liver, and relatively low levels in the kidney and heart, in both A and B subtypes of the Z27 transgenic line, respectively (see Figure 6.15). Phosphor screen images also indicated higher hybridisation levels in the Z27 pituitaries compared to that of the 57C transgenic line.

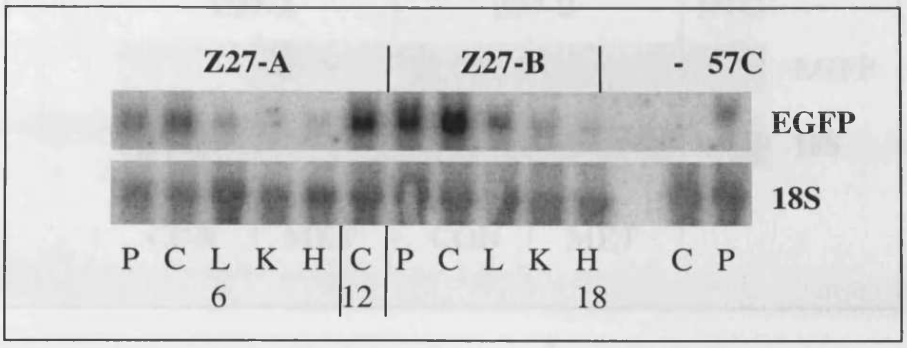


Figure 6.15 Phosphor screen images of total RNA (6, 12 or 18μg, as indicated above), extracted from male **Z27** (A and B) rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific (above) and 18S (below) radio-labelled probe. EGFP-hybridising bands are visible in transgenic samples (Z27-A, Z27-B and 57C), and absent in the non-transgenic sample (-). 18S-hybridising bands indicate levels of RNA loaded. P, C, L, K and H represent pituitary, cortex, liver, kidney and heart samples, respectively.

6.3.3.2.3 Northern blot analysis of transgene up-regulation

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to a (generalised, pharmacological) neural stimulus was investigated using the convulsant metrazole, which has been used in previous studies of endogenous *egr-1* induction (Saffen *et al.*, 1988; Mack *et al.*, 1990; Morita *et al.*, 1996). Control and metrazole-induced transgene expression was determined, by northern analysis, in the cortex, hippocampus and liver of the Z27 (A and B) transgenic line(s) (see Figure 6.16), and in the hippocampus of the Z13 transgenic line (see Figure 6.17). Following metrazole treatment, dramatic increases in transgene expression, compared with control animals, were observed. The lack of apparent transgene expression in the cortex, hippocampus and liver of the control Z27A transgenic rat (see Figure 6.16 and compare with Figure 6.15) may be accounted for by an insufficient exposure time. However, northern analysis failed to detect any transgene expression in the cortex, hippocampus and liver of both the control and metrazole-treated Z25-A transgenic rats (see Figure 6.18).

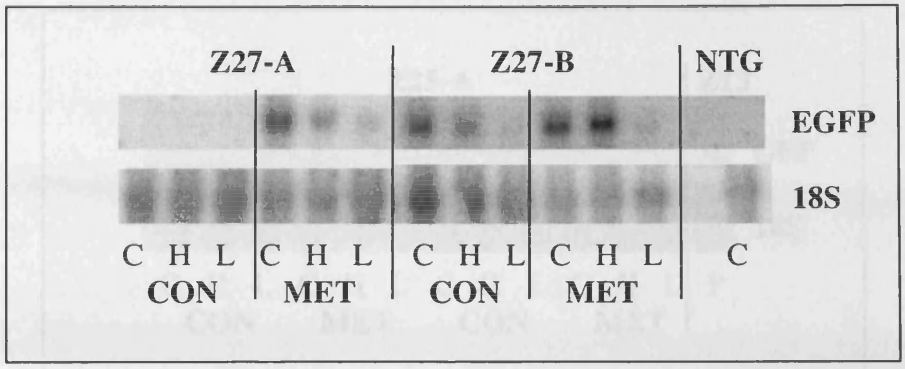


Figure 6.16 Phosphor screen images of total RNA (7μg), extracted from male **Z27** (A and B) rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific (above) and 18S (below) radio-labelled probe. EGFP-hybridising bands are visible in samples taken from sterile water - treated Z27-B transgenic rats (**Z27-B CON**), in addition to samples taken from metrazole-treated Z27 transgenic rats (**Z27-A MET** and **Z27-B MET**), and absent in the samples taken from sterile water - treated Z27-A transgenic rats (**Z27-A CON**) and the non-transgenic sample (**NTG**). 18S-hybridising bands indicate levels of RNA loaded. C, H and L represent cortical, hippocampal and liver samples, respectively.

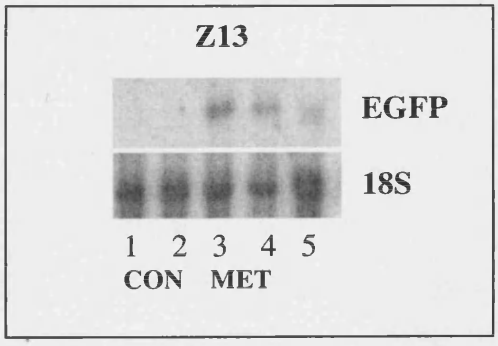


Figure 6.17 Phosphor screen images of total RNA (7μg), extracted from female **Z13** rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific (above) and 18S (below) radio-labelled probe. Lanes 1-4 represent hippocampal samples, and lane 5 represents a pituitary sample. EGFP-hybridising bands are visible in samples taken from metrazole-treated Z13 transgenic rats (**MET**), in addition to the pituitary sample, and absent in the samples taken from sterile water - treated Z13 transgenic rats (**CON**). 18S-hybridising bands indicate levels of RNA loaded.

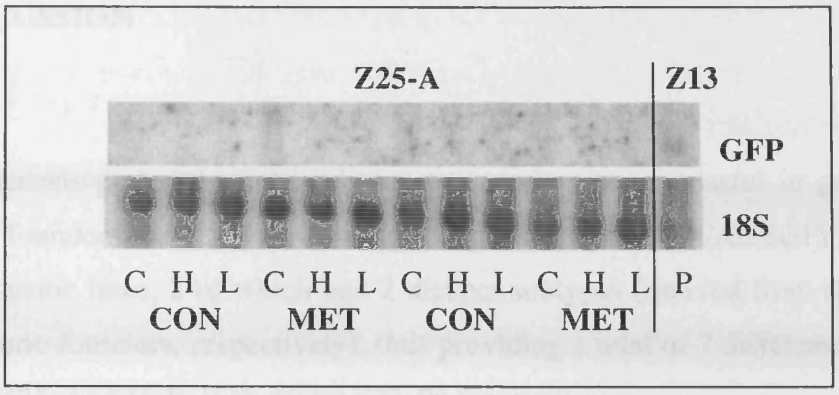


Figure 6.18 Phosphor screen images of total RNA (6μg), extracted from male **Z25-A** rats, resolved on an agarose gel, transferred to a nylon membrane and probed with an EGFP-specific (above) and 18S (below) radio-labelled probe. **CON** represents samples taken from sterile water - treated Z25-A transgenic rats, and **MET** represents samples taken from metrazole-treated Z25-A transgenic rats. C, H, L and P represent cortical, hippocampal, liver and pituitary samples, respectively. EGFP-hybridising bands are only visible in the **Z13** pituitary sample. 18S-hybridising bands indicate levels of RNA loaded.

6.4 DISCUSSION

The transgenesis protocol used in the present study was successful in generating 6 transgenic founders, each demonstrating integration of the *egr1*/d2EGFP transgene, and 5 transgenic lines, 2 of which had 2 distinct subtypes (derived from the Z25 and Z27 chimaeric founders, respectively), thus providing a total of 7 different lines (Z13, Z14, Z16, Z25-A, Z25-B, Z27-A and Z27-B) for analysis.

6.4.1 Optimisation of transgenesis

The present study indicates that the egg survival rate following micro-injection (81%; see appendix F1) was over twice as efficient as that of published data (compared to 36.5-39.4%, Hochi *et al.* 1990, Swanson *et al.* 1992, Charreau *et al.* 1996b). Numerous factors may have contributed to the marked increase in egg survival (see 4.4.1).

In the present study, all (mainly unilateral) OVTs were performed on the same day (day 0) as micro-injection of the fertilised one-cell eggs. Consequently, there was no data to statistically compare efficiencies between day 0 and day 1 OVTs, respectively. It appears that day 0 unilateral OVTs may be more efficient (see appendix F2 for data), since the procedure limits the amount of time the eggs are exposed to conditions outside of their natural environment, and consequently allows the eggs to continue to develop naturally (i.e. to the 2-cell stage) *in vivo* within 1-5 hours subsequent to micro-injection. In addition, day 0 OVTs allow the subsequent developing embryos and surrogate (pseudopregnant recipient) mothers to be at complementary stages of development. It can be argued that unilateral OVTs are more efficient than bilateral OVTs, since less invasive surgery is performed, the amount of time the pseudopregnant recipient mothers are subjected to anaesthesia is restricted, and therefore enables improved post-surgical recovery and entry into a normal gestation period. Again, there was insufficient data to statistically compare efficiencies between unilateral and bilateral OVTs, respectively.

In summary, transgenesis was optimised in the rat, and subsequently resulted in the successful generation of 6 founder transgenic rats. The increase in the overall transgenesis efficiency of the present study (67% - see appendix F3), compared to that of the *mPer1*/d2EGFP study (31%) could be due to various factors such as increased technical experience and the use of a smaller transgene (see 4.4.1).

6.4.2 Transgene expression analysis

The present study used a transgenic approach to demonstrate that proximal regions of the *egr-1* gene can direct tissue-specific and pharmacologically-regulated transcript expression in the rat. The *egr1*/d2EGFP transgene was successfully integrated into the rat genome, as shown by PCR and Southern analysis (see 6.2.4) of genomic DNA derived from founder (or descendant) transgenic rats. The present transgenic rat model study demonstrates that the *egr-1*/d2EGFP transgene (including only proximal 5'-flanking sequences of the *egr-1* gene) confers both tissue-specific constitutive- and inducible transcript expression in the brain and liver, as shown by RT-PCR and northern analysis (see below). Each of these aspects of transgene expression mirror expression of the endogenous *egr-1* gene (see Slade *et al.*, 2002).

The Z25-A (highest apparent copy number) transgenic line expressed transgene transcripts in the pituitary and cortex, as shown by RT-PCR analysis (see Figure 6.12). However, northern blot analysis of the Z25-A transgenic line failed to detect transgene transcript expression in the cortex, hippocampus and liver (see Figure 6.18). This is a surprising result and appears to reflect a relatively low level of transgene expression in this line, that is nevertheless detectable by RT-PCR. The low level of expression may be due to incorporation of the transgene into a relatively inhibitory region of a particular chromosome. The Z27-A (high copy number) and Z27-B (lower copy number) transgenic lines expressed transgene transcripts in the pituitary and cortex, as shown by RT-PCR analysis (see Figure 6.13). Furthermore, northern blot analysis of the Z27-A and Z27-B transgenic lines detected highest levels of transgene transcript expression in the pituitary and cortex, followed by the liver, and relatively low levels in the kidney and heart (see Figure 6.15), and (metrazole-) induction of transgene transcript expression in the cortex, hippocampus and liver (see

Figure 6.16). Northern blot analysis of the Z13 (low copy number) transgenic line also detected (metrazole-) induction of transgene transcript expression in the hippocampus (see Figure 6.17).

These findings parallel the results obtained with an *egr-1* transgenic mouse model (Tsai *et al.*, 2000) in which 5'-flanking sequences of the murine *egr-1* gene were shown to direct both constitutive expression in brain, heart and liver, and also hepatectomy-associated gene induction in liver. The present findings are also consistent with those of the previous *egr1*/d4EGFP transgenic rat model (Slade *et al.*, 2002), but indicate that the previous inferences about the potential importance of the *egr-1* intron in conferring appropriate expression are not valid. However, in order to fully investigate the role of intronic sequences, it would be necessary to compare both constitutive and induced expression between multiple lines of intron (+) and intron (-) lines. This analysis is beyond the scope of the present study.

The findings of the present study indicate that proximal elements within the *egr-1* gene are sufficient to, at least broadly, recapitulate expression of the endogenous *egr-1* gene in the rat. Taken together with extensive *in vitro* evidence of the regulatory capacity of the *egr-1* proximal 5'-flanking sequence (Changelian *et al.*, 1989; Sakamoto *et al.*, 1991; DeFranco *et al.*, 1993; Cohen *et al.*, 1996; Yan *et al.*, 1999; Bernal-Mizrachi *et al.*, 2000), it can be further argued that proximal regions of the *egr-1* gene alone are sufficient to confer an appropriate pattern of expression.

6.4.3 Future Directions

The findings of the present study show that expression of the *egr1*/d2EGFP transgene recapitulates that of the endogenous gene, and reflects observations in the *egr-1* transgenic mouse and rat models (Tsai *et al.*, 2000, and Slade *et al.*, 2002, respectively). In order to establish the relative importance of intronic *egr-1* sequences in conferring both basal and regulated transgene expression, further analysis is required. Therefore, having established robust transcription of the transgene in the present model, it is necessary for future studies to demonstrate that the transgene is expressed robustly at the protein level (see Chapter 7).

CHAPTER 7

A TRANSGENIC RAT MODEL FOR STUDYING THE REGULATION OF EGR-1 GENE EXPRESSION II

B. PROTEIN ANALYSIS

7.1 OVERVIEW AND AIMS OF STUDY

The aim of the present study was to investigate *egr1*/d2EGFP transgene expression at the protein level and its induction in the rat brain and other tissues.

7.1.1 Transgene expression analysis

A wide range of techniques are available for detecting transgene expression. Typically, RNA-based methods such as RT-PCR, northern blot analysis, and *in situ* hybridisation (as demonstrated in the previous chapters) are commonly used to identify the presence of transgene-specific transcripts (Murphy and Carter, 1993; Glover and Hames, 1995). The previous experiments were designed to involve a transgene-specific (non-endogenous) sequence, and therefore allowed transgene expression to be easily identified. For example, the EGFP reporter gene sequences used in the previous 3 studies were convenient since they do not exist in the mammalian genome. However, the aforementioned techniques can only determine transgene transcript expression, therefore, other techniques, such as western analysis and immunohistochemistry (IHC), are required to establish whether these transcripts are translated into mature proteins. This is particularly important in the present study because this model has been designed to permit direct analysis of GFP fluorescence.

It should be noted that in the previous *egr-1*/GFP model, fluorescence could not be detected (Chapter 5).

7.1.1.1 Experimental aims

The aims of the present study are:

1. To investigate the regulation of both endogenous *egr-1* and transgene expression in the Z line transgenic rat by:

   a) Investigating transgene expression in the *Egr1*/d2EGFP (Z) line transgenic rat pituitary gland, and transgene induction within the Z transgenic rat brain and liver, following metrazole treatment, using optimised western blot protocols.

   b) Localising transgene expression in the Z line transgenic rat pituitary gland using IHC, and co-localising endogenous- and trans- gene expression using an optimised dual tyramide signal amplification (TSA) / IHC protocol.

   c) Investigating transgene induction within the Z line transgenic rat brain, following nocturnal photic stimulation, using IHC, and co-localising endogenous- and trans- gene induction within the Z line transgenic rat brain, following nocturnal photic stimulation, using the optimised dual TSA / IHC protocol.

   d) Directly visualising transgene expression in the Z line transgenic rat (following nocturnal photic stimulation), using fluorescence microscopy.

2. To define the regions of regulatory sequence required for conferring physiologically-regulated *egr-1* gene expression in the rat brain and anterior pituitary gland.

7.2 METHODS

7.2.1 Animal procedures

All animal procedures were conducted according to UK Home Office regulations (Schedule 1 humane killing methods), and local ethical review. Sprague-Dawley rats were maintained in approved laboratory conditions on a 14 hour light - 10 hour dark cycle, on a reverse-lighting scheme (lights on at 20:00; lights off at 10:00), with food and water available *ad libitum*. Unless otherwise stated, all determinations of transgene expression were confirmed with duplicate experimental animals/groups.

7.2.2 Transgene protein expression analysis

Breeding lines (Z13, Z14, Z16, Z25-A, Z25-B, Z27-A and Z27-B) of transgenic rats were established and maintained from founders (see chapter 6), and subsequent generations were used for transgene expression analysis. (Non-transgenic litter-mates were used as experimental controls). As mentioned in 7.1.1, techniques such as western analysis and IHC are required to establish whether or not the transgene transcripts are translated into mature proteins.

Western analysis is conceptually similar to Southern and northern analysis; specific proteins are fractionated by size on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane for identification with the appropriate antibodies. The procedure consists of 5 stages:-

i) tissue sampling;
ii) protein extraction from rat tissues;
iii) sample, gel and western blot preparation;
iv) primary and secondary antibody incubations and washes;
v) chemiluminescent detection.

IHC can be used to detect the presence of specific proteins in cells or tissues, by using the appropriate antibodies. The procedure consists of 3 stages:-

i) tissue preparation;

ii) primary and secondary antibody incubations and washes;

iii) detection and visualisation by microscopy.

With respect to the present study, which involves the *egr1*/d2EGFP promoter/reporter transgenic rat model created as described in the previous chapter, fluorescence (transgene EGFP expression) may also be directly detected in freshly fixed tissue sections using a fluorescence microscope. The composition of all media, buffers, reagents and solutions used in experiments are as described in appendix A.

7.2.2.1 Western Analysis

7.2.2.1.1 Fresh frozen sampling of Z line rat tissues (following experimental stimuli)

Male and female adult SD rats (2-4 month old) were killed by stunning and decapitation, according to UK Home Office Regulations (Schedule 1 humane killing methods; 1 hour after an injection of either pentylenetetrazole (metrazole; 50mg/kg, i.p.; Sigma,-Aldrich, Poole, Dorset, UK) or sterile water, where applicable). Various tissues (see 7.2.2.1.1.1 and 7.2.2.1.1.2) were immediately dissected out, placed into a 1.5ml microcentrifuge tube and placed on dry ice for prompt freezing. The tissues were transferred on dry ice, and either placed in a -70°C freezer for temporary storage, or immediately used for protein extraction (7.2.2.1.2).

7.2.2.1.1.1 Brain and pituitary sampling

The roof of the rat skull was excised using a pair of sharp dissecting scissors, and the brain was removed using a pair of blunt curved forceps. The rat brain was laid ventral side up, and bisected in the coronal plane at both the rostral and caudal ends of the optic chiasm, using a scalpel (see appendix E). The resultant coronal brain slice was

then laid flat (caudal end facing down), and a sample of the hippocampus was obtained using a scalpel and a pair of fine curved watchmakers forceps. A sample of the cortex was taken using a scalpel, and then whole pituitary glands were immediately dissected out, using a pair of fine curved watchmakers forceps.

7.2.2.1.1.2 Liver sampling

Each rat was placed on its back, and an incision was made in the skin and abdominal wall with a sharp pair of dissection scissors, to expose the abdominal cavity. A sample of the liver was obtained, using a pair of curved dissecting forceps to grip the tissues, and a sharp pair of dissection scissors to excise a portion of the tissue.

7.2.2.1.2 Protein extraction from rat tissues

Tissue samples were homogenised in an appropriate volume of ice-cold protein extraction buffer, and then centrifuged and gently vortexed, if required, to collect and disperse the homogenate. The homogenate was frozen on dry ice for 5 minutes and then incubated on wet ice for 15 minutes, prior to centrifugation at 14,000 rpm, 4°C, for 10 minutes. The resultant supernatant was removed using a pipette and placed into a fresh 1.5ml microcentrifuge tube. An aliquot of each protein sample was used for quantification (7.2.2.1.2.1), and the remaining sample was then stored at -70°C, ready for Western analysis (see 7.2.2.1.3.2).

7.2.2.1.2.1 Quantification of protein samples

0.5-5µl of protein sample was diluted in sterile water (to a final volume of 800µl) in a cuvette, and 200µl of reagent (Bio-Rad Protein assay) was added. Each cuvette was covered with nescofilm and mixed by inversion. A water blank was also prepared to provide a reference sample. Samples were then quantitated using a calibrated spectrophotometer (Biophotometer, Eppendorf).

7.2.2.1.3 Sample, gel and western blot preparation

7.2.2.1.3.1 Polyacrylamide gel preparation

Gel electrophoresis plates were assembled according to the manufacturer's protocol (Bio-Rad Protean II). A 15% resolving gel was prepared by adding: 1.88ml 40% acrylamide/bisacrylamide mix (Promega; stored at 4°C), 1.88ml 1M Tris (pH 8.8), 50μl 10% SDS, 1.14ml sterile water, 50μl 10% APS (stored at 4°C), and 5μl TEMED (stored at 4°C). The gel mixture was applied between the electrophoresis plates (1cm below where the well-forming comb tip would be positioned), covered with isopropanol, and left to set for 30 minutes. A 5% stacking gel was prepared by adding: 320μl 40% acrylamide/bisacrylamide mix (Promega; stored at 4°C), 320μl 1M Tris (pH 6.8), 25μl 10% SDS, 1.72ml sterile water, 12μl 10% APS (stored at 4°C), and 5μl TEMED (stored at 4°C). The resolving gel was rinsed with distilled water, and blotted with 3MM Whatman paper, prior to the application of the stacking gel and subsequent insertion of the well-forming comb. The stacking gel was left to set for 15 minutes, and then rinsed with distilled water. The plates, containing the respective gels, were assembled in the electrophoresis tank, and running buffer was then added within and around the electrophoresis cell.

7.2.2.1.3.2 Pre-treatment and electrophoresis of protein samples

Protein samples (50μg; 7.2.2.1.2) were diluted to 8μl with protein extraction buffer, immediately prior to electrophoresis. Protein samples were mixed with 1 volume of sample buffer in a 0.5ml microcentrifuge tube, and boiled for 3 minutes on a thermal cycler, together with a 5μl aliquot of the pre-stained broad-range (6-175kDa) protein marker (NEB), and 1ng of rEGFP (27kDa recombinant EGFP, Clontech; used as a positive control). Samples were centrifuged at 14,000 rpm, RT, for 10 seconds, prior to immediate loading into the polyacrylamide gel, and were resolved at 150V, for 1-2 hours. The polyacrylamide gel was then removed from the plates by immersion in transfer buffer.

7.2.2.1.3.3 Western blot preparation

The protein within the polyacrylamide gel was subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham) using a Trans-blot system (Bio-Rad Protean II), as follows. An appropriate-sized piece of PVDF membrane was immersed in methanol, rinsed in water, and then allowed to equilibriate in transfer buffer for at least 5 minutes. The transblotting plastic frame was laid down, and a porous pad, 2 sheets of 3MM paper (soaked in transfer buffer), the polyacrylamide gel (on a piece of 3MM paper soaked in transfer buffer), the membrane, and 3 sheets of 3MM paper, were applied. Any air bubbles were removed by gentle rolling of a plastic tube. The final porous pad was then applied, and the frame closed and placed in the transfer tank. The Bio-Cooling unit was added to the transfer tank, and topped up with transfer buffer, prior to running the Trans-blot system at 100V for 1 hour, with a stirring magnet.

7.2.2.1.4 Antibody incubations and washes

Subsequent to protein transfer, the PVDF membranes were equilibrated in TBST for 15 minutes, and then blocked with 10ml of blocking solution (5% (w/v) milk powder in TBST), for 45 minutes. Blots were then rinsed with TBST, prior to incubation in 10ml of the primary antibody (1:200-1:1000 dilution of Living Colours A.V. GFP peptide antibody (#8367-2; Clontech), 1:4000 dilution of Egr-1 specific polyclonal anti-sera (C19; Santa Cruz Biotechnology), or, 1:1000-1:17500 dilution of GFP monoclonal antibody (#8362-1; Clontech)) for 45 minutes. Blots were subsequently rinsed with TBST, washed in TBST for 7 minutes, twice, and then incubated in 10ml of the secondary antibody (1:5000-1:10000 anti -rabbit or -mouse IgG, peroxidase-linked species specific whole antibody (from donkey or sheep; NA 934 or NA 931, respectively; Amersham) for 30 minutes. Following this, blots were rinsed with TBST, washed in TBST for 5 minutes, 3 times, and then TBS for 5 minutes.

7.2.2.1.5 Chemiluminescent detection

The membranes were finally developed using the ECL-Plus chemiluminescent detection system (Amersham), as follows. Excess TBS from the blots was removed by blotting the back of the membrane on 3MM paper. The blots were then incubated (on cling film) with a 40:1 mixture of solutions A and B of the ECL-Plus system (Amersham, RPN 2132), for 5 minutes at room temperature. Excess solution was blotted from the membrane which was placed between 2 pieces of acetate, prior to exposure to X-ray film (MXB; Kodak) for appropriate lengths of time (from 1 second to 3 minutes). The X-ray films were subsequently developed.

7.2.2.2 Immunohistochemistry (IHC)

7.2.2.2.1 Tissue preparation

7.2.2.2.1.1 Perfusion of tissues

Male and female adult SD rats (2-4 month old) were deeply anaesthetised (1 hour following nocturnal photic stimulation, where applicable) with an i.p. injection of sodium pentobarbitone (150mg/kg), and perfused (5ml/min) intracardially via the ascending aorta with 40ml of 0.01M phosphate buffered saline (PBS) at 37°C, then 40ml of ice-cold 0.01M PBS, followed by 200ml of ice-cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) for 20-30 minutes. Rats killed during the dark period were anaesthetised in the dark, and had their heads covered with a light-proof hood throughout perfusion.

7.2.2.2.1.2 Fixation of tissues

Brains and pituitary glands were removed from the perfused animal (7.2.2.2.1.1), post-fixed with 4% PFA in 0.1M PB for 90 minutes at 4°C, and cryoprotected overnight with 10% sucrose in 0.1M PB containing 0.01% sodium azide, at 4°C. A section containing the SCN was isolated as described (see 3.2.1.1), loosely wrapped in foil, and frozen by submersion in cold isopentane (maintained at -40°C with dry ice). Pituitary glands were submerged in Cry-M-Bed (Bright; supported by a small cylindrically-shaped piece of foil) and frozen on dry ice.

7.2.2.2.1.3 Tissue sectioning

Tissues (7.2.2.2.1.2) were sectioned at 10μm on a cryostat and freeze-thaw mounted onto slides, as described in 2.3.4.1.2.

7.2.2.2.2 Single-label EGFP Immunohistochemistry

The slides (7.2.2.2.1.3) were rinsed 4 times with PBS buffer, incubated in PBS buffer for 5 minutes, permeabilised in methanol at -20°C for 2 minutes, and then rinsed 4 times and incubated in PBS buffer for 5 minutes, twice. Tissue sections were first blocked with 10% normal goat serum (NGS, Vector Laboratories) in PBS buffer containing 0.15% v/v Triton-X (Sigma; PBS-T) for 20 minutes at RT, in a humidified container. Subsequently, excess serum was blotted away and the sections were incubated with the primary antibody (an IgG fraction of a polyclonal antibody raised against GFP [isolated directly from *Aequorea victoria*] in rabbit, A-11122, Molecular Probes Inc, Eugene, OR, diluted 1:200 with PBS-T), for an hour at RT, and then washed 4 times and incubated in PBS buffer for 5 minutes. The sections were incuba-

ted with the secondary fluorescent antibody (Alexa Fluor 488 goat anti-rabbit IgG, A-21206; Molecular Probes Inc, Eugene, OR, diluted 1:500 with PBS-T), for 30 minutes at RT, in the dark, and then washed 4 times and incubated in PBS buffer for 5 minutes, twice. The sections were mounted in Vectashield mounting medium for fluorescence (with DAPI; Vector Laboratories) and viewed with a Leica DM-RD fluorescence microscope. Images were captured as described (see 2.3.4.5.3.2). Negative controls for this technique included IHC of non-transgenic sections (using the complete protocol), IHC of sections with exclusion of the primary antibody, and IHC of sections with exclusion of the secondary antibody.

7.2.2.2.3 Double-label EGFP:Egr-1 Immunohistochemistry

For simultaneous detection of EGFP and Egr-1, double-label immunohistochemistry was performed. For visualisation of EGFP, the tyramide signal amplification (TSA) method was applied (Shindler and Roth, 1996), using a commercially available kit (NEL 701A, PerkinElmer Life Sciences, Boston, MA). 10μm sections were washed by rinsing 3 times with TNT buffer and incubation in TNT buffer for 5 minutes. The slides were immersed in 0.3% H_2O_2 in methanol at RT for 20 minutes and washed as described above. Sections were blocked in TNB buffer for 30 minutes at RT, in a humidified container. Subsequently, excess buffer was blotted away, and sections were first incubated with anti-GFP rabbit IgG (A-11122, Molecular Probes Inc, Eugene, OR; diluted 1:2000 in TNB), for an hour at RT, then biotinylated goat anti-rabbit IgG (diluted 1:500 in TNB) for 30 minutes at RT, and then streptavidin-HRP (PerkinElmer Life Sciences, Boston, MA; diluted 1:100 in TNB) for 30 minutes at RT, with washes in between each step (see above). The sections were then incubated in fluorescein (FITC)-labelled tyramide (diluted 1:50 in amplification diluent; PerkinElmer Life Sciences, Boston, MA) for 10 minutes at RT, and washed as described.

For visualisation of Egr-1, tissue sections were then treated as described for single-label IHC (7.2.2.2.2) from the blocking step onwards, using Egr-1 rabbit polyclonal antibody (C-19, Santa Cruz; diluted 1:200 in PBS-T) as the primary antibody, and Cy3 goat anti-rabbit Ig (Sigma C-2306; diluted 1:50 in PBS-T) as the secondary antibody. The sections were mounted in Vectashield mounting medium for fluorescence (with DAPI; Vector Laboratories) and viewed with a Leica DM-RD fluorescence microscope. Images were captured as described (see 2.3.4.5.3.2).

Controls for this technique included dual TSA:IHC of non-transgenic sections (using the complete protocol), single-TSA and single-IHC analysis of transgenic sections, dual TSA:IHC with the exclusion of each of the primary antibodies, and dual TSA:IHC with the exclusion of FITC-labelled tyramide (unamplified control). Controls for cross-reactivity of the second secondary antibody with the primary antibody were also included by treating transgenic sections with the normal concentration of the primary antibody (positive control) and a 1:10 dilution (as used for the TSA method; LeSauter *et al.*, 2003) of the normal concentration of the primary antibody (negative control). In this case, 1:200 and 1:2000 dilutions of the anti-GFP rabbit IgG (Molecular Probes Inc.) were applied in positive and negative controls, respectively, with a 1:50 dilution of Cy3 goat anti-rabbit Ig (Sigma C-2306).

7.2.2.3 Direct detection of fluorescence by fluorescence microscopy

7.2.2.3.1 Perfusion of tissues

Rats were perfused as described in 7.2.2.2.1.1.

7.2.2.3.2 Fixation of tissues

Brains and pituitary glands were removed from perfused rats (7.2.2.3.1.1), post-fixed with 4% PFA in 0.1M PB for 90 minutes at 4°C, and incubated overnight in 0.01M PBS, at 4°C.

7.2.2.3.3 Tissue sectioning

A sample of the brain (7.2.2.3.1.2) containing the SCN was isolated as described (see 3.2.1.1), and incubated in ice-cold 0.01M PBS, ready for sectioning. Pituitary glands (7.2.2.3.1.2) were submerged in a small cylindrically-shaped piece of foil containing ultra-low gelling temperature agarose (Sigma A2576), and incubated on ice, ready for sectioning. A fresh blade was screwed onto the vibratome (an in-house model similar to the Leica VT1000 S vibratome used in 4.2.5.2.2, but with slower blade speed), before attaching the pre-frozen platform to the machine and surrounding it with ice and water. The cold brain/pituitary sample was mounted onto the chuck using 'superglue' (Loctite) and screwed down into the platform, which was then filled with ice-cold 0.01M PBS. Initially, the mounted brain was approached with thick cuts (200μm); the thickness control was adjusted to 50μm prior to approaching the desired region for tissue collection. Free-floating sections were transferred from the platform of the vibratome, with a fine paintbrush, to a petri dish filled with ice-cold 0.01M PBS. The sections were then mounted onto slides with a drop of Vectashield mounting medium for fluorescence (with DAPI; Vector Laboratories), ready for detection of fluorescence (7.2.2.3.2).

7.2.2.3.4 Direct detection of EGFP fluorescence by fluorescence microscopy

The sections (7.2.2.3.1.3) were viewed with an Olympus BX61 fluorescence microscope (with Olympus BX-UCB mercury lamp), and images were captured using an F-view camera (Olympus) and AnalySIS software (Soft Imaging Systems, Münster, Germany). Controls for this technique included non-transgenic tissues.

7.3 RESULTS

7.3.1 Western blot analysis

7.3.1.1 Western blot analysis of transgene expression in the Z transgenic pituitary gland

Rat pituitary glands were used for initial western blot analysis of transgene expression since a previous study had established that *egr-1* is highly expressed in the anterior pituitary gland (Slade and Carter, 2000). Initial western blot analyses using the Living Colours A.V. GFP peptide antibody (Clontech; see 7.2.2.1.4) failed to detect EGFP (transgene) expression in Z line transgenic pituitary glands (data not shown); the Egr-1 specific polyclonal antisera (Santa Cruz Biotechnology; see 7.2.2.1.4) was used to confirm successful transfer of protein from polyacrylamide gels to membranes (data not shown). Subsequent western blot analyses using the GFP monoclonal antibody (Clontech; see 7.2.2.1.4) revealed specific detection of d2EGFP (Clontech; 31kDa) in various tissues of the Z transgenic lines (see below).

Western blot analysis revealed a 31kDa d2EGFP immunoreactive protein band in the Z25-B (see Figure 7.1), Z27-A (see Figure 7.2) and Z27-B (see Figures 7.1 and 7.2) rat pituitary glands, respectively, with the highest levels detected in the Z25-B rat pituitary gland. Two distinct bands are clearly visible in Figure 7.2; a finding that has previously been reported by the manufacturer (Clontech). However, this doublet is not visible, or less distinct, in Figure 7.1 (see lanes 3 and 7, respectively), due to differences in the resolution of the gel. Different levels of the 31kDa d2EGFP immunoreactive protein band in the two Z27-A samples (taken from different subjects) shown in Figure 7.2 may be due to oestrus cyclical differences (Slade and Carter, 2000). The d2EGFP immunoreactive protein band (31kDa) appears larger than the positive control band (27kDa) due to the addition of a PEST sequence (see 6.1.1). Western blot analysis, however, failed to detect transgene expression in the Z13, Z14, Z16 and Z25-A rat pituitary glands, respectively (see Figure 7.1).

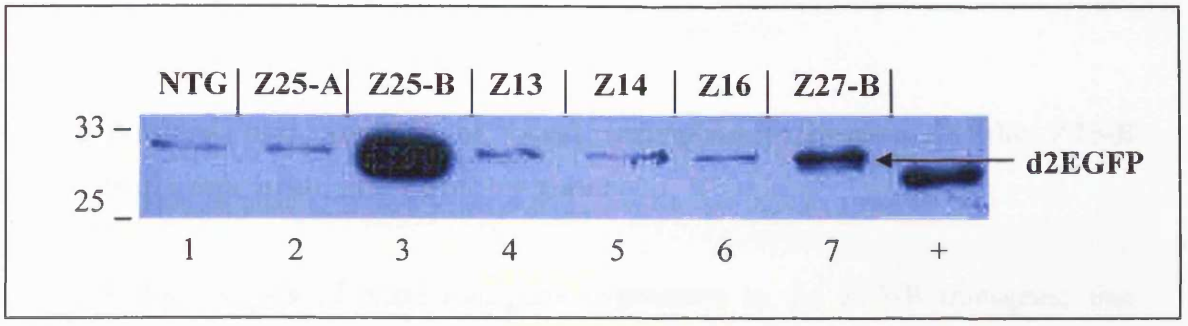


Figure 7.1 Film autoradiograph of western blot analysis of protein samples (50µg) extracted from female non-transgenic (NTG) and **Z25-A**, **Z25-B**, **Z13**, **Z14**, **Z16** and **Z27-B** transgenic rat pituitary glands, resolved on a polyacrylamide gel (**1.75 hours**), transferred to a PVDF membrane, incubated with the primary (GFP monoclonal) antibody (**1:10000**; Clontech), and then incubated with the secondary (peroxidase-linked mouse) antibody (1:10000; Amersham). (d2)EGFP-specific bands (31kDa) are visible in the Z25-B and Z27-B samples (lanes 3 and 7, respectively), and the positive control (lane marked '+': 1ng recombinant EGFP (27kDa); Clontech), and absent in the NTG, Z25-A, Z13, Z14, and Z16 samples (lanes 1, 2, 4, 5 and 6, respectively). The (kDa) scale bar on the left represents the pre-stained broad-range protein marker (NEB).

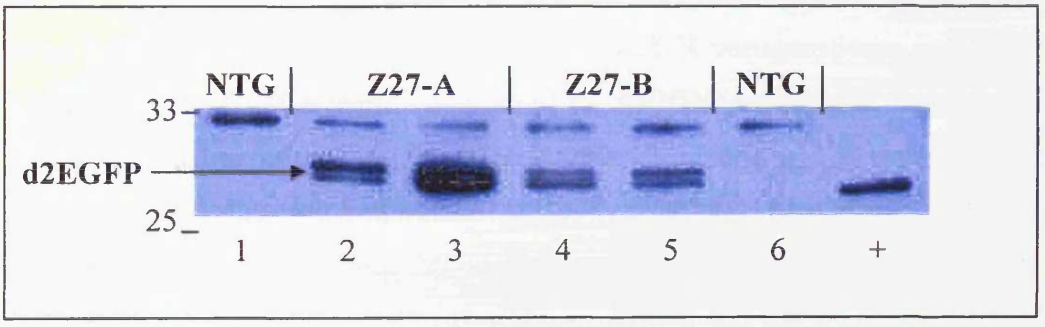


Figure 7.2 Film autoradiograph of western blot analysis of protein samples (50µg) extracted from female non-transgenic (NTG) and **Z27-A** and **Z27-B** transgenic rat pituitary glands, resolved on a polyacrylamide gel (**2 hours**), transferred to a PVDF membrane, incubated with the primary (GFP monoclonal) antibody (**1:15000**; Clontech), and then incubated with the secondary (peroxidase-linked mouse) antibody (1:10000; Amersham). (d2)EGFP-specific bands (31kDa) are visible in the Z27-A and Z27-B samples (lanes 2 and 3, and 4 and 5, respectively, all taken from different subjects), and the positive control (lane marked '+': 1ng recombinant EGFP (27kDa); Clontech), and absent in the NTG sample (lane 1). The (kDa) scale bar on the left represents the pre-stained broad-range protein marker (NEB).

7.3.1.2 Western blot analysis of basal transgene expression in the Z25-B transgenic brain and pituitary gland

Western blot analysis of basal transgene expression in the Z25-B transgenic line detected a 31kDa d2EGFP immunoreactive protein band in the pituitary gland (see Figure 7.3), also shown in Figure 7.1. Western blot analysis, however, failed to clearly detect transgene expression in the cortex and hippocampus of this particular transgenic line (see Figure 7.3).

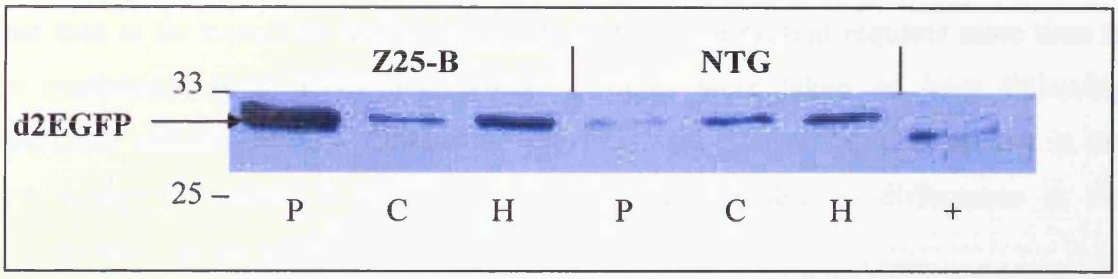


Figure 7.3 Film autoradiograph of western blot analysis of protein samples (50µg) extracted from female non-transgenic (NTG) and **Z25-B** transgenic rats, resolved on a polyacrylamide gel (**1.75 hours**), transferred to a PVDF membrane, incubated with the primary (GFP monoclonal) antibody (**1:10000**; Clontech), and then incubated with the secondary (peroxidase-linked mouse) antibody (1:10000; Amersham). P, C and H represent pituitary, cortex and hippocampal samples, respectively. (d2)EGFP-specific bands (31kDa) are visible in the Z25-B pituitary sample and the positive control (lane marked '+': 1ng recombinant EGFP (27kDa); Clontech), and absent in the Z25-B cortical and hippocampal and NTG samples. The (kDa) scale bar on the left represents the pre-stained broad-range protein marker (NEB).

Note: Due to time restrictions of this thesis, the western blots incorporated in this thesis were not reprobed with a control antibody and quantified.
It is anticipated that future experiments will include this procedure.

7.3.1.3 Western blot analysis of transgene up-regulation

The capacity of *egr-1* genomic sequences within the transgene to mediate up-regulation in response to a (generalised, pharmacological) neural stimulus was investigated using the convulsant metrazole, as in previous studies (see 5.3.2 and 6.3.3.2.3, respectively). Control and metrazole-induced transgene expression was determined, by western analysis, in the liver, cortex and hippocampus of the Z27-A transgenic line (see Figure 7.4), and in the pituitary gland (also shown in Figure 7.2), cortex and hippocampus of the Z27-B transgenic line (data not shown). Following metrazole treatment, increases in transgene expression, compared with control animals, were observed. However, the magnitude of this up-regulation is less than that seen at the transcript level (see Figure 6.16), since protein requires more time to be synthesised (both RNA and protein samples were taken an hour following metrazole administration). Unlike the previous analyses, no band is present in the non-transgenic sample in Figure 7.4. This may be due to differences in the concentration of the primary antibody.

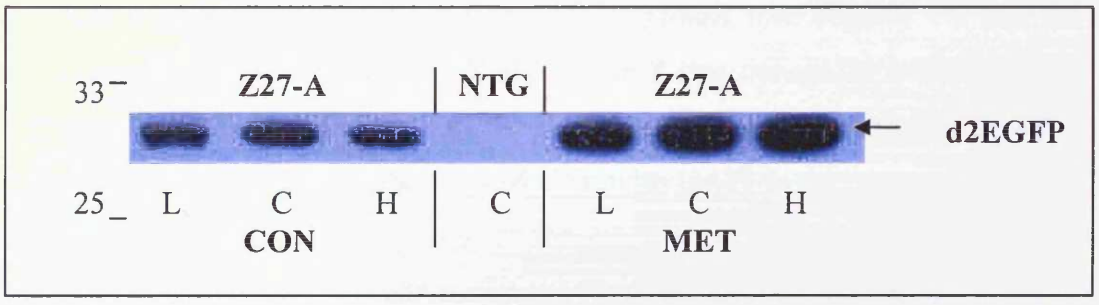


Figure 7.4 Film autoradiograph of western blot analysis of protein samples (50μg) extracted from female non-transgenic (NTG) and **Z27-A** transgenic rats, resolved on a polyacrylamide gel (**1.5 hours**), transferred to a PVDF membrane, incubated with the primary (GFP monoclonal) antibody (**1:17500**; Clontech), and then incubated with the secondary (peroxidase-linked mouse) antibody (1:10000; Amersham). d2EGFP-specific bands (31kDa) are visible in the samples taken from sterile water-treated Z27-A transgenic rats (**Z27-A CON**), in addition to samples taken from metrazole-treated Z27-A transgenic rats (**Z27-A MET**), and absent in the **NTG** sample. The (kDa) scale bar on the left represents the pre-stained broad-range protein marker (NEB). L, C and H represent liver, cortex and hippocampal samples, respectively.

7.3.2 Immunohistochemical analysis of transgene expression

IHC was used to detect transgene expression in various tissues of the Z transgenic lines (see below). The tissues were initially analysed by single-label EGFP IHC to confirm specific binding of the antibodies (see 7.2.2.2.2), detect basal transgene expression and confirm nocturnal light-inducibility of the transgene. Following this, double-label EGFP:Egr-1 TSA:IHC was performed (and optimised; see 7.4.1) for the simultaneous visualisation of EGFP and Egr-1 within the given tissue.

7.3.2.1 Single-label EGFP IHC in the Z line transgenic pituitary gland

Single-label IHC analysis of transgene expression in the pituitary gland revealed that expression (specific binding of the antibodies to immunoreactive d2EGFP (transgene) protein) was restricted to the anterior lobe of the pituitary gland of the Z16, Z25-B, Z27-A and Z27-B transgenic lines (see Figure 7.5). Similar to previous findings (see 5.3.5), transgene (protein) expression within the anterior lobe was confined to a sub-population of cells, often associated into small groups (see Figures 7.5 and 7.6). Close inspection of immunoreactive EGFP in the Z line transgenic anterior pituitary gland revealed partial confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in the 'merged' images in Figures 7.5 and 7.6), and also filling of the cytoplasmic and cellular processes (as shown by green labelling surrounding the DAPI in the 'merged' images in Figures 7.5 and 7.6).

The highest levels of immunoreactive EGFP were detected in the Z25-B, Z27-A and Z27-B transgenic anterior pituitary glands (see Figure 7.5 **h**, **f** and **e**, respectively, and Figure 7.6), and the lowest levels in the Z13, Z14 (data not shown, but similar to that of Z16) and Z16 transgenic anterior pituitary glands (see Figure 7.5 **i**). No immuno-reactive EGFP was detected in the Z25-A transgenic line pituitary gland line (data not shown), in non-transgenic pituitary glands (see Figure 7.5 **a** and **b**), in negative control sections (see Figure 7.5 **c** and **d**, for examples), and in the posterior pituitary of all of the Z transgenic lines (see Figure 7.5 **g** and Figure 7.6, for examples). The apparent staining of the intermediate lobe of the Z27-B transgenic pituitary gland (see

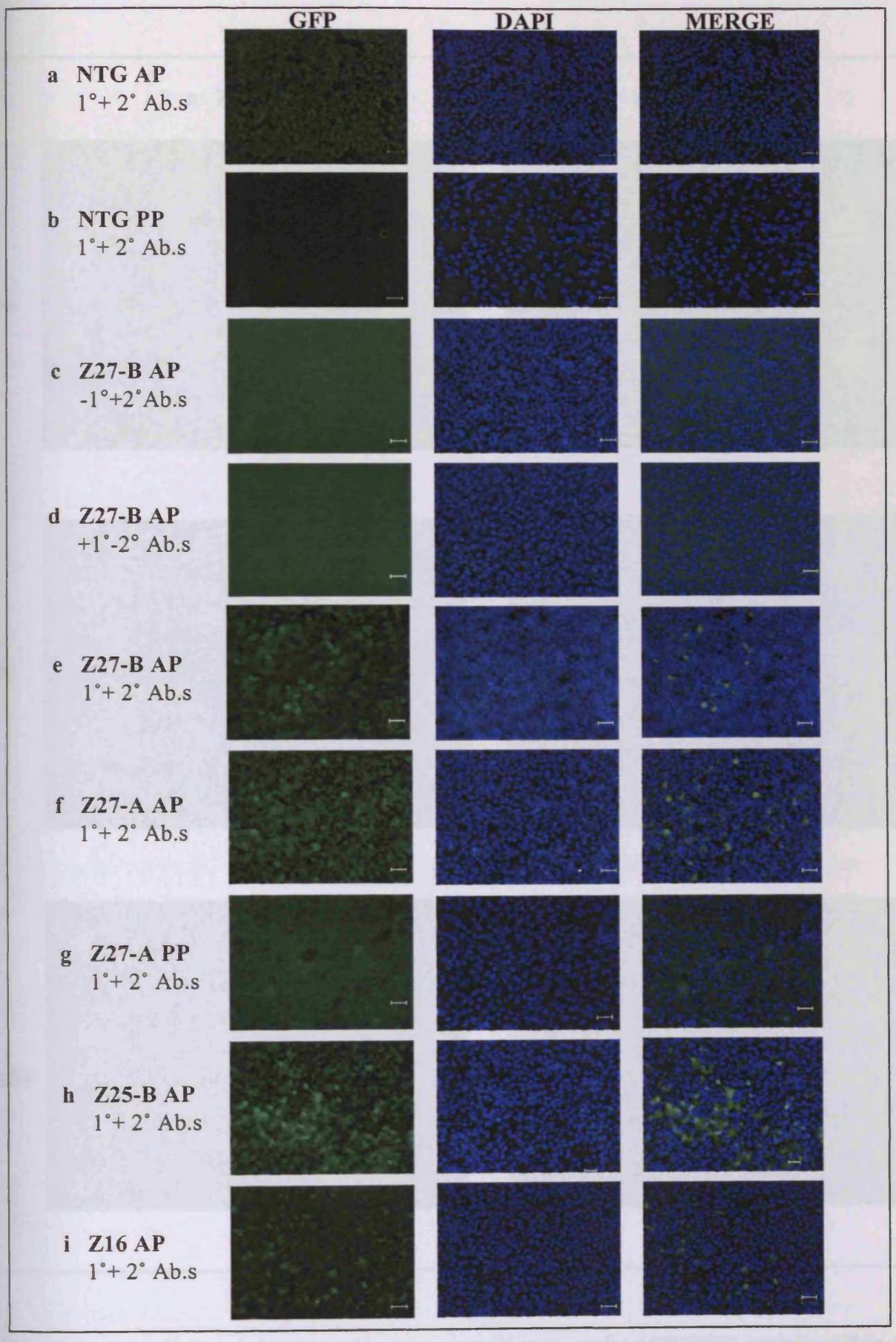


Figure 7.5 High resolution images of immunoreactive transgene (d2EGFP) protein within **Z27-B, Z27-A, Z25-B** and **Z16** transgenic and non-transgenic (**NTG**) female pituitary gland sections (10μm), following IHC with/without (+/-) **GFP** (Molecular Probes Inc.) as the primary antibody (**1° Ab**) and Alexa Fluor 488 (Molecular Probes Inc.)-conjugated secondary antibody (**2° Ab**). **AP** and **PP** represent the anterior and posterior pituitary, respectively; '**MERGE**' represents an overlay of the **GFP** and **DAPI** images. Scale bar = 25 μm. See text for further discussion.

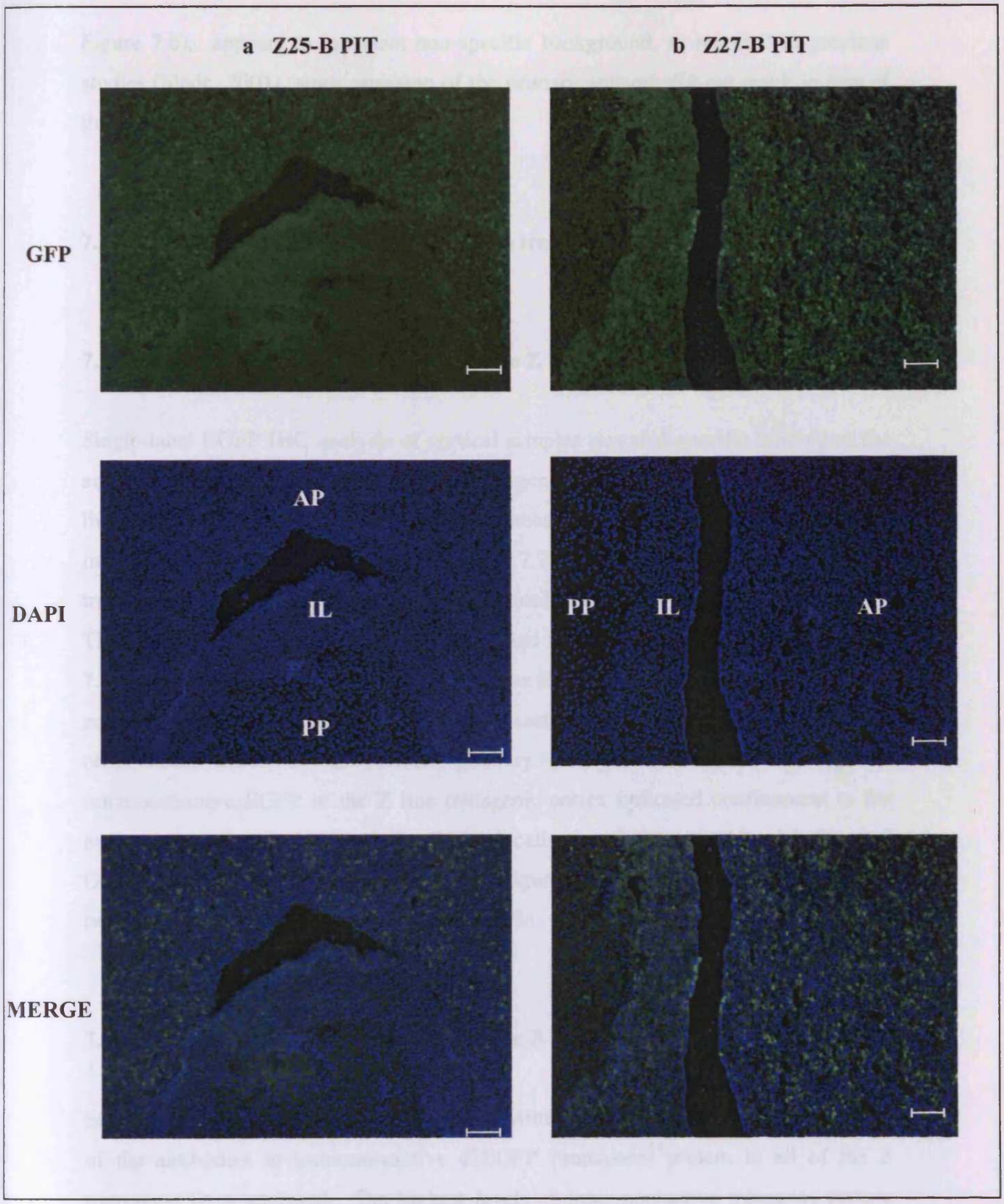


Figure 7.6 Lower resolution images of immunoreactive transgene (d2EGFP) protein within **Z25-B** and **Z27-B** transgenic female pituitary gland (**PIT**) sections (10μm), following IHC with **GFP** and Alexa Fluor 488-conjugated (Molecular Probes Inc.) antibodies. **AP, IL** and **PP** represent the anterior, intermediate and posterior lobes of the pituitary, respectively; '**MERGE**' represents an overlay of the **GFP** and **DAPI** images. Scale bar = 100μm **(a)** and 50μm **(b)**. See text for further discussion.

Figure 7.6), appears to represent non-specific background, as reported in previous studies (Slade, 2001), since omission of the primary antisera did not result in loss of this staining.

7.3.2.2 Single-label EGFP IHC in the Z line transgenic brain

7.3.2.2.1 Single-label EGFP IHC in the Z line transgenic cortex

Single-label EGFP IHC analysis of cortical samples revealed specific binding of the antibodies to immunoreactive d2EGFP (transgene) protein in all of the Z transgenic lines analysed. The highest levels of immunoreactive transgene protein were detected in the Z27-B transgenic cortex (see Figure 7.7 **d**), followed by the Z13 and Z14 transgenic cortex (Z13 data not shown, but similar to that of Z14; see Figure 7.7 **a**). The lowest levels were detected in the Z16 and Z25-B transgenic cortex (see Figure 7.7 **b** and **c**, respectively). No immunoreactive EGFP was detected in non-transgenic cortical samples, and in IHC negative control sections (data not shown). In contrast to observations made in the anterior pituitary (see 7.3.2.1), close inspection of immunoreactive EGFP in the Z line transgenic cortex indicated confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in the 'merged' images in Figure 7.7), and suggests that EGFP may be primarily confined to the nucleus in the brain.

7.3.2.2.2 Single-label EGFP IHC in the Z line transgenic SCN

Single-label EGFP IHC analysis of SCN (daytime) samples revealed specific binding of the antibodies to immunoreactive d2EGFP (transgene) protein in all of the Z transgenic lines analysed. The highest levels of immunoreactive transgene protein were detected in the Z27-B transgenic SCN (see Figure 7.8 **f** and **g**), and very low levels in the Z16 and Z25-B transgenic SCN (see Figure 7.8 **d** and **e**, and **b** and **c**, respectively). No immunoreactive EGFP was detected in non-transgenic SCN samples (see Figure 7.8 **a**), and in IHC negative control sections (data not shown).

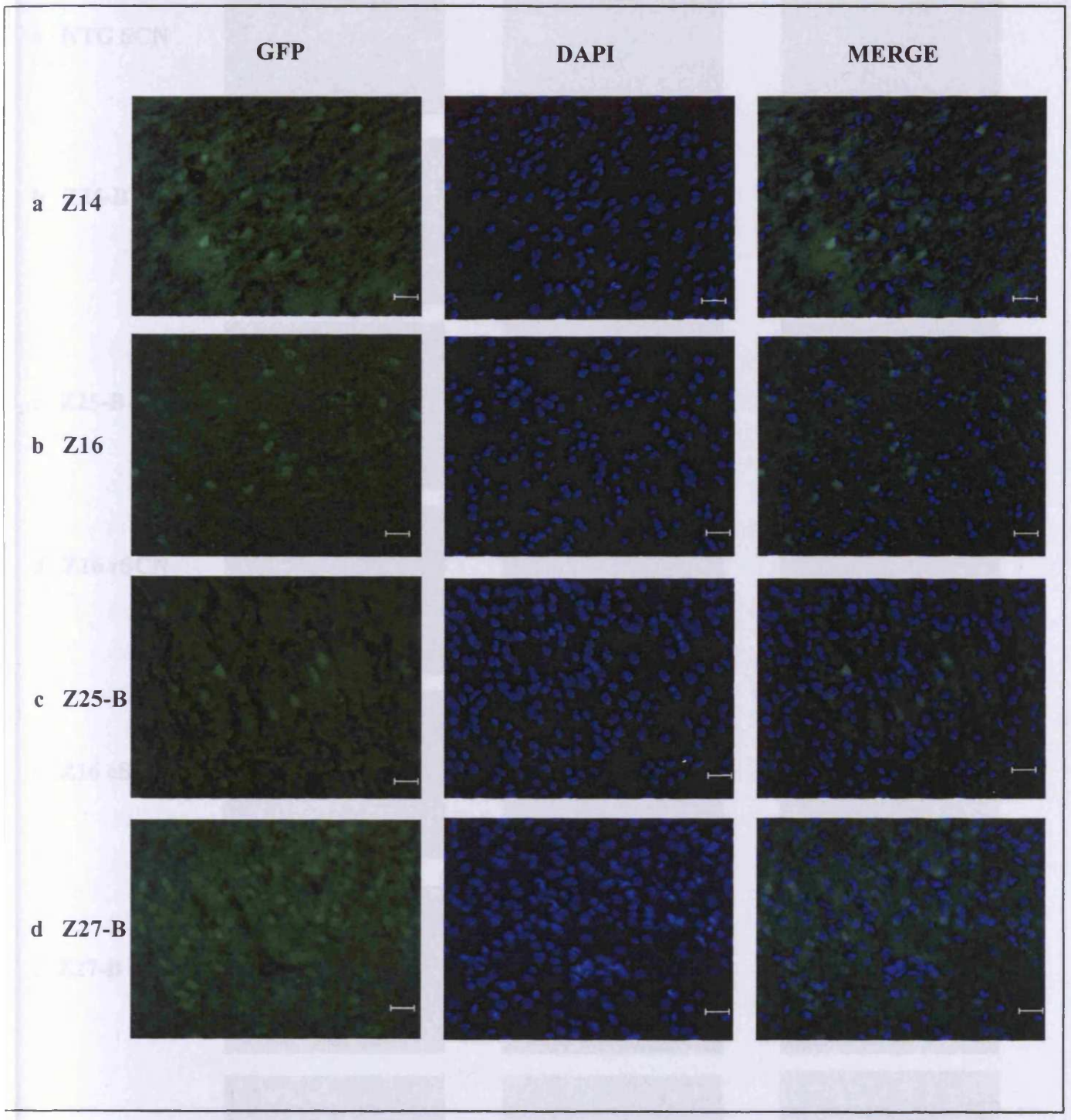


Figure 7.7 High resolution images of immunoreactive transgene (d2EGFP) protein within **Z14, Z16, Z25-B** and **Z27-B** transgenic male cortical sections (10μm), following IHC with **GFP** and Alexa Fluor 488 (Molecular Probes Inc.)-conjugated antibodies. '**MERGE**' represents an overlay of the **GFP** and **DAPI** images. Scale bar = 25 μm. See text for further discussion.

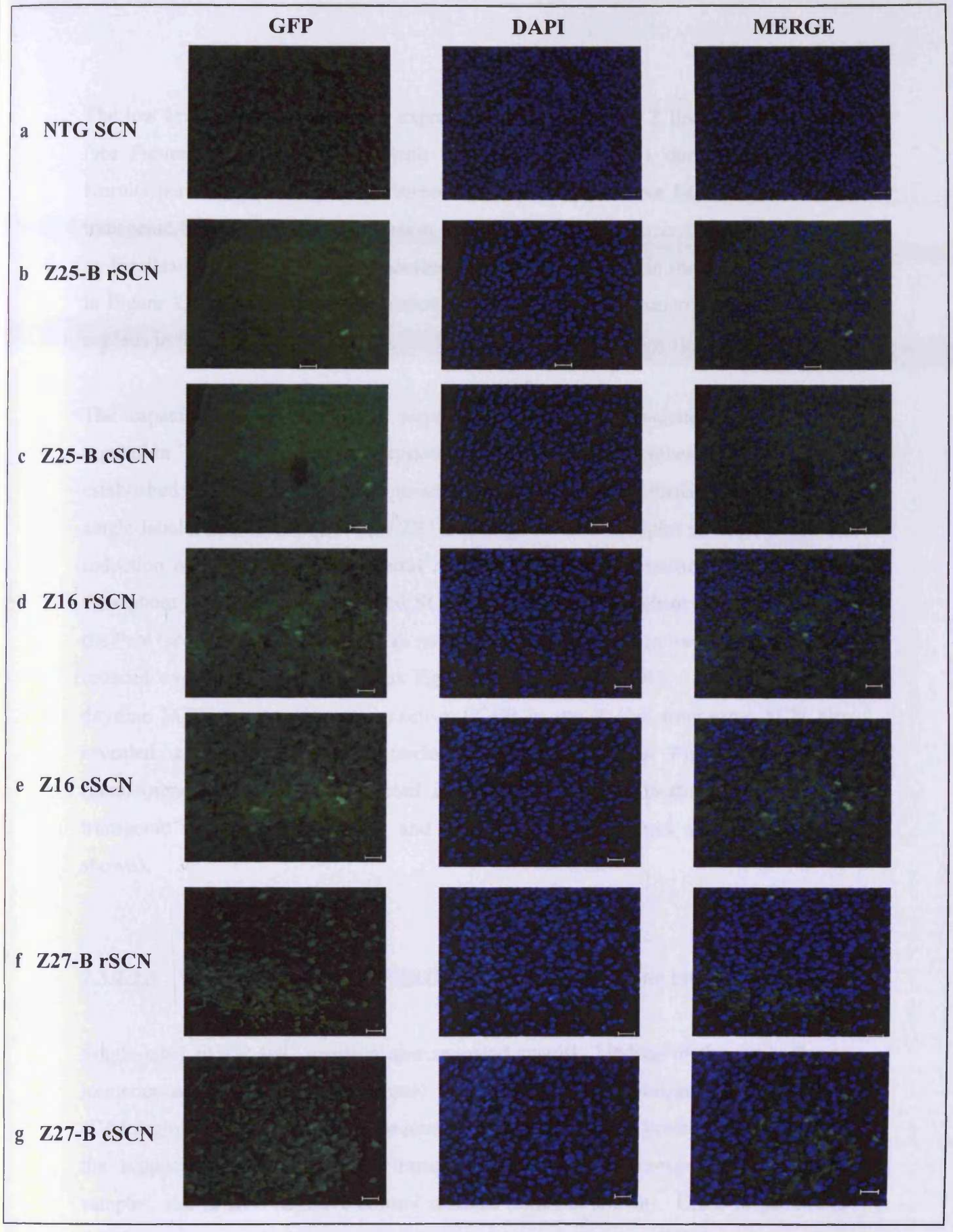


Figure 7.8 High resolution images of immunoreactive transgene (d2EGFP) protein within **Z25-B, Z16** and **Z27-B** transgenic and non-transgenic (**NTG**) male rostral (**r**) and caudal (**c**) SCN sections (10μm), following IHC with **GFP** and Alexa Fluor 488 (Molecular Probes Inc.)-conjugated antibodies. '**MERGE**' represents an overlay of the **GFP** and **DAPI** images. Scale bar = 25 μm (50 μm in the NTG SCN image). (The third ventricle is located to the left hand side of each image.) See text for further discussion.

The low levels of daytime EGFP expression observed in the Z line transgenic SCN (see Figure 7.8) are consistent with low endogenous Egr-1 during the day (see Kornhauser *et al.*, 1996). Close inspection of immunoreactive EGFP in the Z line transgenic SCN indicated confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in the 'merged' images in Figure 7.8), and supports the notion that EGFP may be primarily confined to the nucleus in the brain (see 7.3.2.2.1).

The capacity of *egr-1* genomic sequences within the transgene to mediate up-regulation in response to a physiological stimulus was investigated using an established circadian, light-pulse paradigm. Following a nocturnal photic stimulus, single-label EGFP IHC in Z16 and Z27-B transgenic SCN samples revealed a discrete induction of transgene in the ventral region of rostral SCN sections, which spread throughout the SCN in more caudal SCN sections, and in a subset of neurons within the PeN (see Figure 7.9; Z16 images not shown). These observations mirror the light-induced expression of endogenous Egr-1 (Slade *et al.*, 2001). As seen with the daytime SCN samples, immunoreactive EGFP in the Z line transgenic SCN also revealed confinement to the nuclear compartment (see Figure 7.9). No immunoreactive EGFP was detected in SCN samples of non-stimulated and non-transgenic subjects, respectively, and in IHC negative control samples (data not shown).

7.3.2.2.3 Single-label EGFP IHC in the Z line transgenic hippocampus

Single-label EGFP IHC analysis also revealed specific binding of the antibodies to immunoreactive d2EGFP (transgene) protein in the Z16 transgenic hippocampus (CA1 region; see Figure 7.10). No immunoreactive transgene protein was detected in the hippocampus of the other transgenic lines, in non-transgenic hippocampal samples, and in IHC negative control sections (data not shown). Close inspection of the Z line transgenic hippocampus revealed immunoreactive EGFP in the nuclear compartment and also cellular processes (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in the 'merged' images in Figure 7.10).

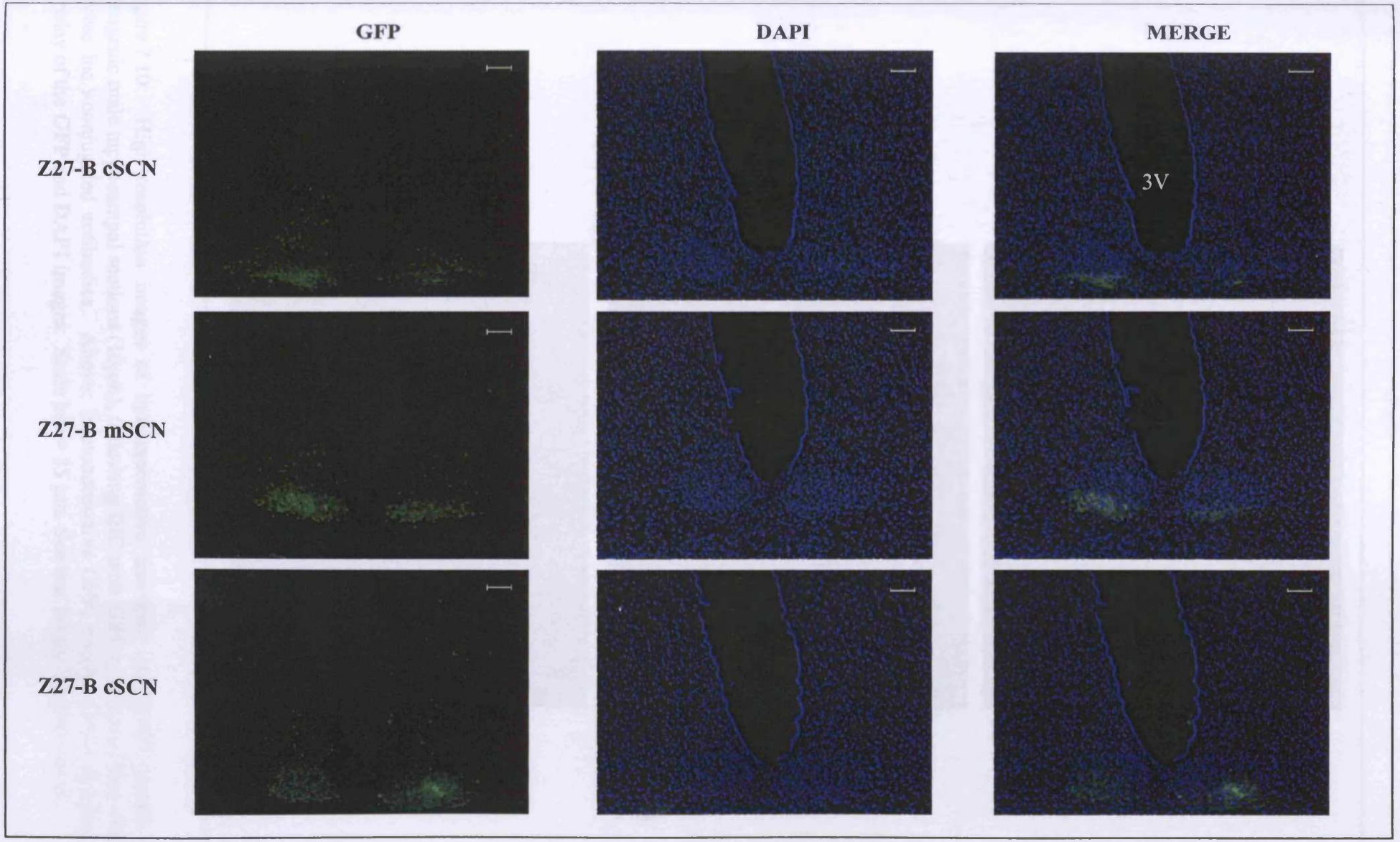


Figure 7.9 Low resolution images of immunoreactive transgene (d2EGFP) protein within **Z27-B** transgenic male rostral (**r**), medial (**m**) and caudal (**c**) SCN sections (10μm), sampled at subjective night (01:00h; 6 hours after lights off), following an hour of photic stimulation, and IHC with **GFP** and Alexa Fluor 488 (Molecular Probes Inc.)-conjugated antibodies. '**MERGE**' represents an overlay of the **GFP** and **DAPI** images. 3V represents the third ventricle. Scale bar = 100 μm. See text for further discussion.

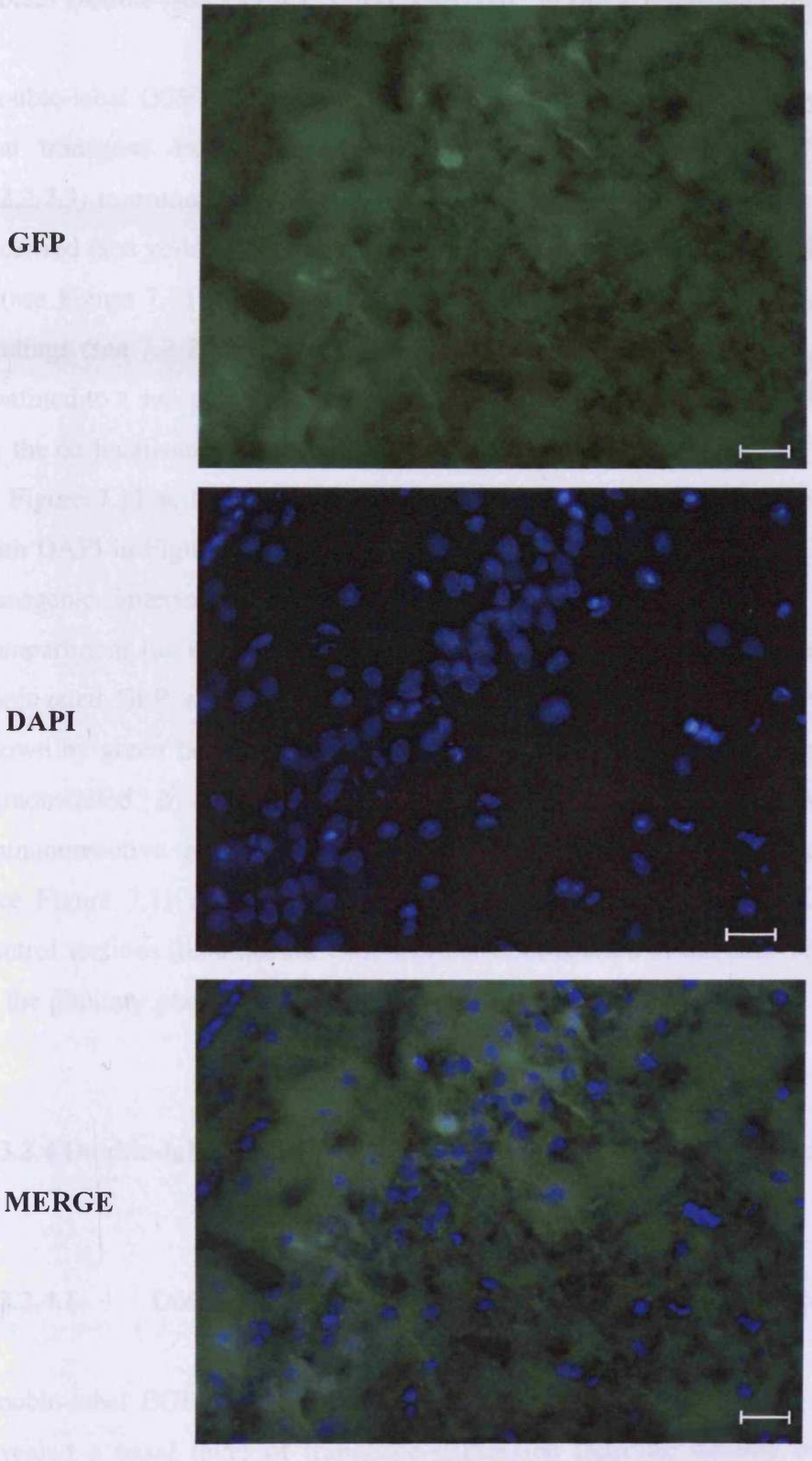


Figure 7.10 High resolution images of immunoreactive transgene (d2EGFP) protein within **Z16** transgenic male hippocampal sections (10μm), following IHC with **GFP** and Alexa Fluor 488 (Molecular Probes Inc.)-conjugated antibodies. Above: immunoreactive GFP; Centre: DAPI staining; Below: an overlay of the **GFP** and **DAPI** images. Scale bar = 25 μm. See text for further discussion.

7.3.2.3 Double-label EGFP:Egr-1 TSA:IHC in the Z transgenic pituitary gland

Double-label EGFP:Egr-1 TSA:IHC in the Z27-B transgenic pituitary gland revealed that transgene expression (specific binding of the antibodies (GFP-FITC; see 7.2.2.2.3) to immunoreactive d2EGFP (transgene) protein; see Figure 7.11 **a**) was co-localised (see yellow labelling in Figure 7.11 **d**) with endogenous (Cy3-labelled) Egr-1 (see Figure 7.11 **b**) in the anterior lobe of the pituitary gland. Similar to previous findings (see 7.3.2.1), transgene (and Egr-1) expression within the anterior lobe was confined to a sub-population of cells, often associated into small groups (as shown by by the co-localisation (light green/blue labelling) of FITC-conjugated GFP with DAPI in Figure 7.11 **e**, and the co-localisation (purple labelling) of Cy3-conjugated Egr-1 with DAPI in Figure 7.11 **f**). Close inspection of immunoreactive EGFP in the Z line transgenic anterior pituitary gland revealed partial confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of FITC-conjugated GFP with DAPI in Figure 7.11 **e**), and also filling of the cytoplasm (as shown by green labelling surrounding the DAPI in Figure 7.11 **e**); observations also demonstrated by single-label EGFP IHC (see Figures 7.5 and 7.6). No immunoreactive transgene protein was detected in the posterior lobe of the pituitary (see Figure 7.11 **a**), in non-transgenic pituitary glands, and in TSA:IHC negative control sections (data not shown). Further optimisation of the dual TSA/IHC protocol in the pituitary gland, however, is required to confirm these findings.

7.3.2.4 Double-label EGFP:Egr-1 TSA:IHC in the Z line transgenic brain

7.3.2.4.1 Double-label EGFP:Egr-1 TSA:IHC in the Z line transgenic cortex

Double-label EGFP:Egr-1 TSA:IHC in Z27-B and Z16 transgenic cortical samples revealed a basal level of transgene expression (specific binding of the antibodies (GFP-FITC; see 7.2.2.2.3) to immunoreactive d2EGFP (transgene) protein; see Figures 7.11 **a** and 7.12 **a**, respectively), which was co-localised (see yellow labelling in Figures 7.11 **d** and 7.12 **d**, respectively) with endogenous (Cy3-labelled) Egr-1 (see Figures 7.11 **b** and 7.12 **b**, respectively). Similar to previous findings (see Figure 7.7)

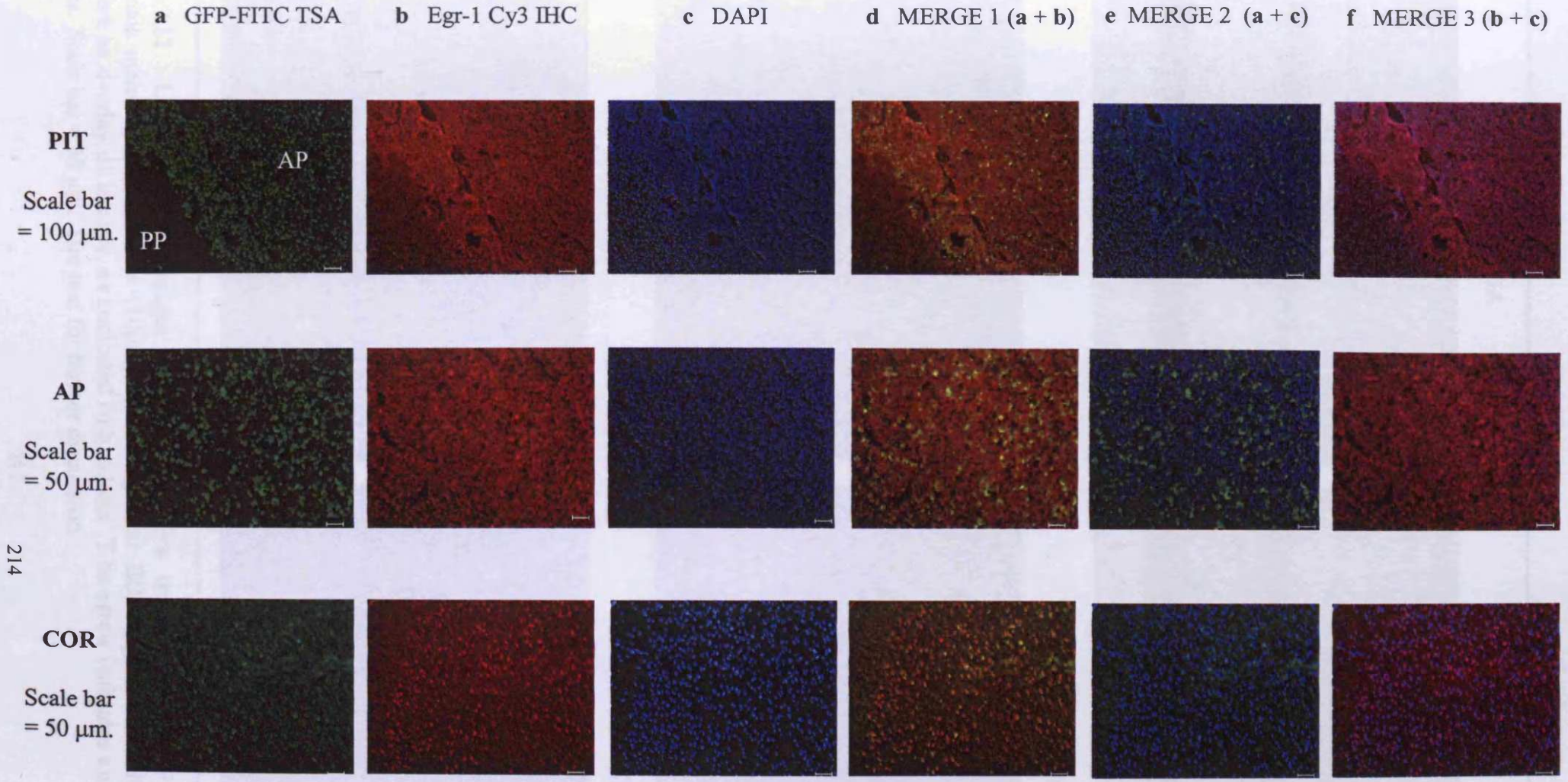


Figure 7.11 Low resolution images of immunoreactive transgene (d2EGFP) protein within **Z27-B** transgenic female anterior pituitary gland (**AP**), pituitary gland (**PIT**) and cortical (**COR**) sections (10µm), following dual EGFP:Egr-1 TSA:IHC. **PP** represents the posterior pituitary. 'MERGE' images represent an overlay of images, as indicated in brackets. See text for further discussion.

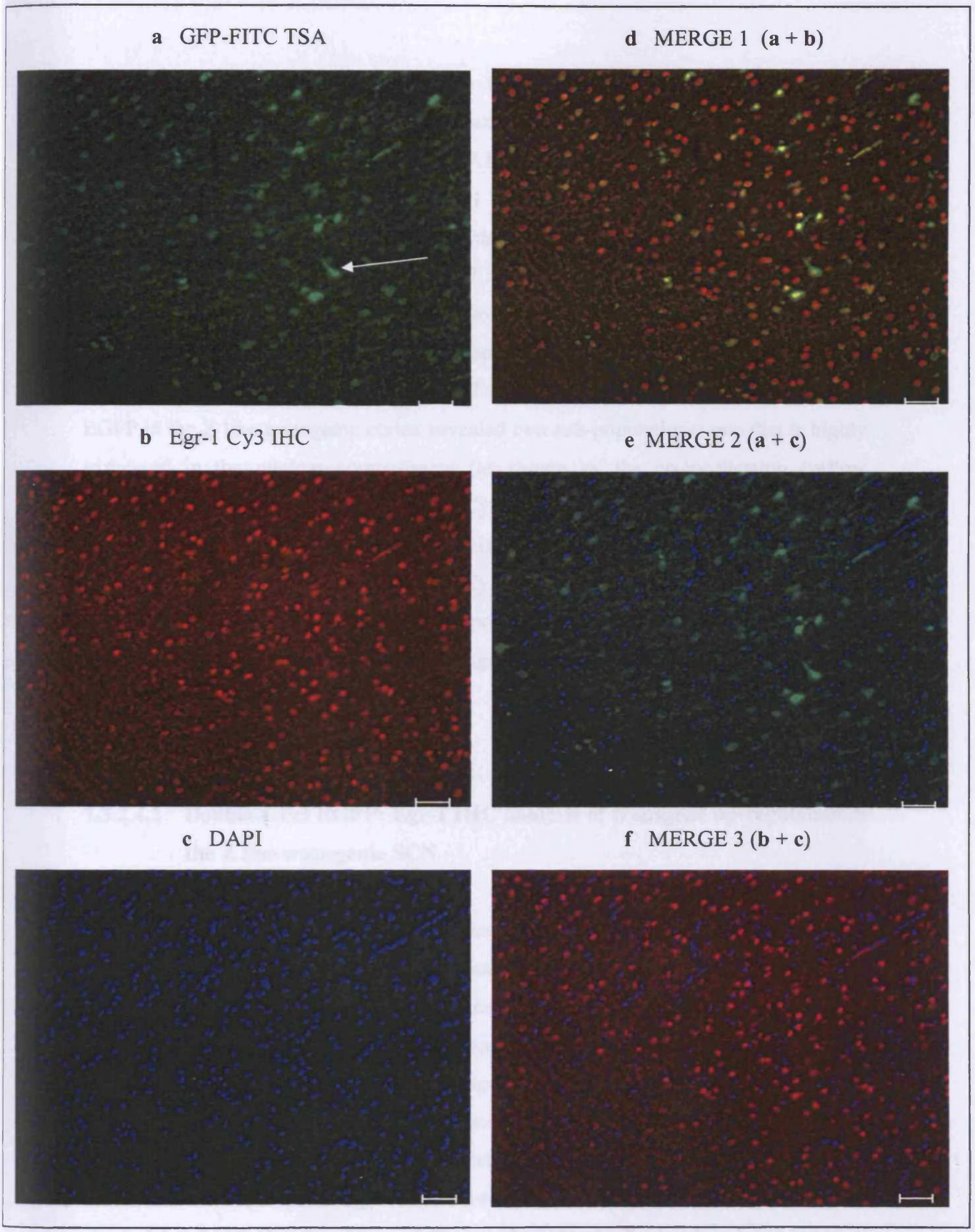


Figure 7.12 Low resolution images of immunoreactive transgene (d2EGFP) protein within **Z16** transgenic male cortical sections (10μm), following dual EGFP:Egr-1 TSA:IHC. 'MERGE' images represent an overlay of images, as indicated in brackets. The arrow indicates an EGFP-stained cellular process. Scale bar = 50 μm. See text for further discussion.

transgene (and Egr-1) expression within the cortex was apparent in the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of FITC-conjugated GFP with DAPI in Figures 7.11 **e** and 7.12 **e**, and the co-localisation (purple labelling) of Cy3-conjugated Egr-1 with DAPI in Figures 7.11 **f** and 7.12 **f**). This supports the hypothesis that EGFP may be primarily confined to the nuclear compartment in the brain (see 7.3.2.2.1, 7.3.2.2.2 and 7.3.2.2.3). At the same time, EGFP-expressing cellular processes are also clearly observed in the cortex of this line (see Figure 7.12), indicating that EGFP is not confined to the nucleus. Note that these processes are Egr-1 negative (see Figure 7.12). Close inspection of immunoreactive EGFP in the Z line transgenic cortex revealed two sub-populations: one that is highly expressed in the nuclear compartment (as shown by the co-localisation (yellow labelling) of FITC-conjugated GFP with Cy3-conjugated Egr-1 in Figures 7.11 **d** and 7.12 **d**), one that is expressed at lower levels (as shown by the co-localisation (orange labelling) of FITC-conjugated GFP with Cy3-conjugated Egr-1 in Figures 7.11 **d** and 7.12 **d**). No immunoreactive transgene protein was detected in Z25-B and Z27-A transgenic cortical samples, in non-transgenic cortical samples, and in TSA:IHC negative control sections (data not shown).

7.3.2.4.2 Double-label EGFP: Egr-1 IHC analysis of transgene up-regulation in the Z line transgenic SCN

The capacity of *egr-1* genomic sequences within the transgene to mediate up-regulation in response to a physiological stimulus was investigated using an established circadian, light-pulse paradigm. Following this stimulus, double-label EGFP:Egr-1 FIHC in Z16 and Z27-B transgenic SCN samples revealed a discrete induction of transgene in the ventral region of rostral SCN sections, which spread throughout the SCN in more caudal SCN sections (see Figure 7.13 **a**, and Figures 7.14 **a** and 7.15 **a**, respectively), and in a subset of neurons within the PeN (see Figures 7.13 **a** and 7.14 **a,** respectively). These observations mirrored those demonstrated by single-label EGFP IHC (see Figure 7.9), in addition to the light-induced expression of endogenous Egr-1 (see Figure 7.13 **b,** and Figures 7.14 **b** and 7.15 **b**, respectively).

The highest levels of co-localisation of the transgene with endogenous Egr-1 were detected in the Z27-B transgenic SCN samples (see yellow labelling in Figures 7.14 **d** and 7.15 **d**; compared to that of the Z16 transgenic SCN samples in Figure 7.13 **d**).

EGFP (and Egr-1) expression within the Z16 and Z27-B transgenic SCN was primarily confined to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of FITC-conjugated GFP with DAPI in Figures 7.13 **e**, and 7.14 **e** and 7.15 **e**, respectively, and the co-localisation (purple labelling) of Cy3-conjugated Egr-1 with DAPI in Figures 7.13 **f**, and 7.14 **f** and 7.15 **f**, respectively). This further supports the hypothesis that EGFP may be more confined to the nucleus in the brain (see 7.3.2.2.1, 7.3.2.2.2, 7.3.2.2.3 and 7.3.2.4.1). Close inspection of immunoreactive EGFP in the Z line transgenic SCN revealed two sub-populations (similar to that of seen in the cortex; see 7.3.2.4.1); one that is highly expressed in the nuclear compartment (as shown by the co-localisation (yellow labelling) of FITC-conjugated GFP with Cy3-conjugated Egr-1 in Figures 7.13 **d**, 7.14 **d** and 7.15 **d**), and one that is expressed at lower levels (as shown by the co-localisation (orange labelling) of FITC-conjugated GFP with Cy3-conjugated Egr-1 in Figures 7.13 **d**, 7.14 **d** and 7.15 **d**). The light stimulus was not associated with transgene induction in other areas of the neural axis including the hippocampus and cortex (not shown), and SCN / PeN induction was not observed in non-stimulated animals sampled at 01:00h (see Figures 7.13 and 7.14). In addition, no immunoreactive transgene protein was detected in nocturnal photically-stimulated Z25-B and Z27-A transgenic SCN samples, in non-transgenic SCN samples, and in TSA:IHC negative control sections (data not shown).

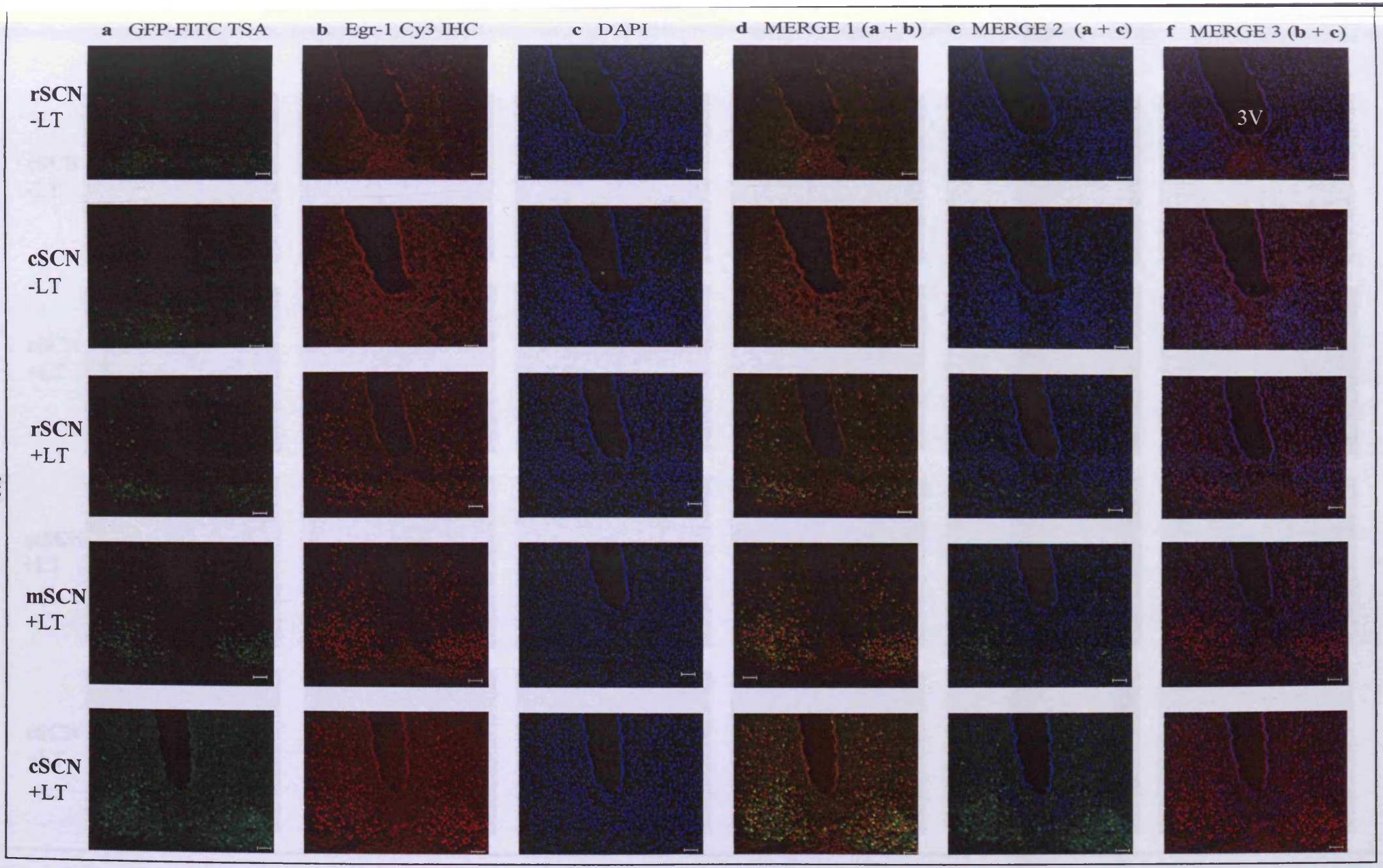


Figure 7.13 Low resolution images of immunoreactive transgene (d2EGFP) protein within **Z16** transgenic male rostral (**r**), medial (**m**) and caudal (**c**) SCN sections (10μm), sampled at subjective night (01:00h; 6 hours after lights off), following an hour of photic stimulation (+LT; non-stimulated = -LT), and dual EGFP:Egr-1 TSA:IHC. 'MERGE' represents an overlay of images, as indicated in brackets. 3V represents the third ventricle. Scale bar = 50 μm. See text for further discussion.

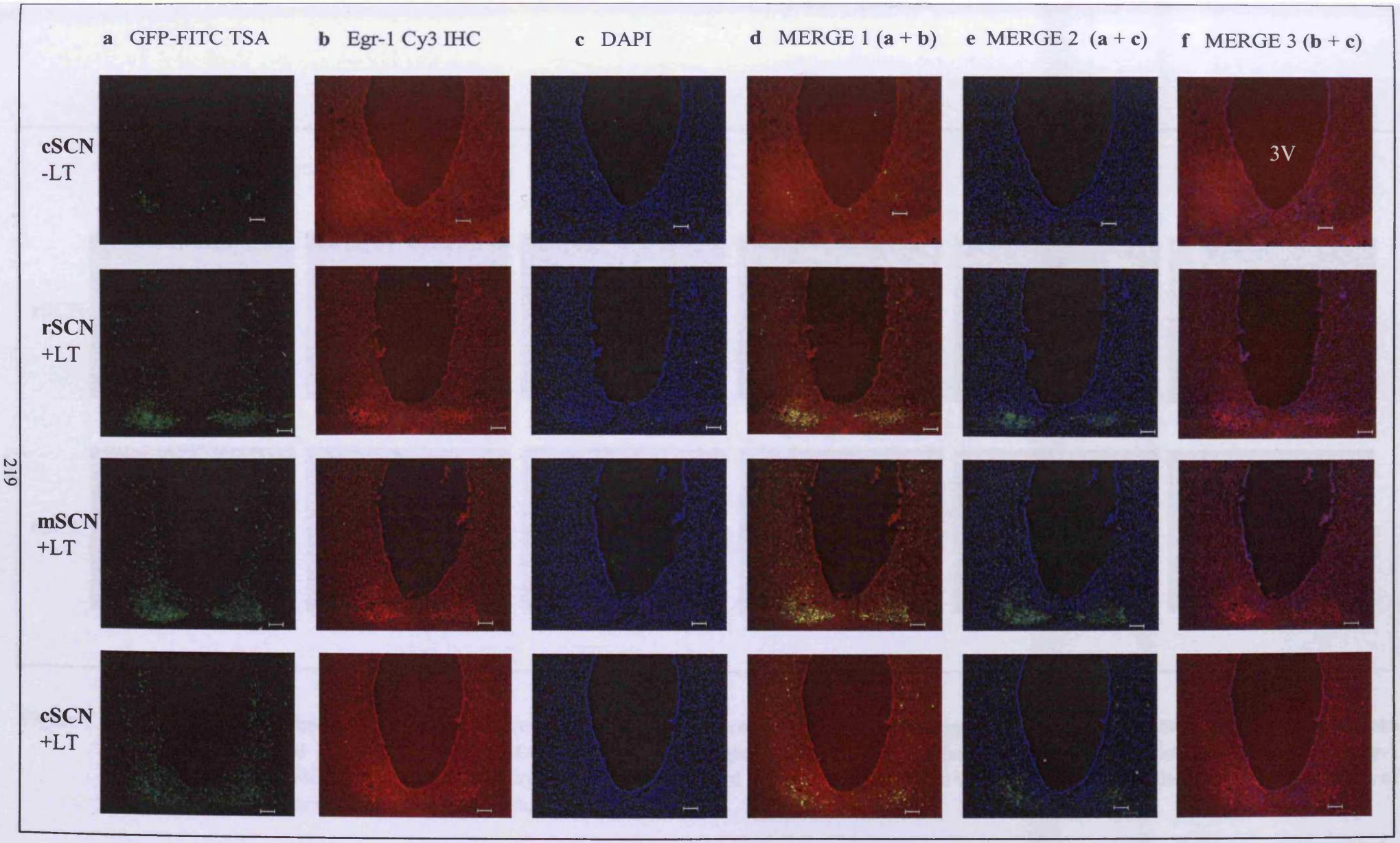


Figure 7.14 Low resolution images of immunoreactive transgene (d2EGFP) protein within **Z27-B** transgenic male rostral (**r**), medial (**m**) and caudal (**c**) SCN sections (10μm), sampled at subjective night (01:00h; 6 hours after lights off), following an hour of photic stimulation (+LT; non-stimulated = -LT), and dual EGFP:Egr-1 TSA:IHC. 'MERGE' represents an overlay of images, as indicated in brackets. 3V represents the third ventricle. Scale bar = 100 μm. See text for further discussion.

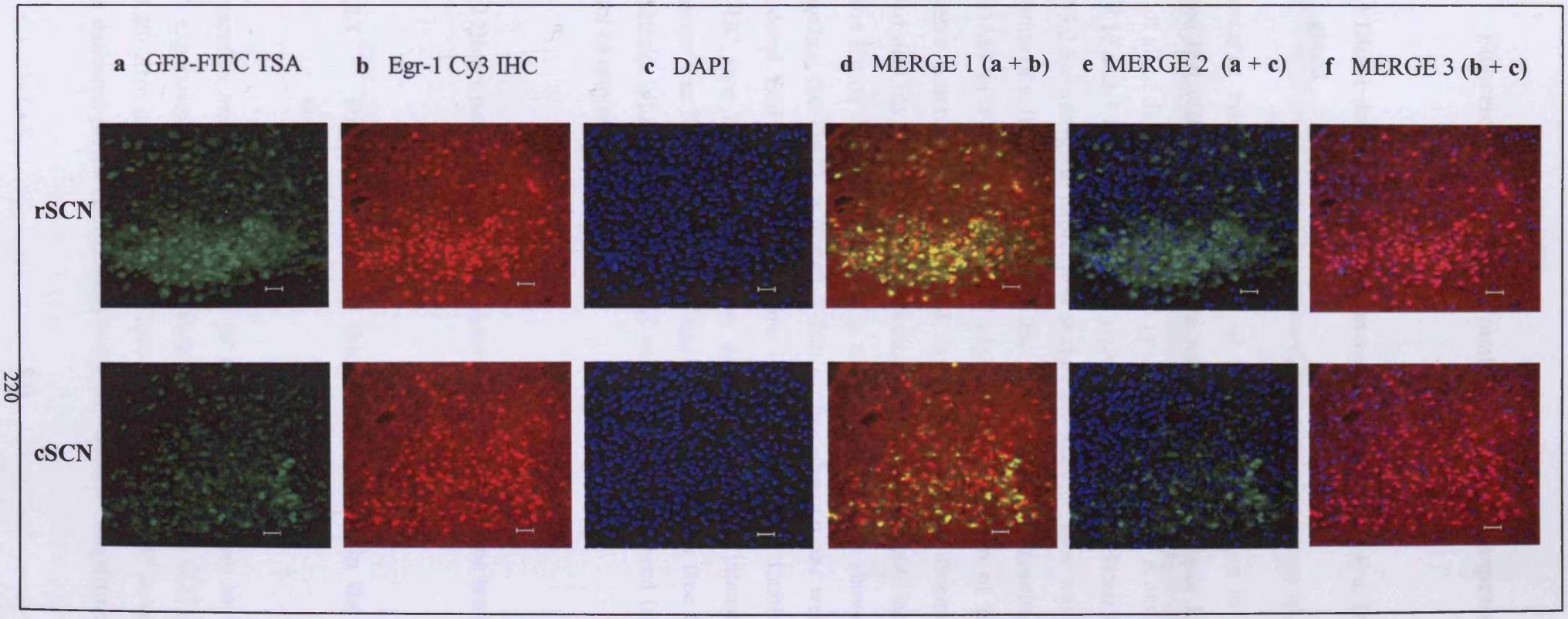


Figure 7.15 High resolution images of immunoreactive transgene (d2EGFP) protein within **Z27-B** transgenic male rostral (**r**) and caudal (**c**) SCN sections (10μm), sampled at subjective night (01:00h; 6 hours after lights off), following an hour of photic stimulation, and dual EGFP:Egr-1 TSA:IHC. 'MERGE' represents an overlay of images, as indicated in brackets. (The third ventricle is located on the left hand side of each of image). Scale bar = 25 μm. See text for further discussion.

7.3.3 Fluorescence microscopic analysis of transgene expression

7.3.3.1 Direct detection of fluorescence (GFP) in the Z line transgenic pituitary gland

Fluorescence microscopic analysis of transgene expression in the pituitary gland revealed that (GFP) expression was restricted to the anterior lobe of the pituitary gland of all of the Z transgenic lines analysed (Z25-B, Z27-A and Z27-B; see Figures 7.16, 7.17 and 7.18; Z27-A data not shown). Similar to previous findings (see 7.3.2.1 and 7.3.2.3), transgene expression within the anterior lobe was confined to a sub-population of cells, often associated into small groups (see simultaneous visualisation with DAPI in Figures 7.16 and 7.17). Close inspection of EGFP in the Z25-B transgenic anterior pituitary gland revealed partial confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in Figure 7.17), and also filling of the cytoplasm (as shown by green labelling surrounding the DAPI in Figure 7.17); similar observations were made using both single-label EGFP IHC (see Figures 7.5 and 7.6), and double-label EGFP:Egr-1 TSA:IHC (see Figure 7.11), albeit at a lower signal intensity. No transgene expression was detected in non-transgenic pituitary glands (see Figure 7.16), and in the posterior pituitary of all of the Z transgenic lines analysed (see Figures 7.16 and 7.17 for examples).

7.3.3.2 Direct detection of fluorescence (GFP) in the Z line transgenic brain

7.3.3.2.1 Direct detection of fluorescence (GFP) in the Z line transgenic cortex

Fluorescence microscopic analysis of transgene expression in the cortex revealed (GFP) expression in all of the Z transgenic lines analysed (Z27-B: see Figures 7.19 and 7.20; Z16: data not shown). Close inspection of EGFP in the Z27-B transgenic cortex indicated partial confinement to the peri-nuclear compartment (as shown by the

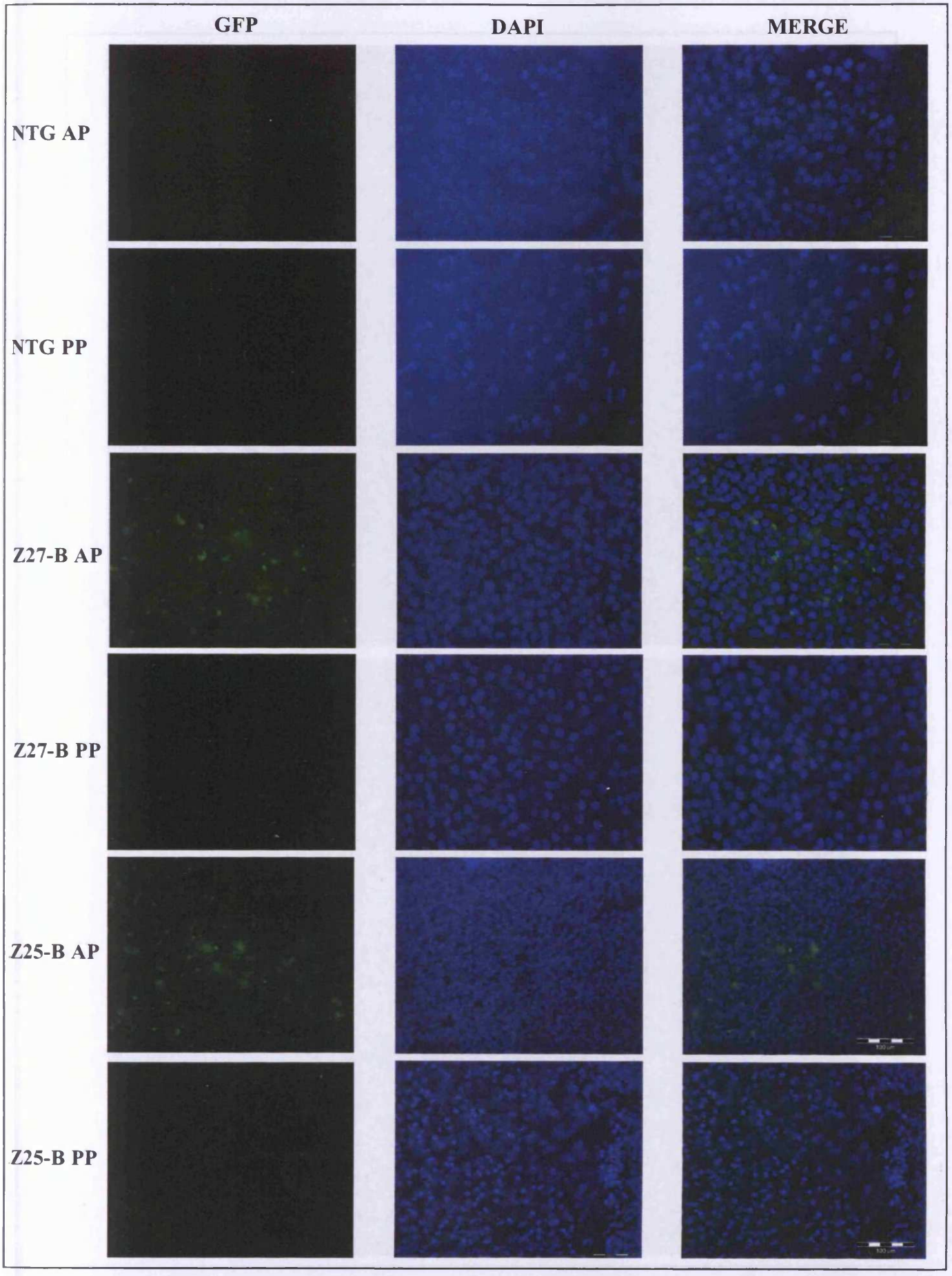


Figure 7.16 Fluorescence microscopic images of direct fluorescence (**GFP**) in Z transgenic female pituitary gland sections (50μm). **AP** and **PP** represent the anterior and posterior pituitary, respectively; **NTG** represents non-transgenic samples. Scale bar = 50μm (**NTG** and **Z27-B** samples) and 100μm (**Z25-B** samples). See text for further discussion.

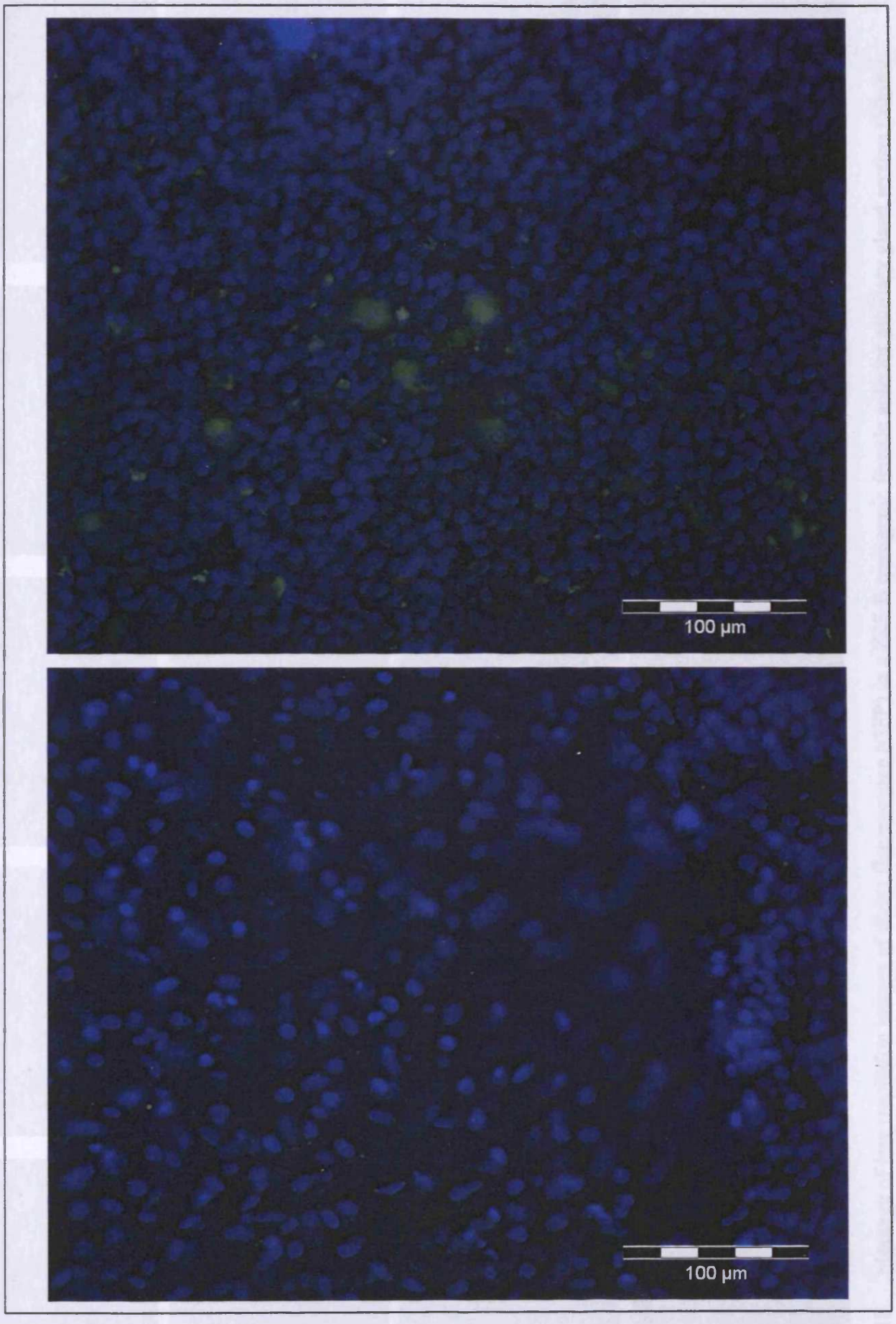


Figure 7.17 Low resolution fluorescence microscopic images of direct fluorescence (GFP) in the **Z25-B** transgenic female pituitary gland sections (50µm). Above: merged GFP and DAPI images of the anterior pituitary gland. Below: merged GFP and DAPI images of the posterior pituitary. Scale bar = 50µm. See text for further discussion.

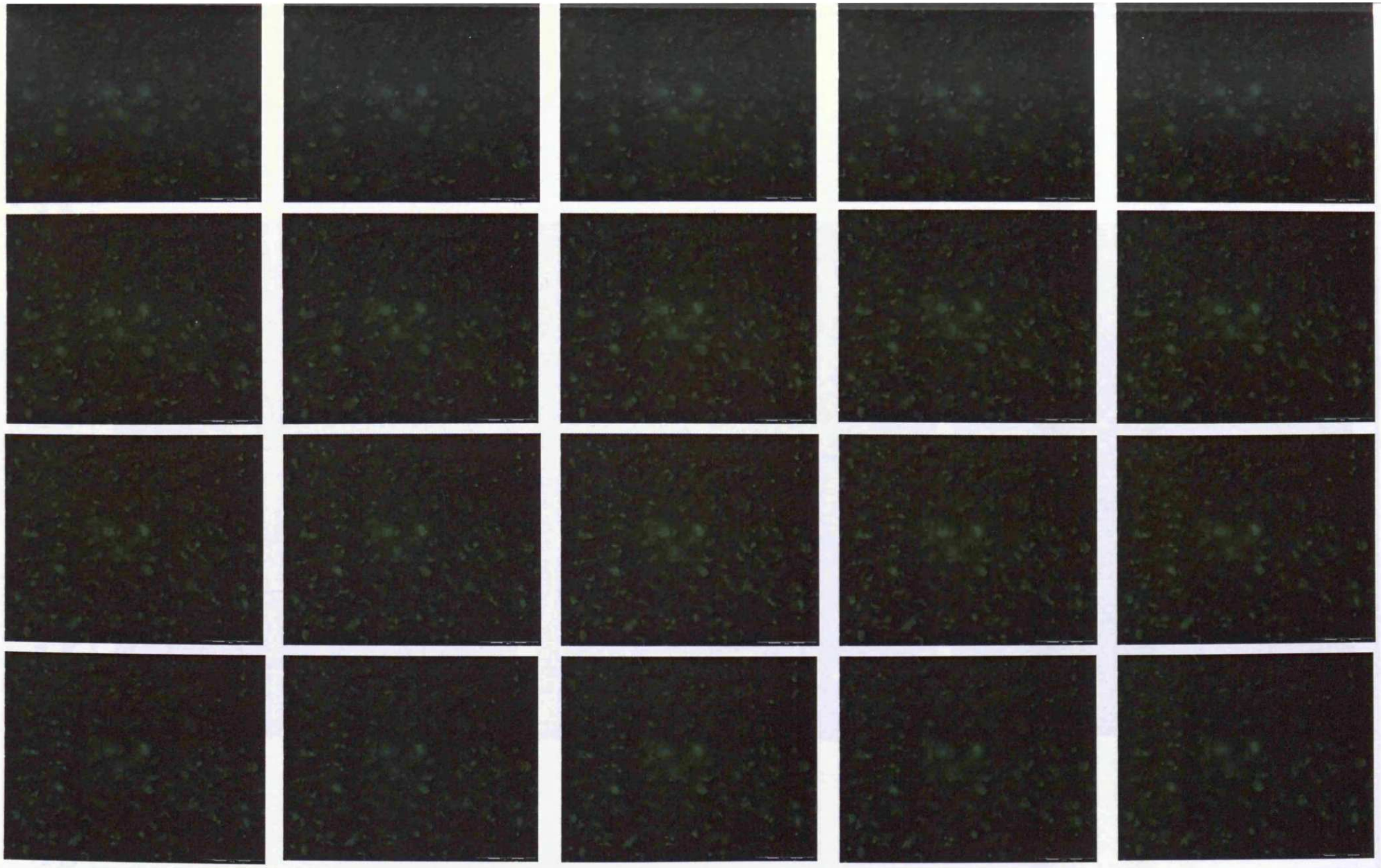


Figure 7.18 Montage of low resolution images of direct fluorescence (GFP) in a **Z25-B** transgenic female anterior pituitary gland section (50μm). Scale bar = 100μm. Each image represents a 1μm layer (in a series of 20; see above, left to right, top to bottom) taken from the section. See text for further discussion.

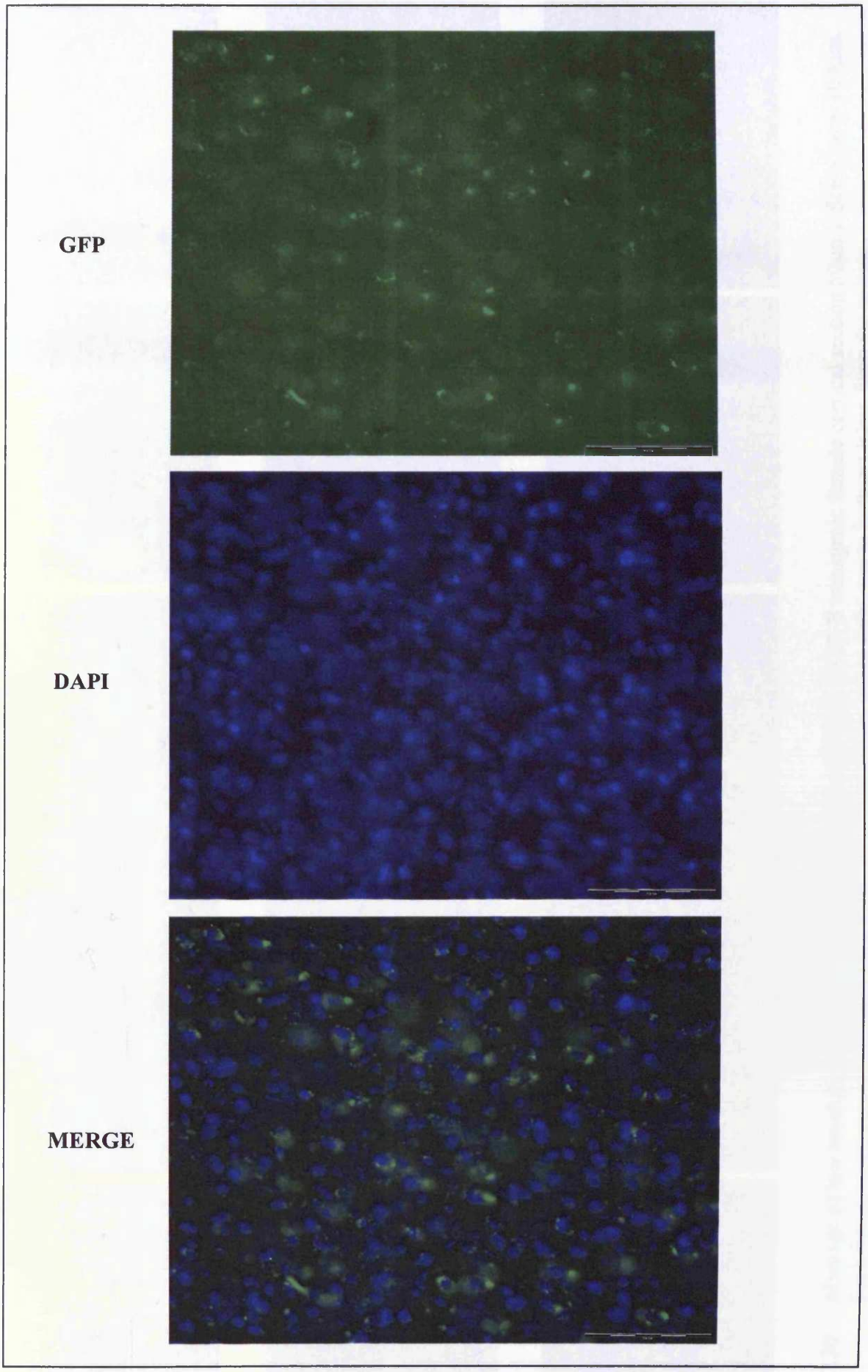


Figure 7.19 Fluorescence microscopic images of a **Z27-B** transgenic female cortical section (50μm). Above: direct fluorescence (GFP); Centre: DAPI staining; Below: an overlay of the GFP and DAPI images. Scale bar = 100μm. See text for further discussion.

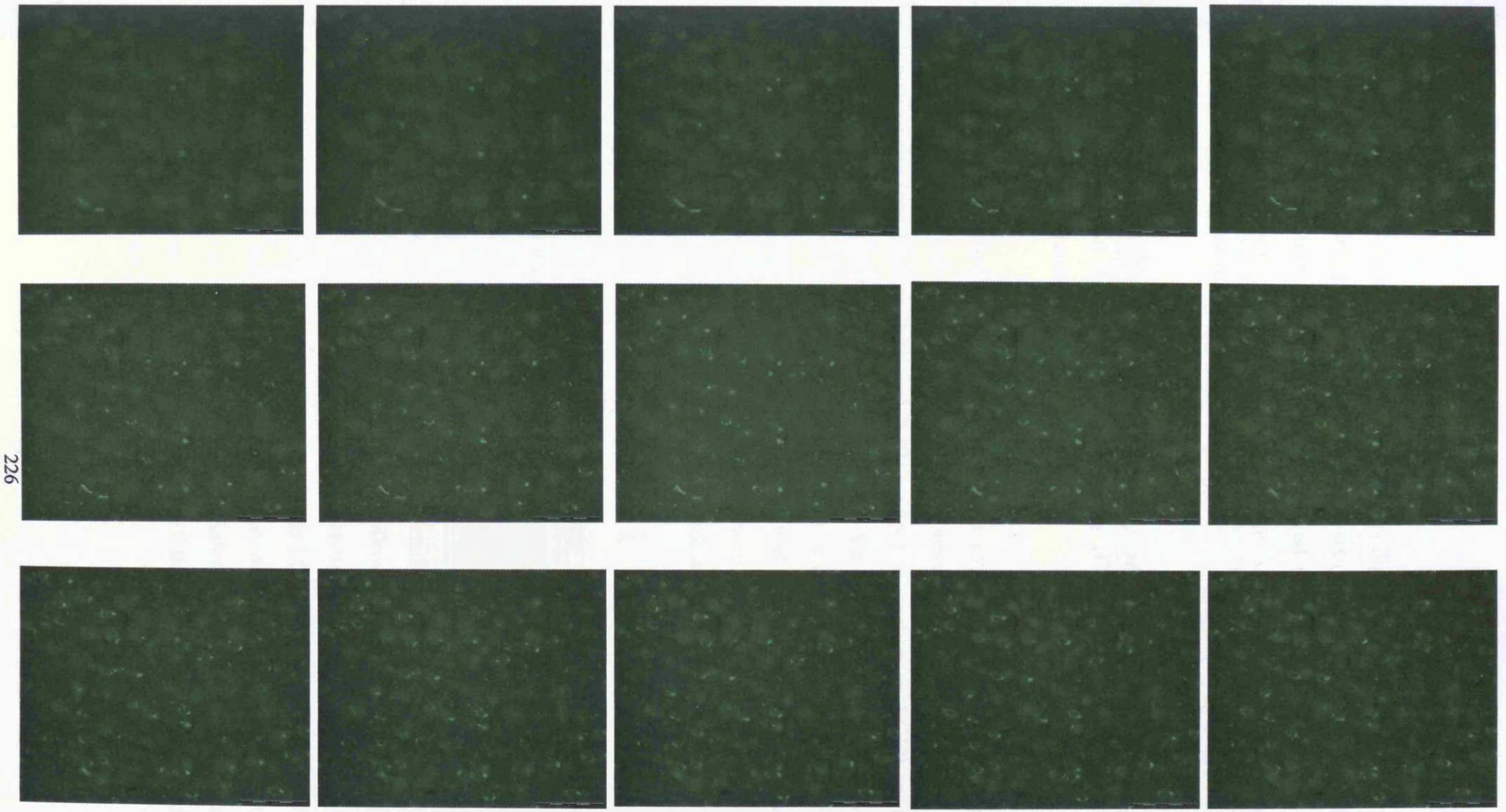


Figure 7.20 Montage of low resolution images of direct fluorescence (GFP) in a **Z27-B** transgenic female cortical section 50μm). Scale bar = 100μm. Each image represents a 1μm layer (in a series of 15; see above, left to right, top to bottom) taken from the section. See text for further discussion.

simultaneous visualisation of GFP with DAPI in Figure 7.19). These observations were distinct from those derived from IHC analysis (see Figures 7.7, 7.11 and 7.12), where immunoreactive EGFP expression appeared to be primarily confined to the nuclear compartment. No transgene expression was detected in non-transgenic cortical samples (data not shown).

7.3.3.2.2 Direct detection of fluorescence (GFP) in the Z line transgenic hippocampus

Fluorescence microscopic analysis of transgene expression in the Z16 transgenic hippocampus revealed high levels of GFP expression in the CA1 region of the hippocampus (see Figures 7.21 and 7.22). Close inspection of EGFP in the Z16 transgenic hippocampus revealed confinement to the nuclear compartment (as shown by the co-localisation (light green/blue labelling) of GFP with DAPI in the 'merged' images in Figure 7.21). These observations mirrored those derived from IHC analysis (see Figure 7.10), albeit at a higher signal intensity. No transgene expression was detected in non-transgenic hippocampal samples (data not shown).

7.3.3.2.3 Direct fluorescence microscopic analysis of transgene up-regulation in the Z line transgenic SCN

The capacity of *egr-1* genomic sequences within the transgene to mediate up-regulation in response to a physiological stimulus was investigated using an established circadian, light-pulse paradigm. Following this stimulus, fluorescence microscopic analysis in the Z16 and Z27-B transgenic lines revealed a discrete induction of transgene in the ventral region of the SCN sections (see Figure 7.23 for example). These findings broadly mirror that of Figures 7.13, 7.14 and 7.15, respectively, albeit at a much lower signal intensity. The induction of GFP fluorescence was more clearly observed in the Z16 transgenic line; further studies are required to validate the Z27-B data (not shown).

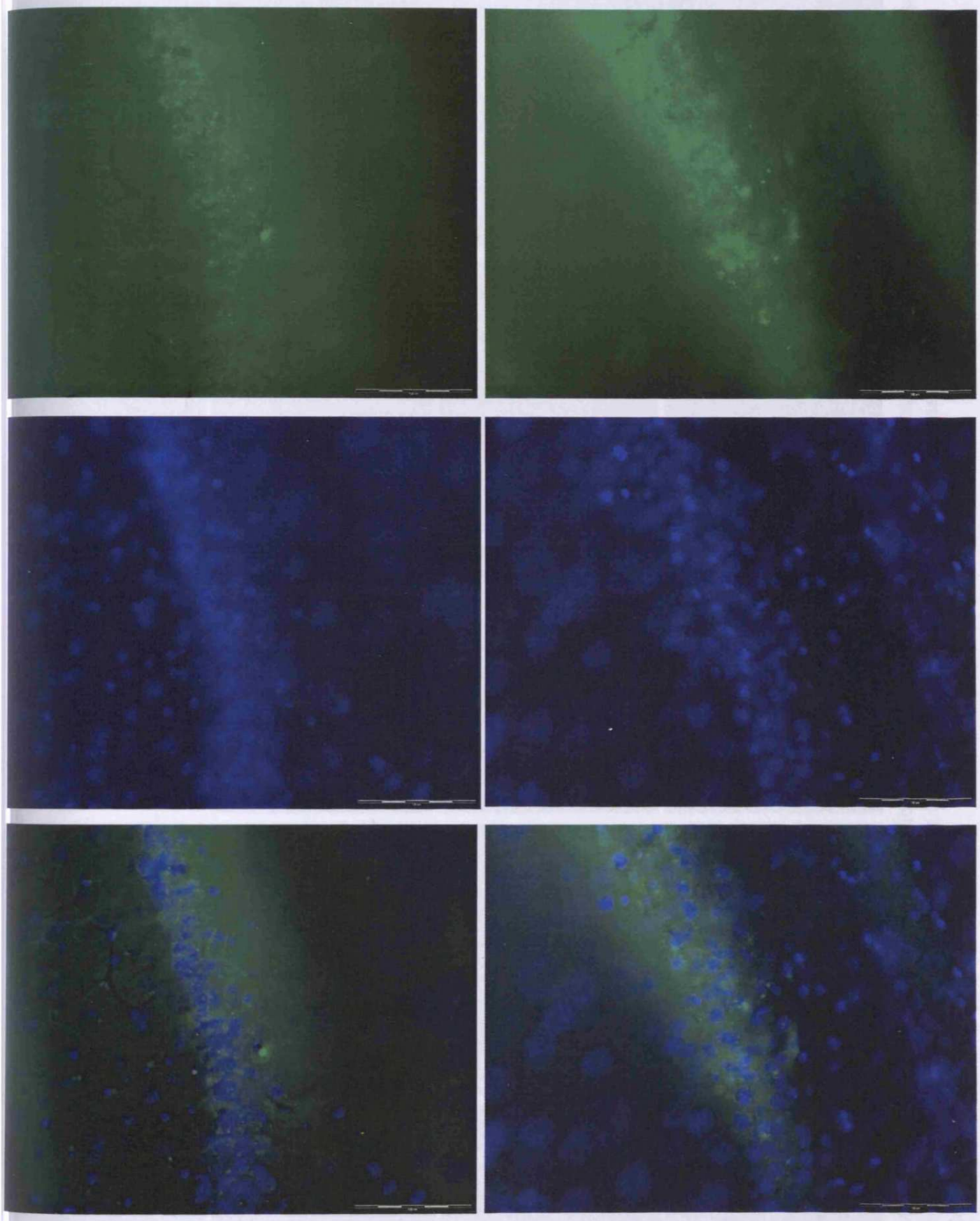


Figure 7.21 Fluorescence microscopic images of **Z16** transgenic female hippocampal sections (50μm). Above: direct fluorescence (GFP); Centre: DAPI staining; Below: merged GFP and DAPI images. Scale bar = 100μm. See text for further discussion.

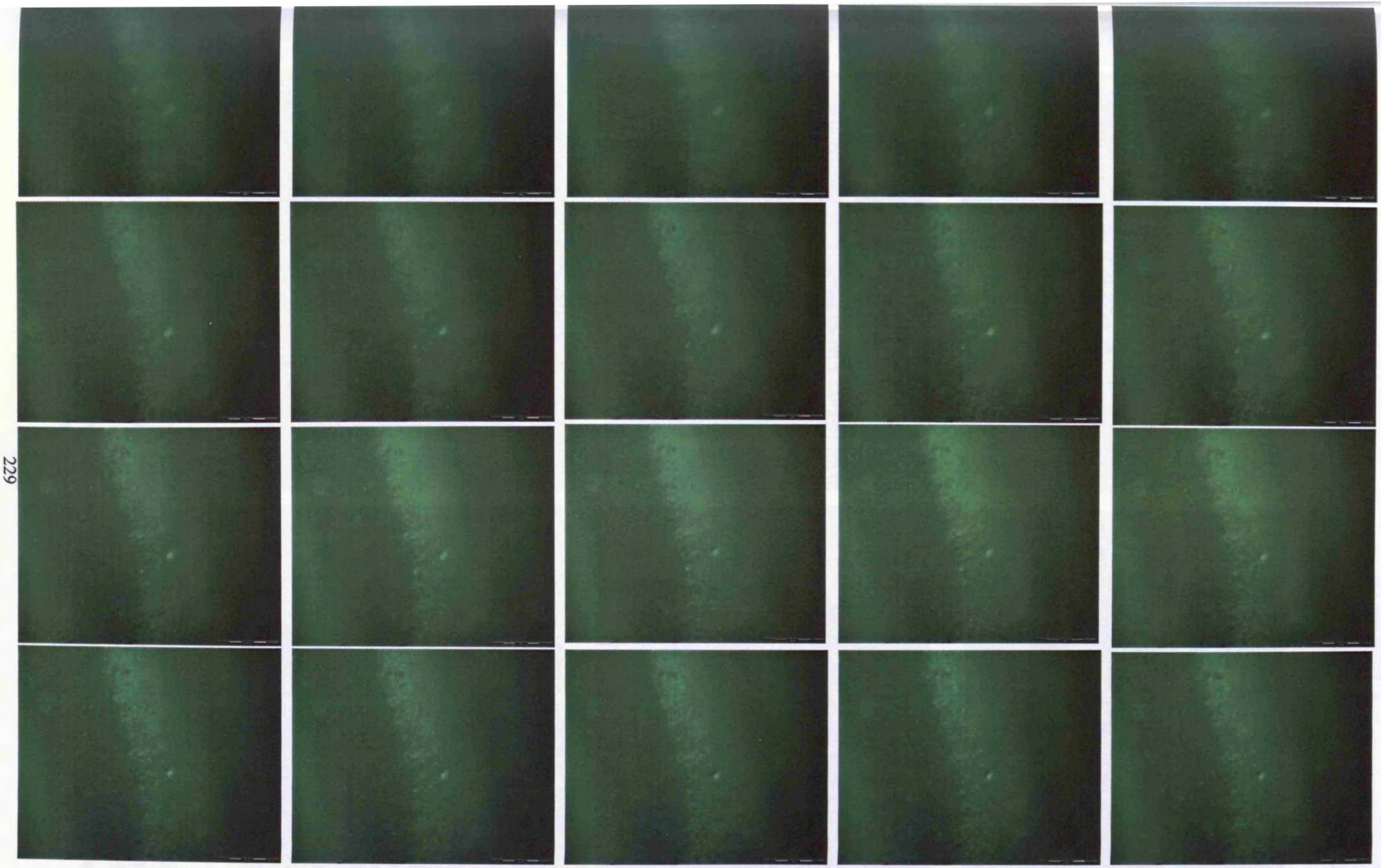


Figure 7.22 Montage of low resolution images of direct fluorescence (GFP) in a **Z16** transgenic female hippocampal section (50μm). Scale bar = 100μm. Each image represents a 1μm layer (in a series of 20; see above, left to right, top to bottom) taken from the section. See text for further discussion.

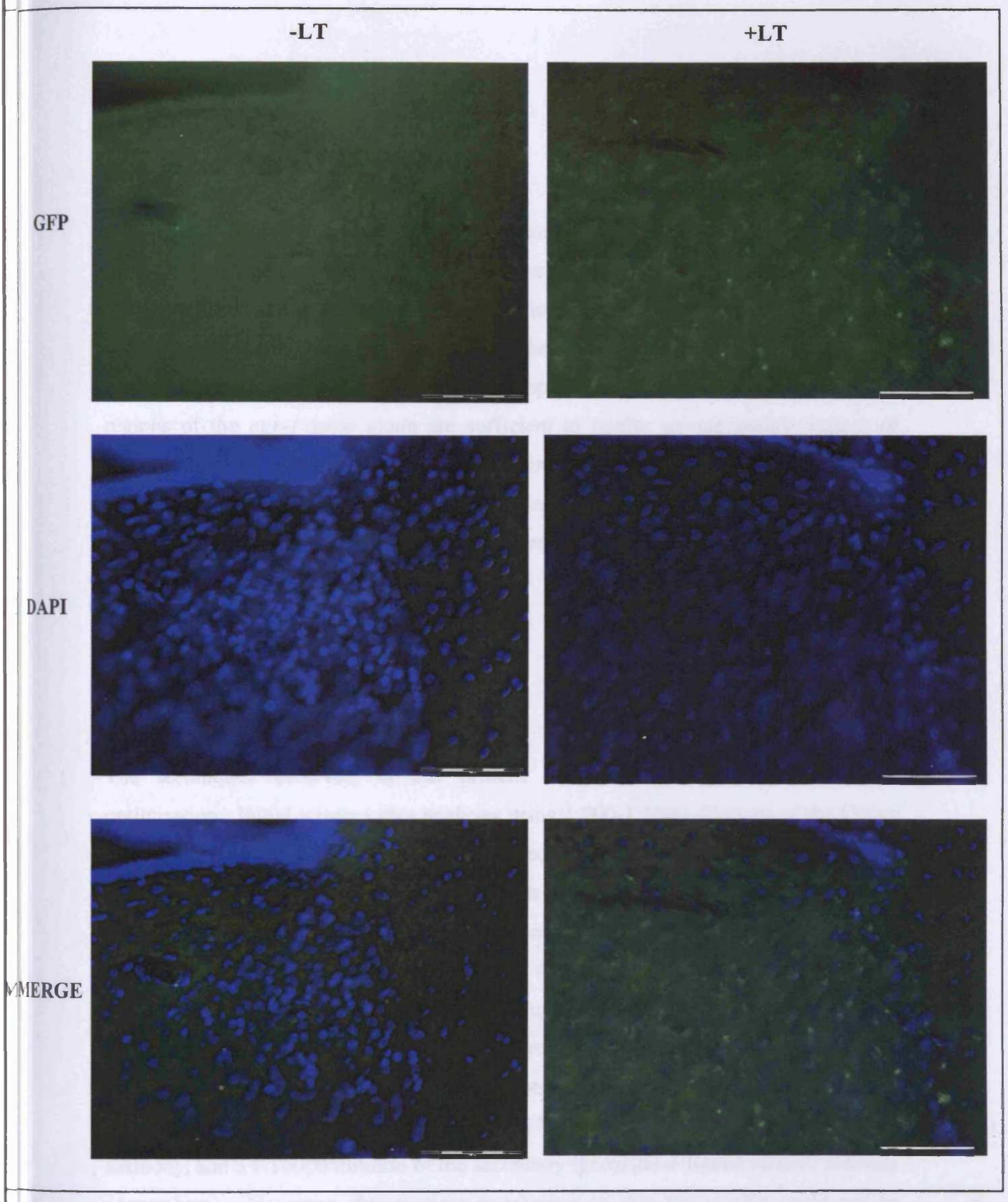


Figure 7.23 Fluorescence microscopic images of direct fluorescence (GFP) in **Z16** transgenic female SCN sections (50μm) sampled at subjective night (01:00h; 6 hours after lights off), following an hour of photic stimulation (**+LT**; non-stimulated = **-LT**). Above: direct fluorescence (GFP); Centre: DAPI staining; Below: an overlay of the GFP and DAPI images. Scale bar = 100μm. (The third ventricle is located in the top left corner of each image). See text for further discussion.

7.4 DISCUSSION

The present transgenic rat study demonstrates that the *egr-1*/d2EGFP transgene (including only proximal 5'-flanking sequences of the *egr*-1 gene) confers both tissue-specific constitutive- and inducible expression at the protein level, in the brain, pituitary gland and liver. The findings of the present study are in agreement with studies conducted at the mRNA level (see Chapter 6 and 7.4.2) showing that proximal regions of the *egr-1* gene alone are sufficient to confer an appropriate pattern of expression. This conclusion may be drawn since *egr1*/d2EGFP transgene expression recapitulates that of the endogenous gene, and reflects observations in the *egr-1* transgenic mouse and (*egr-1* intron-containing) rat models (Tsai *et al.*, 2000, and Slade *et al.*, 2002, respectively).

7.4.1 Optimisation of protocols

The techniques involved in the present study were subjected to extensive optimisation. Initial western blot analyses using 1:200-1:1000 dilutions of the Living Colours A.V. GFP peptide antibody (Clontech) failed to detect EGFP (transgene) expression in Z line transgenic pituitary glands (data not shown). Subsequent western blot analyses used 1:1000-1:17500 dilutions of the GFP monoclonal antibody (Clontech), resulting in detection of d2EGFP (Clontech; 31kDa) in various tissues of the Z transgenic lines (see 7.3.1). These findings, however, also revealed non-specific bands (see 7.3.1). Therefore, based on the present analyses it may be suggested that the optimal conditions for EGFP western blots are a 2 hour resolution of the protein samples, followed by incubation with a 1:17500 dilution of the GFP monoclonal antibody, and a 1:10000 dilution of the secondary (peroxidase-linked mouse) antibody (Amersham). However, due to time restrictions of this thesis, this could not be confirmed by further experiment.

Initially, fresh frozen samples (as described in 2.3.4.1.1) were used for IHC analysis. No immunoreactive transgene (GFP) expression was detected in the various tissues analysed, thus, it was decided that animals would be perfused and fixed in paraformaldehyde prior to IHC analysis. Extensive optimisation of the dual TSA:IHC technique was also performed, with initial analyses involving the alteration of concentrations of reagents used in the technique. For example, 1:200-1:20000 dilutions of the first primary antibody (anti-GFP rabbit IgG, Molecular Probes), 1:100-1:500 dilutions of the second primary antibody (Egr-1 rabbit polyclonal C19 antibody, Santa Cruz), and 1:50-1:200 dilutions of the second secondary antibody (Cy3 goat anti-rabbit Ig, Sigma) were used. Subsequent to optimisation of this technique, no cross-reactivity was detected between the respective antibodies (see 7.2.2.2.3). Also, the optimised dual EGFP:Egr-1 TSA:IHC technique was validated by incomplete co-localisation of EGFP and Egr-1 in the Z line transgenic brain (see Figures 7.11-7.15). The direct detection of fluorescence in the Z line transgenic tissues, in the SCN in particular, by fluorescence microscopy, is currently still being optimised. Future studies of the Z line transgenic SCN may involve isolation of the hypothalamus from the brain, and using ultra-low gelling temperature agarose as a mounting medium, similar to that described for the mounting of pituitary gland samples (see 7.2.2.3.3). This may allow more intact SCN sections to obtained.

7.4.2 Transgene expression analysis

Various methods of protein detection (western blot analysis, IHC and direct fluorescence microscopy) were used to investigate transgene expression in the present study, and were successful in confirming robust expression of the *egr-1*/d2EGFP transgene at the protein level. However, the presence of numerous transgenic lines (Z13, Z14, Z16, Z25-A, Z25-B, Z27-A and Z27-B), as described in chapter 6, has meant that investigations carried out in the present study have had to be selective, due to breeding dynamics and time restrictions of this thesis. Although protein analyses in the present study were more extensive than the transcript analyses previously performed (see Chapter 6), the present findings have generally shown consistency between the various protein detection methods, and also with transcript level results (see Chapter 6 and Table 7.1). For example, northern blot analysis of the Z25-A

transgenic line (highest apparent copy number; see Figure 6.10) failed to detect transgene transcript expression in the cortex, hippocampus and liver (following a pharmacological stimulus; see Figure 6.18), which was thought to reflect a relatively low level of transgene expression in this line, that is nevertheless detectable in the pituitary gland and cortex by RT-PCR (see Figure 6.12). The present study failed to detect immunoreactive transgene protein in pituitary glands of this line, as shown by both western blot analysis and single-label IHC (see Figure 7.1, and 7.3.2.1). For this reason, studies of the remaining transgenic lines were given precedence.

RT-PCR and northern blot analysis detected transgene transcript expression in the Z27-A and Z27-B lines (high and low copy numbers, respectively; see Figure 6.10), in tissues such as the pituitary gland (see Figures 6.13 and 6.15), and in the cortex, hippocampus and liver following a pharmacological stimulus (see Figure 6.16). Similarly, western blot analysis revealed immunoreactive transgene protein in the Z27-A and Z27-B pituitary glands (see Figure 7.2), and up-regulation in the Z27-A and Z27-B cortex and hippocampus following a pharmacological stimulus (see Figure 7.4; Z27-B data not shown). Further protein analysis by IHC, and analysis using direct fluorescence microscopy, have confirmed transgene protein expression in sub-populations of cells in the anterior lobe of the pituitary gland of the Z27-A and Z27-B lines (see Figures 7.5, 7.11 and 7.16). A similar distribution of Egr-1 protein expression has been observed in a previous study (Knight *et al.*, 2000). In addition, dual EGFP:Egr-1 TSA:IHC revealed co-localisation of immunoreactive transgene protein with endogenous Egr-1 (see Figure 7.11). Furthermore, protein analysis by IHC, and analysis using direct fluorescence microscopy, have confirmed transgene protein expression in the Z27-B cortex (see Figure 7.7, 7.11, 7.19 and 7.20), and also daytime transgene protein expression in the Z27-B SCN (see Figure 7.8). Nocturnal photic stimulation was shown to induce transgene expression in the ventral region of the Z27-B SCN, which was co-localised with endogenous Egr-1 by double-label EGFP:Egr-1 TSA:IHC (see Figures 7.14 and 7.15). For this reason, the Z27-B line was selected for analysis by direct fluorescence microscopy, following a nocturnal photic stimulus. Although direct fluorescence detection experiments in the Z27-B SCN have not yet been duplicated, and the experimental conditions for SCN analysis have not yet been optimised (with respect to SCN sectioning), preliminary findings are looking promising.

ANALYSIS \ TRANSGENIC LINE		Z13	Z14	Z16	Z25-A	Z25-B	Z27-A	Z27-B
DNA:	**SOUTHERN**	↓	↓	↓	↑	-	↑	↓
RNA:	**RT-PCR**				- ↓ P C	↑↑ P C	- ↓ P C	- ↓ P C
	NORTHERN	- - P **H**			(C) (H) (L)		↑ ↑ ↓ - ↓ ↓ P **C H L** K H*	↑ ↑ - - ↓ ↓ P **C H L** K H*
PROTEIN:	**WESTERN**				(P)	↑ P	- ↑ ↑ ↑ P **C H L**	- P
	IMMUNOHISTO-CHEMISTRY	↓ - P C	↓ - P C	↓ ↓ ↓ - P C **S** H	(P)	↑ ↓ ↓ P C S	↑ P	↑ ↑ ↑ P C **S**
	FLUORESCENCE MICROSCOPY			↓ ↑ C **S** H		↑ P	↑ P	↑ ↑ P C **S**

Table 7.1 The table above contains a summary of the data obtained from all the analyses performed on the various Z transgenic lines.

↑ / - / ↓ represent high / intermediate / low copy numbers, respectively;
P, C, H, L, K, H*, S represent pituitary, cortex, hippocampus, liver, kidney, heart, SCN, respectively;
↑ / - / ↓ / ( ) represent high / intermediate / low / no basal expression levels, respectively;
letters in **bold** indicate transgene inducibility with metrazole;
letters in **underlined bold** indicate transgene inducibility with light.

Similar to the Z27-A and Z27-B lines, RT-PCR analysis (see Figure 6.12) detected transgene transcript expression in the pituitary gland and cortex of the Z25-B line (high copy number; see Figure 6.10). Western blot analysis also revealed immunoreactive transgene protein in the Z25-B pituitary gland (see Figure 7.1), localised to the anterior lobe, as shown by IHC (see Figure 7.6) and by direct fluorescence (see Figures 7.16, 7.17 and 7.18). However, in contrast to Z27-B, western blot analysis failed to detect immunoreactive transgene protein in the Z25-B cortex (see Figure 7.3), but this was nevertheless detected at very low levels by single-label IHC (see Figure 7.7). Furthermore, daytime transgene protein expression in the Z25-B SCN was detected at very low levels (see Figure 7.8); nocturnal photic stimulation failed to induce transgene expression in the Z25-B (and Z27-A) SCN.

The Z16 line has been the most extensively analysed of the low copy number transgenic lines (see Figure 6.9), at the protein level. Nocturnal photic stimulation of this line resulted in up-regulation of immunoreactive transgene protein in the SCN (see Figure 7.13), similar to that seen in the Z27-B SCN (see Figures 7.14 and 7.15), which was co-localised with endogenous Egr-1 by dual EGFP:Egr-1 TSA:IHC (see Figure 7.13). High basal levels of immunoreactive transgene protein were also detected in the CA1 region of the Z16 hippocampus, initially by single-label IHC (see Figure 7.10), and then by analysis using direct fluorescence microscopy (see Figures 7.21 and 7.22). This reflects the pattern of constitutive *egr-1* expression (see Herdegen and Leah, 1998). Western blot analysis (Figure 7.1) failed to detect immunoreactive transgene protein in the Z13 and Z14 pituitary glands, but was nevertheless detected in very low levels by IHC (data not shown). Immunoreactive transgene protein was also detected in the Z14 cortex, by IHC, as illustrated in Figure 7.7.

The findings of the present study indicate that immunoreactive transgene expression is confined to a sub-population of cells in the anterior pituitary, partially within the nuclear compartment, and, unlike Egr-1 protein, also in the cytoplasm (see Figures 7.5, 7.6, 7.11, 7.16 and 7.17). Therefore, these models (in particular, the Z25-B, Z27-A and Z27-B lines) may aid in the identification of activated cell-types that could not be discriminated through direct analysis of nuclear transcription factor protein. In

general, the findings of the present study also indicate that immunoreactive transgene expression in the brain is more confined to the nuclear compartment (cortex - see Figure 7.7; SCN - see Figures 7.8 and 7.9; hippocampus - see Figure 7.10), compared with the pituitary. Closer inspection revealed two sub-populations of transgene expression within the nuclear compartment: one that is highly expressed, and one that is expressed at lower levels (cortex - see Figures 7.11 and 7.12; SCN - see Figures 7.13, 7.14 and 7.15). Direct detection of fluorescence analyses of the respective tissues appear to support these hypotheses (anterior pituitary gland - see Figures 7.16 and 7.17; hippocampus - see Figure 7.21; SCN - see Figure 7.23). However, an inconsistency in cortical transgene expression was detected by direct fluorescence microscopy (see peri-nuclear expression in Figure 7.19), in comparison to those seen in the IHC analyses (see Figures 7.7, 7.11 and 7.12). Further studies are required to understand this finding, which may be related to the absence of tissue fixation (to slides) in the direct fluorescence studies.

The significance of these findings is further discussed in Chapter 8.

7.4.3 Conclusions

Taken together, the findings of the present study indicate that transgene copy numbers, to some extent, is inversely related to transgene expression. For example, a lack of transgene expression (mRNA or protein) was observed in tissues of the Z25-A (highest apparent copy number) transgenic line. Interestingly, the 93-F (highest apparent copy number) transgenic line, also failed to express the *egr-1*/d4EGFP transgene in studies by Slade (2001). The high copy number transgenic lines (Z25-B and Z27-A) appear to be characterised by abundant transgene expression in the anterior pituitary gland, and the lower copy number transgenic line (Z16) by low daytime expression in the SCN, and up-regulation of transgene expression following nocturnal photic stimulation. The relationship between copy numbers and transgene expression is further discussed in Chapter 8.

In conclusion, the higher copy number transgenic lines may provide models for studying the regulation of Egr-1 in the pituitary gland, and the lower copy number transgenic lines for studying light-stimulated regulation of Egr-1 in the SCN. Concurrent studies in the Z16 and Z27-B transgenic retina and pineal gland also indicate light-dark responses in these tissues, with increases in transgene expression detected in samples taken in the dark period (Man *et al.*, 2004; data not shown). The Z16 transgenic line may also provide a model in which hippocampal function may be investigated, such as in learning and memory paradigms. Further analyses of the Z13 and Z14 (low copy number) transgenic lines are required before any conclusions may be drawn regarding these particular lines. Since GFP fluorescence can be detected directly from tissues of the various Z transgenic lines, these transgenic models may serve as convenient and dynamic models for studying the role and regulation Egr-1 and Egr-1-expressing cells in the rat. The future implications of these models are further discussed in Chapter 8.

CHAPTER 8

GENERAL DISCUSSION

8.1 Outcomes of this thesis

The earliest studies performed in this thesis on the regulation of vasopressin transgene expression in the SCN failed to detect the hGH reporter both in the more extensively analysed JP-59 line, and then in the JP-17 line (Chapter 3). Since the completion of these studies, the creators of these transgenic lines have confirmed the findings of this thesis with respect to transgene (non-) expression in the JP-59 transgenic SCN (Wells *et al.*, 2003). However, recent findings indicate that transgene expression is detectable in the JP-17 transgenic SCN, as determined by ISH, following a longer exposure to autoradiographic film (Wells *et al.*, 2003). Since there was limited access to the JP-17 transgenic line, less extensive analyses of this line were performed, and thus the negative findings may have been due to a lack of optimisation of ISH with respect to this line. In addition, a recent study has revealed that transgenic rats expressing a VP-EGFP fusion gene (Ueta *et al.* 2004), which incorporates similar sequences to that used by Zeng *et al.* (1994; see Figure 3.3**h**), but replaces CAT with the EGFP reporter gene, have shown expression of the EGFP gene and strong fluorescence in the SON, the PVN and the SCN. Thus, it appears that the efficiency of the transgene in directing expression to the SCN, at levels comparable to that of the respective endogenous gene, may be influenced by the reporter gene selected. However, additional studies would be required to investigate this notion further.

Four *mPer1*/d2EGFP (Y) transgenic lines were then successfully generated (Chapter 4). However, extensive analyses of three of these lines failed to detect reporter gene expression. Although these findings were disappointing, and unexplained, the experience gained with respect to transgenesis was valuable, and contributed to an increased efficiency in later studies (Chapter 6). The observed contrast between

protein-level expression of the d2EGFP variant compared with the d4EGFP cannot be fully explained on the basis of the present studies but is noteworthy because there is no published data on expression of the d4EGFP in an *in vivo* system. In contrast, translation of the d2EGFP protein has been successfully demonstrated in four transgenic models (present study; Kuhlman *et al.*, 2000; Bi *et al.* 2002; Dorsky *et al.*, 2002).

Analysis of the *egr-1*/d4EGFP (57C, Chapter 5) transgenic line generated interesting expression data. However, despite robust transcription of the transgene, direct GFP fluorescence was undetectable. The latter part of this thesis involved the generation of seven *egr-1*/d2EGFP (Z) transgenic lines. These lines have demonstrated robust expression of the transgene at both transcript and protein levels (Chapters 6 and 7, respectively), and permit the direct detection of GFP fluorescence. For this reason, the potential significance of the *egr-1*/d2EGFP transgenic model will be further discussed (see below).

8.2 Expression of the *egr-1*/d2EGFP transgene

Analysis of the *egr-1*/d2EGFP transgenic lines has confirmed the results of previous transgenic studies (Slade *et al.*, 2002; Tsai *et al.*, 2000) in that transgene expression was observed to be tissue-specific (e.g. in the anterior pituitary gland, cerebral cortex, and CA1 hippocampal area) and inducible (e.g. in the hippocampus). An additional finding of the present study, which is in agreement with the results of Tsai *et al.* (2000), is that the *egr-1* intron would not appear to be necessary to direct this pattern of expression. Clearly, further studies of both additional transgene constructs, and extensive comparisons of tissue and cellular expression patterns would be required to fully rule out any contribution of the *egr-1* intron. However, although the pattern of transgene expression was generally comparable between the different *egr-1*/d2EGFP transgenic lines, marked differences have been observed in individual lines. These observations broadly confirm previous transgenic animal studies, but the extensive analysis of multiple lines performed here has provided an interesting perspective that deserves further discussion.

The results indicate that expression of the *egr-1*/d2EGFP transgene is inversely related to copy number, at least above a certain number of integrated copies of transgene (e.g Z25-A line). Previous studies of the *egr-1*/d4EGFP transgenic model (Slade, 2001) also provided evidence of this phenomenon. In contrast, a previous study in this laboratory using a quite different construct (AA-NAT-CAT; Burke *et al.*, 1999) demonstrated abundant transgene expression in animals with 80 and 170 copies of integrated transgene. However, the findings of the present study, and those of Slade (2001) are in agreement with a literature (see Garrick *et al.*, 1998 and Henikoff, 1998) that reports reduced transgene expression in association with large concatameric arrays of repeated transgene copies. A reduction in copy number at a transgene integration site has been shown to be associated with a decrease in chromatin compaction and methylation, and a consequent increase in transgene expression (Garrick *et al.*, 1998). Hence, it is suggested that this mechanism of transgene silencing is also observed here for the Z25-A line.

In addition to the copy number/expression phenomenon discussed above, a correlation between copy number and tissue-specificity of expression has also been observed. Thus, the transgenic lines with apparent copy numbers next highest relative to the Z25-A line (Z25-B and Z27-A) are characterised by abundant transgene expression in the pituitary gland, but relatively lower levels in the brain (compared to the lower copy number Z16 line). This is interesting because it suggests that a copy number-related mechanism of transgene silencing, perhaps related to chromatin configuration, may be more active in the brain than peripheral tissues. It should be noted that the transgenic lines studied here were generated by pronuclear microinjection, resulting in random transgene integration, and thereby introducing potential position effects (see 3.4.2). Further study of multiple lines would be required to investigate this phenomenon further. Alternatively, an approach similar to that used by Garrick *et al.*, (1998) in which position effects were eliminated by use of the Cre/lox system could be applied in mice.

These findings demonstrate the value of generating multiple different lines of transgenic rats/mice, in order that expression can be fairly judged. The use of larger transgene constructs (e.g. BACs, see Wells and Carter, 2001) may serve to eliminate position effects, and produce copy number-independent expression. Despite these observations, the *egr-1*/d2EGFP transgenic lines remain interesting models for further analysis.

8.3 Future applications of the *egr-1*/d2EGFP transgenic model

8.3.1 Studies in the brain

Analyses of the *egr-1*/d2EGFP transgenic model, are in agreement with previous studies that demonstrated stimulus-specific induction in the rat brain: metrazole-related induction in the hippocampus (Slade *et al.*, 2002), and nocturnal photic stimulation in the SCN and PeN (Slade *et al.*, 2001; 2002). The lower copy number (Z16, Z27-B) transgenic lines appear to be the most suitable models for studying Egr-1 in the brain, in particular, the light-stimulated regulation of Egr-1 in the SCN.

The *mPer1*-d2EGFP transgenic mouse model (Kuhlman *et al.*, 2000) has already provided a model in which SCN organisation may be analysed dynamically, on a circadian time scale. IHC studies of this model have demonstrated rhythmic expression of *Per1* mRNA, GFP and PER protein (LeSauter *et al.*, 2003). Further analysis of samples taken at peak expression times revealed expression of *Per1* mRNA, GFP and PER protein in the rostral SCN, but not in a small 'hole-like' region in the mid-ventrolateral and caudo-lateral SCN (LeSauter *et al.*, 2003; Karatsoreos *et al.*, 2004). Recent studies have indicated that this region includes GRP-immunopositive cells that express both light-induced *mPer1* and Fos (Karatsoreos *et al.*, 2004). These findings indicate that there are some SCN cells that are rhythmic, with respect to clock gene expression, and others that are solely light-responsive. Thus, further studies of the present *egr-1*/d2EGFP transgenic model studies may also permit the morphological characterisation of discrete sub-populations of cells within light-regulated brain nuclei, and provide insights into the cellular progression of photic responses.

In addition, the Z16 transgenic line may be an appropriate model for investigating hippocampal function in learning and memory paradigms, such as in different spatial memory tasks in a water maze (see Jenkins *et al.*, 2003). Indeed, this transgenic line is currently being used by colleagues in the School of Psychology (Cardiff University), for studying hippocampal function in behavioural tasks. A recent transgenic mouse model has been generated in which EGFP expression is controlled by the c-*fos* promoter (Barth *et al.*, 2004). This model will also have many applications in neurobehavioural studies.

8.3.2 Studies in the anterior pituitary gland

The higher copy number (Z25-B, Z27-A) transgenic lines appear to be the most suitable models for studying the regulation of Egr-1 in the pituitary gland. The findings of the present study are in agreement with a previous study that localised Egr-1 to a sub-population of anterior pituitary cells in the rat (and mouse) by IHC (Knight *et al.*, 2000). Dual ISH/IHC has also demonstrated that the *egr-1*/d4EGFP transgene is primarily restricted to the nuclei of LH beta-subunit-expressing cells (see Figure 5.11). This particular analysis has not been performed on the *egr-1*/d2EGFP transgenic lines, but based upon analyses carried out thus far, similar findings are expected, and would reflect the demonstration of Egr-1 protein in rat gonadotrophs (Knight *et al.*, 2000). The full extent of the cellular specificity of transgene expression in the anterior pituitary will, however, require considerable further analysis in which, firstly, any small populations of transgene-positive / LH β-subunit-negative cell type(s) (see Chapter 5) are characterised, and secondly the co-localisation of transgene with Egr-1 is quantitated.

In theory, the GFP-expressing cell population in the Z-line anterior pituitary glands should be amenable to analysis by fluorescence-activated cell sorting (FACS; e.g Magoulas *et al.*, 2000). Using this approach, it may be possible to isolate the GFP/Egr-1-expressing cellular population for biochemical analysis. In addition, it may be possible to isolate viable Egr-1/GFP cells for primary culture studies, as

suggested for GH/GFP cells by Magoulas *et al.* (2000). These studies may provide novel insights into the neuroendocrine gonadotrophin axis, which would complement our previous work on oestrogenic regulation of Egr-1 in the rat pituitary gland (Man and Carter, 2003). FACS analysis and sorting is, of course, available via conventional approaches, but the use of endogenous GFP fluorescence omits the need for antisera and permeabilisation treatments. Application of FACS to the *egr-1*/d2EGFP model may also be interesting with respect to the analysis of potential sub-populations of cells that show different fluorescence intensities (see Okabe *et al.,* 1997).

8.4 General applications of GFP-expressing transgenic models

In addition to applications of the *egr-1*/d2EGFP transgenic model discussed above, other potential applications are also evident. One application of a transgenic model that expresses GFP in the brain is in electrophysiological studies. An example of such a study involves transgenic mice that express GFP in GnRH neurons (Spergel *et al.*, 1999). This particular study used a combination of fluorescence and infra-red differential interference contrast (IR-DIC) video microscopy to acquire physiological recordings of action potentials in GnRH neurons of brain slices of these mice. Another example is provided by a recently generated transgenic mouse model that targets GFP to GHRH cells (Balthasar *et al.*, 2003). This study used patch clamp recordings to demonstrate spontaneous action potentials, and epifluorescence stereomicroscopy to reveal a network of GHRH cells and their terminals at the sub-cellular, single cell, and multi-cellular levels in the intact brain. The *mPer1*/d2EGFP transgenic mouse model (Kuhlman *et al*, 2000) has provided a convenient and dynamic model for demonstrating the circadian expression and localisation of *Per1* mRNA and PER1 protein in the SCN (by IHC and fluorescence microscopy - Kriegsfeld *et al.,* 2003; LeSauter *et al.,* 2003) and in the retina (by IHC - Witkovsky *et al.,* 2003). Further studies, using fluorescence microscopy and electrophysiology, have revealed that phase-resetting light pulses induce *Per1* and persistent spike activity in a distinct subset of SCN neurons (Kuhlman *et al.,* 2003), and that there is a positive, linear correlation of *Per1* transcription with neuronal spike frequency output (Quintero *et al.*, 2003). Recent studies of this model used whole-cell current-clamp recordings to show that SCN neurons exhibit robust rhythms in resting membrane

potential that are maintained in constant conditions (Kuhlman and McMahon, 2004). The population of Egr-1 SCN cells could be studied in similar paradigms. Also, the population of PeN Egr-1-positive neurons that may be functionally linked to GH secretion (Tanaka *et al.*, 1999; Davies *et al.*, 2004) could be investigated using these approaches.

Another application of transgenic rodents expressing GFP is in organ transplantation studies. For example, one study performed organ transplantation of heart, intestine, liver, and pancreas from GFP-transgenic rats to normal rats, and used flow cytometry and PCR analysis to detect donor cell migration (Hakamata *et al.*, 2001). Another example is provided by a recent study in which neural progenitor cells were transplanted from neonatal GFP transgenic mouse brains, to diseased and normal (rat and mouse host) retina, suggesting a potential use in retinal regeneration studies (Mizumoto *et al.*, 2003). The general significance of the transgenic animals discussed here is that they may provide models for the study of the cellular and molecular basis of human and animal diseases.

APPENDIX A

COMPOSITION OF MEDIA, BUFFERS, REAGENTS AND SOLUTIONS

All buffers, media, reagents and solutions were made with Elgastat reverse osmosis purified water, unless otherwise stated.

Solutions and labware used in RNA procedures were treated with 0.1% diethylpyrocarbonate (DEPC; Sigma D5758), mixed well by vigorous shaking, and incubated at room temperature (RT) overnight. Solutions and labware were autoclaved at 121°C for 15 minutes, and the resultant sterile 0.1% DEPC-treated solution was poured off from labware.

Solutions sterilised using 0.2μm filters are described here as 'sterile-filtered' or 'filter-sterilised.'

MEDIA

Aquamount (BDH): Water-based mounting media for use on slides and coverslips.

DPX (BDH): Xylene-based mounting media for use on slides and coverslips.

Vectashield (Vector Laboratories): Mounting medium for fluorescence (with DAPI) for use on slides and coverslips.

LB broth

Mix 1 Luria-Bertani (LB) broth tablet (Sigma) per 50ml water and autoclave at 121°C for 15 minutes. Add appropriate sterile-filtered antibiotic, if required, to the broth and store at 4°C.

LB agar

Mix 1 LB agar tablet (Sigma) per 50ml water and autoclave at 121°C for 15 minutes. Cool the solution to 55°C and add sterile-filtered antibiotic, if required. Pour into petri dishes (to ~0.5cm depth), on a level surface and allow to solidify. Store LB agar plates at 4°C.

M2 manipulation media

Add 130µl sodium lactate (Sigma L-7022), 50ml M2 stock medium (Sigma – M7167), and 25µl of pencillin/streptomyocin stock solution. Filter sterilise the media and aliquot into 12 x 0.5ml, 6 x 7ml and 1 x 3ml vials. Store at 4°C.

Penicillin G / Streptomyocin stock solution (for M2 and M16 culture media)
Dissolve 12mg penicillin in 40µl sterile water, and 10mg streptomyocin in 40µl sterile water. Centrifuge briefly, add the penicillin to the streptomyocin, and then make up the volume to 100µl with sterile water (final concentration 0.12mg/µl Penicillin / 0.1mg/µl streptomyocin). Store at –20°C.

M2 manipulation media + hyaluronidase

Add 3ml M2 media to a 30mg vial of hyaluronidase (Sigma H-4272) and aliquot into 6 x 450µl vials. Store at -20°C.

M16 maintenance media

Add 5µl of pencillin/streptomyocin stock solution to 10 ml of M16 stock medium (Sigma M7292) and filter sterilise. Aliquot into 13 x 750µl vials and store at 4°C.

BUFFERS

Buffer 1

Buffer contains 100mM Tris-HCl and 150mM NaCl.

Adjust pH to 7.5, and store at RT.

Buffer 2

Buffer contains 100mM Tris-HCl, 100mM NaCl and 50mM $MgCl_2$.

Adjust pH to 9.5, and store at RT.

Buffer 3

Buffer contains 10mM Tris-HCl and 1mM EDTA.

Adjust pH to 8.0, and store at RT.

Depurination Buffer

Add 974.2ml water to 25.8ml concentrated HCl (final concentration 0.25M HCl) and mix by inversion. Store at RT.

Denaturation Buffer

Add 87.7g NaCl and 20.0g NaOH. Add water to a final volume of 1L (final concentration 1.5M NaCl/ 0.5M NaOH) and mix by inversion. Store at RT.

0.5M EDTA (pH 8.0)

Dissolve 186.1g of disodium ethylene diamine tetra acetate dehydrate (EDTA) in 800ml water, and adjust the pH to 8.0 with NaOH pellets. Add water to a final volume of 1L. Autoclave for 15 minutes and store at RT.

Hybridisation Buffer

Add 500ml 1M sodium phosphate (NaPi), 2ml 0.5M EDTA pH 8.0, 350ml 20% SDS and 150ml formamide. Mix gently to avoid frothing. Store at RT.

***In situ* hybridisation buffer I**

Add 0.95ml 1M Tris (pH 7.4), 0.19ml 0.25M EDTA (pH 8.0), 3.75ml 4M NaCl, 23.8ml deionised formamide, 9.52ml 50% dextran sulphate, 0.95ml 50x Denhardt's solution and 1.02ml DEPC-water. Mix well by inversion and store at -20°C in 1ml aliquots.

50× Denhardt's Solution

(Sigma D2532) Store at -20°C in 1ml aliquots.

50% Dextran sulphate

Add 7ml DEPC-water to 5g dextran sulphate (MW 500,000; Sigma D6001). Dissolve mixture at 68°C and add DEPC-water to a final volume of 10ml. Mix thoroughly and store at 4°C.

***In situ* hybridisation buffer II**

Prepare as for *in situ* hybridisation buffer I, and add 1.6ml nucleic acid mix. Store at -20°C in 1ml aliquots.

3:1 LB

Add 5ml deionised formamide, 0.5ml 20x MAE, 1.7ml formaldehyde and 0.3ml DEPC and store at -20°C.

20x MAE (MOPS-acetate-EDTA) **buffer**

Buffer contains 400mM MOPS, 100mM NaAc and 20mM EDTA Adjust the pH to 7.0 with NaOH. Cover in foil (to protect from light) and store at 4°C.

MITE (micro-injection TE) **buffer**

Buffer contains 10 mM Tris-HCl (pH 7.4) and 0.2 mM EDTA (pH8.0) and filter-sterilise. Store at 4 °C.

10x NLB (Northern loading buffer)

Buffer contains 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, and 1mg/ml ethidium bromide in 1x MAE buffer and store at -20°C.

OLB (Oligo-labelling buffer) **5x buffer**

Mix solutions A, B and C in a ratio of 2:5:3 (v/v/v) and store in 100µl aliquots at -20°C.

Solution O 1.25M Tris HCl (pH 7.0) and 125mM $MgCl_2$

Solution A Add 18µl β-mercapethanol (14.3M), 5µl dTTP (100mM), 5µl dATP (100mM), 5µl dGTP (100mM) and 1ml Solution O

Solution B 2M HEPES (pH 6.6)

Solution C Random hexamer oligonucleotides (Pharmacia Pd (N)6) at 90 OD units/ml in TE

10x Orange-G loading buffer

Buffer contains 50% (v/v) glycerol, 10mM EDTA, 10mM Tris-HCl (pH 7.6) and 1 mg/ml orange-G (Sigma; a nucleic acid tracking dye containing 7-hydroxy-8-phenylazo-1,3-naphthalenedisulphonic acid).

PBS (phosphate buffered saline) – (DEPC-treated) – for ISH

Add 5ml 1M sodium phosphate (NaPi) buffer, 4.5g NaCl and 495ml water (and 500µl DEPC). Mix the solution well by shaking and incubate at RT overnight. Autoclave for 15 minutes and store at RT.

0.01M PBS – for perfusion

Add 2.883g $Na_2HPO_4.2H_2O$, 0.598g $NaH_2PO_4.2H_2O$, 18.000g of NaCl and 0.400g of KCl. Add volume of water (ddH_2O) to 2L and mix well. Adjust pH to 7.2-7.4.

PBS Buffer – for IHC

Add 8.0g NaCl (150mM), 1.3g dibasic (Na_2) sodium phosphate (9.1mM), and 0.2g monobasic (NaH_2) sodium phosphate (1.7mM) and mix well. Adjust the buffer to pH7.4 with NaOH and add water to a final volume of 1L.

PBS-T (0.15% v/v Triton X in PBS buffer) – for IHC

Add 9.985ml PBS buffer and 15μl Triton-X (Sigma) and mix well.

Protein extraction buffer

Buffer contains 20mM HEPES, pH 7.9, 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA and 25% glycerol. Store at 4°C. Immediately prior to use, add 0.5mM DTT and 5μl/ml Protease inhibitor cocktail (Sigma P8340, stored at -20°C).

10x RNase buffer

Add 5ml 1M Tris (pH7.5), 5ml 0.5M EDTA (pH 8.0), 14.61g NaCl, and 40ml DEPC-treated water. Autoclave for 15 minutes and store at RT.

Running buffer

Add 3g TRIS base, 14.4g glycine and 1g of SDS (or 5ml of 20% SDS). Add water to a final volume of 1L and mix by inversion.

Sample buffer (Transduction Labs, 2X)

Add 250μl Tris (pH 6.8), 250μl 20% SDS, 100μl glycerol, 20μl B-mercapto-ethanol, 60μl 0.1%bromophenol blue and 370μl water. Stored at -20°C in aliquots.

1M NaPi (sodium phosphate) - (DEPC-treated)

Add 288.5ml 1M Na_2HPO_4 and 211.5ml 1M NaH_2PO_4 (and 500µl DEPC). Mix the solution well, by shaking, at pH 7.0, 25°C and incubate at RT overnight. Autoclave for 15 minutes and store at RT.

1M Na_2HPO_4 (di-sodium hydrogen orthophosphate)

Dissolve 70.48g Na_2HPO_4 in 500ml water by shaking.

1M NaH_2PO_4 (sodium di-hydrogen orthophosphate)

Dissolve 60g NaH_2PO_4 in 500ml water by shaking.

10x Southern loading buffer

Buffer contains 20% (w/v) Ficoll 400, 0.1M EDTA (pH 8.0), 1.0% (w/v) SDS, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol.

20x SSC (saline sodium citrate) - (DEPC-treated)

Add 87.65g sodium chloride, 44.1g sodium citrate and 400ml water. Adjust the pH of the solution to pH 7.0 and add water to a final volume of 500ml (and add 500µl DEPC). Shake well to mix and incubate at RT overnight and autoclave for 15 minutes. Store at RT.

20x SSPE (saline sodium phosphate EDTA) - (DEPC-treated)

Add 210.4g NaCl, 200ml 1M NaPi and 40ml 0.5M EDTA. Add water to a final volume of 1L, (and add 1ml DEPC). Shake well to mix and incubate at RT overnight and autoclave for 15 minutes. Store at RT.

STE (SDS-Tris-EDTA) **buffer**

Buffer contains 50mM Tris HCl (pH 8.0), 100mM EDTA (pH 8.0) and 0.5% (w/v) SDS. Mix gently to avoid frothing and store at RT.

10% Sucrose in 0.1 M PB (Phosphate Buffer) **with 0.01% NaN_3** – for perfusion

Add 180ml Solution A to 70ml Solution B. Adjust pH to 7.4 and add 250ml ddH_2O. Add 50g sucrose and 50mg NaN_3 to the solution and store at 4°C.

Solution A

Dissolve 35.6g $Na_2HPO_4.2H_2O$ in 1L H_2O.

Solution B

Dissolve 31.2g $NaH_2PO_4.2H_2O$ in 1L H_2O.

50x TAE (Tris-acetate-EDTA) **buffer**

Add 242g Tris base, 57.1ml Glacial acetic acid (Sigma) and 37.2g EDTA.
Add water to a final volume of 1L, mix by inversion and store at RT.

5x tailing buffer (Amersham Pharmacia Biotech)

Buffer (pH7.2) contains 500 mM potassium cacodylate, 10 mM $CoCl_2$ and 1 mM 2-mercaptoethanol. Store at -20°C.

TBS (Tris buffered saline) (pH 8.0)

Buffer contains 10mM Tris and 150mM NaCl and mix by inversion.

TBST (TBS with Tween)

Buffer contains 0.1% Tween-20 in TBS.

TE (Tris-EDTA) **buffer** (pH 7.6)

Buffer contains 10 mM Tris-HCl, 1 mM EDTA and autoclave. Store at RT.

TE (Tris-EDTA) **buffer** (pH 8.0)

Add 10ml 1M Tris-HCl (pH 8.0) and 2ml 0.5M EDTA (pH 8.0).
Add water to a final volume of 1L and store at RT.

TNB (Tris-HCl NaCl-) **blocking buffer**

Heat 0.1M Tris HCl (pH 7.5), 0.15M NaCl and 0.5% blocking agent (0.5g from #NEL701A TSA Kit, Perkin Elmer) to 60°C and stir to dissolve. Store at -20°C in aliquots.

TNT (Tris-HCl NaCl-Triton-X) **wash buffer**

Buffer contains 0.1M Tris HCl (pH 7.5), 0.15M NaCl and 0.3% Triton X. Mix by gentle inversion and store at 4°C.

(Southern) **Transfer Buffer**

Add 175.3g NaCl and 20.0g NaOH. Add water to a final volume of 2L (final concentration 1.5M NaCl/ 0.25M NaOH) and mix by inversion. Store at RT.

(Western) **Transfer buffer** (pH ~8.3)

Add 3g TRIS base, 14.4g glycine and 200ml 20% methanol. Add water to a final volume of 1L and mix by inversion.

1M Tris-HCl (pH 8.5)

Add 121.1g Tris base and ~750ml water. Adjust pH to 8.5 with concentrated HCl and add water to a final volume of 1L. Store at RT.

(Southern/Northern) **Wash Buffer**

Add 50ml 1M sodium phosphate (NaPi), 945ml water and 5ml 20% SDS (final concentration 50mM NaPi/ 0.1% SDS). Mix gently to avoid frothing and store at RT.

REAGENTS

BSA (bovine serum albumin) (10mg/ml; Promega)

Store at -20°C.

DNA Phenol (pH 8.0; Sigma P4557)

Store at 4°C.

1M DTT/0.1M NaAc

Filter-sterilise a solution of 1M DTT (Sigma D9779) and 0.1M sodium acetate (pH 5.2) into a sterile plastic tube. Store at -20°C in 500µl aliquots.

5M DTT/0.1M NaAc

Filter a solution of 5M DTT (Sigma D9779) and 0.1M sodium acetate (pH 5.2) into a sterile plastic tube. Store at -20°C in 500µl aliquots.

FITC (fluorescein; Fluorophore tyramide 'stock solution')

Reconstitute each vial (provided in #NEL701A TSA Kit, Perkin Elmer) containing solid fluorescein tyramide in 0.3ml DMSO. Store at 4°C (stable for up to 3 months), and thaw before use.

Klenow large fragment DNA polymerase (5U/µl, Promega)

Store at -20°C.

1M Levasimole

Dissolve 24mg levamisole (Sigma, L9756) in 100µl for a stock solution, and store at 4°C. Use at 1µl per ml NBT/BCIP solution.

LM-1 hypercoat emulsion

(Amersham Pharmacia Biotech RPN40) Store at 4°C in manufacturer's box.

NBT / BCIP (Nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl-phosphate p-toluidinium salt)

Dissolve 1 tablet (Roche 1697471) in 10ml distilled water to make a solution comprising 0.4mg/ml NBT / 0.19mg/ml BCIP / 100mM Tris buffer, pH 9.5 / 50 mM Mg SO_4.

NGS (normal goat serum) (Vector Laboratories)

Store at -20°C in 500µl aliquots.

Nucleic acid mix

Add 250µl single stranded salmon sperm (Sigma D9156), 250µl yeast tRNA (Sigma R8508) and 500µl DEPC-water. Store at –20°C in 50µl aliquots.

Single stranded salmon sperm DNA (ssDNA) (9.9mg/ml; Sigma D9156)

Store at -20°C in 250µl aliquots.

Yeast tRNA (11.9mg/ml; Sigma R8508)

Store at -20°C in 250µl aliquots.

P/C/IAA (Phenol/Chloroform/Isoamylalcohol) (25:24:1, pH 8.0; Sigma P3803)

Store at 4°C.

Proteinase K (25µg /µl)

Make a stock solution of proteinase K (Melford Laboratories) by adding 125mg Proteinase K to 5ml water and filter-sterilise. Store at -20°C in 250µl aliquots.

Proteinase K/STE (500µg/ml)

Immediately prior to use, add 20µl Proteinase K (25µg/µl) per 1ml STE and mix gently.

RNA Phenol (pH 4.3±0.2; Sigma P4682)

Store at 4°C.

RNase A (30mg/ml; Sigma R4642)

Store at -20°C in 50µl aliquots.

3M NaAc (sodium acetate) (pH 5.2)

Dissolve 408.1g sodium acetate trihydrate in 800ml water, and adjust pH to 5.2 with glacial acetic acid. Add water to a final volume of 1L, and store at RT.

Terminal Deoxynucleotidyl Transferase (TdT)

(Amersham Pharmacia Biotech E2230Y) A primer-dependent DNA polymerase that catalyzes the incorporation of deoxynucleotides into the 3'-OH termini of single or double-stranded DNA. Store at -20 °C.

Triton-X (Sigma)

Store at RT.

Tween-20 (Sigma)

Store at RT.

SOLUTIONS

0.25% Acetic Anhydride / 0.1M Triethanolamine – DEPC-treated

Add 7.46ml triethanolamine (Sigma T1377), 4.5g NaCl, and 2.1ml HCl; adjust the pH of the solution to pH 8.0 and add water to a final volume of 500ml. Add 500µl DEPC, shake well to mix, incubate at RT overnight and autoclave for 15 minutes. Store at RT. Add a neat stock of acetic anhydride (Sigma A6404) to a final concentration of 0.25% (v/v) immediately before use.

Acid cleaning solution

Add 20ml sulphuric acid, 200ml water and 20g potassium dichromate. Shake well to mix and store at RT.

ACSF (artificial cerebral spinal fluid)

Add 134mM NaCl, 16mM $NaHCO_3$, 2mM KCl, 1.25mM KH_2PO_4, 5mM $MgSO_4$ and 10mM $C_6H_{12}O_6$. Bubble the solution with O_2 (for ~2 minutes), add 1mM $CaCl_2$, and store at -20°C.

DEPC-treated water

Add 500µl of DEPC (Sigma D5758) to 500ml of water (0.1% solution). Shake well to mix and incubate at RT overnight. Autoclave for 15 minutes and store at RT.

D19 developer (Kodak; Sigma P5670)

Heat 3.8 litres of double distilled water to 52°C and add 1 pack of D19 powder (595g) until dissolved. Filter the solution (using Whattman filter paper and a funnel) into dark bottles covered with foil. Store at 4°C for up to 6 months.

Fixer (Kodak; Sigma P6557)

Dissolve 1 pack of fixer powder (680g) in 2.84 litres of water, then make volume of mixture up to 3.8 litres. Filter the solution (using Whattman filter

paper and a funnel) into dark bottles covered with foil. Store at 4°C for up to 6 months.

GTC-A Solution

Solution contains 4M GTC (guanidium isothiocyanate; 189.1g/400ml), 0.5% (v/v) Sarkosyl (5ml/L) and 25mM sodium citrate (pH 7, 25ml of 1M/L). Filter-sterilise and store at RT. Add 72µl β-mercaptoethanol per 10ml (final concentration of 0.1M) immediately prior to use.

OLB-C Stop solution

Add 50µl 5M NaCl, 250µl 1M Tris HCl (pH 7.5), 50µl 0.5M EDTA, 156.5µl 20% SDS and 125µl 100mM dCTP. Add sterile water to a final volume of 12.5ml and store at 4°C in 500µl aliquots.

PAF (paraformaldeyde) **- for ISH**

(for 3 plastic coplin jars, each with a capacity to hold ~35ml solution)
Heat 4g paraformaldehyde in 30ml saturated picric acid (Sigma 925-40) to 65°C, titrate until clear with 10M NaOH (~6 drops) and cool to 40°C. Add DEPC-treated water to a final volume of 100ml then add an equal volume of 0.2M (NaPi).

4% PFA (paraformaldehyde) - **for perfusion**

Add 4g paraformaldehyde to 50ml H_2O and heat to 60°C (with stirring). Add 5-6 drops of 1M NaOH, or until solution clears. Cool solution and add 36ml Solution A and 14ml Solution B. Filter solution (through Whatman 3MM filter paper) and store at 4°C.

Solution A 35.6g $Na_2HPO_4.2H_2O$ in 1L H_2O.

Solution B 31.2g $NaH_2PO_4.2H_2O$ in 1L H_2O.

Sephadex G-50

Slowly add 30g Sephadex G-50 (DNA grade, Amersham Pharmacia Biotech 17-0573-02) to 250ml TE (pH 8.0) in a 500ml bottle and auto-clave for 15 minutes. Allow to stand, then decant off supernatant. Replace with an equal volume of TE (pH 8.0) and store at 4°C.

APPENDIX B

COMMERCIAL KITS

DIG RNA labelling kit

See:

http://www.roche-applied-science.com/pack-insert/1277073a.pdf

Qiaex II Agarose Gel Extraction Kit (Qiagen)

See:

http://www.qiagen.com/literature/Handbooks/PDF/DNA_cleanup/INT/QIAEX_II/qiaexII_agarose.pdf

Riboprobe® *In Vitro* Transcription System (Promega)

See:

http://www.promega.com/tbs/tm016/tm016.pdf

Superscript™ Pre-amplification System (Invitrogen)

See:

http://www.invitrogen.com/content.cfm?pageid=93&search_term=11904-018&search_type=3&startrow=1

TSA™ Fluorescein System (PerkinElmer)

See:

http://las.perkinelmer.com/catalog/Product.aspx?ProductID=NEL701A001KT

Wizard® Plus SV Mini-Preps DNA Purification System (Promega)

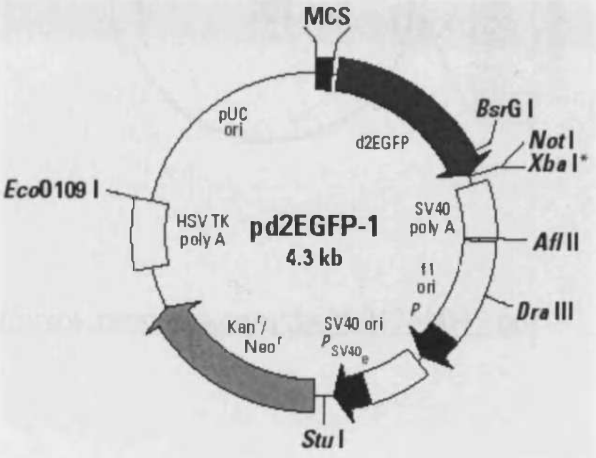
See:

http://www.promega.com/tbs/tb225/tb225.pdf

APPENDIX C

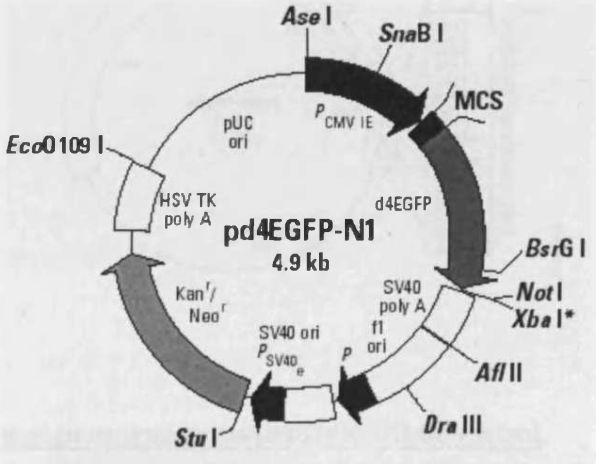
VECTORS

pd2EGFP-1 (Clontech)



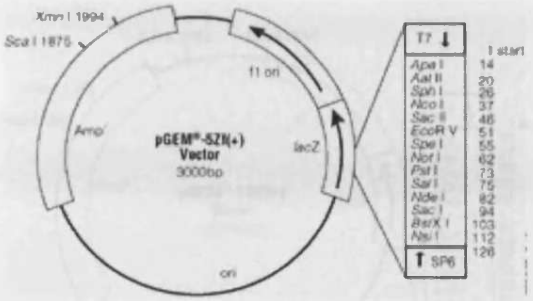
See http://www.clontech.com/techinfo/vectors/vectorsD/pd2EGFP-1.shtml

pd4EGFP-N1 (Clontech)



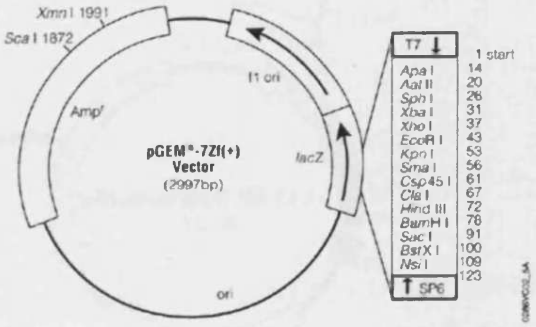
See http://www.clontech.com/techinfo/vectors/vectorsD/pd4EGFP-N1.shtml

pGEM-5Zf(+) (Promega)



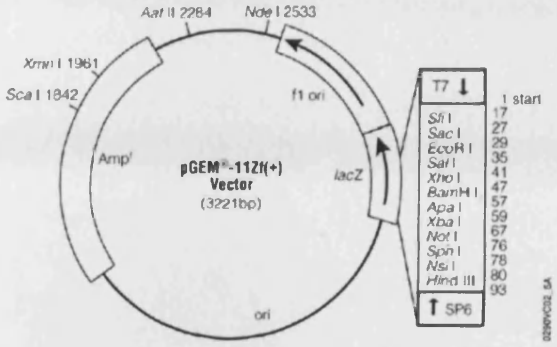
See http://www.promega.com/tbs/tb047/tb047.pdf

pGEM-7Zf(+) (Promega)



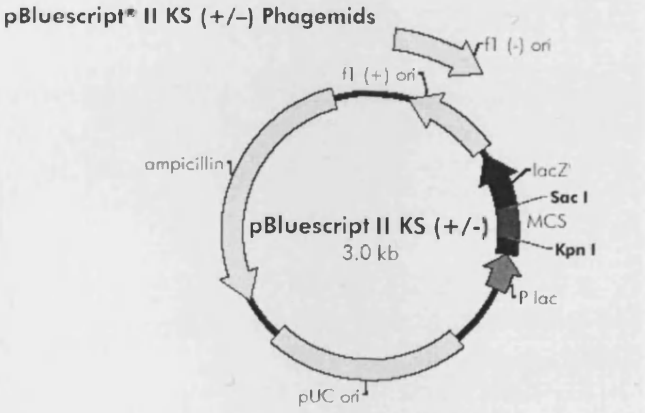
See http://www.promega.com/tbs/tb069/tb069.html

pGEM-11Zf(+) (Promega)



See http://www.promega.com/tbs/tb075/tb075.html

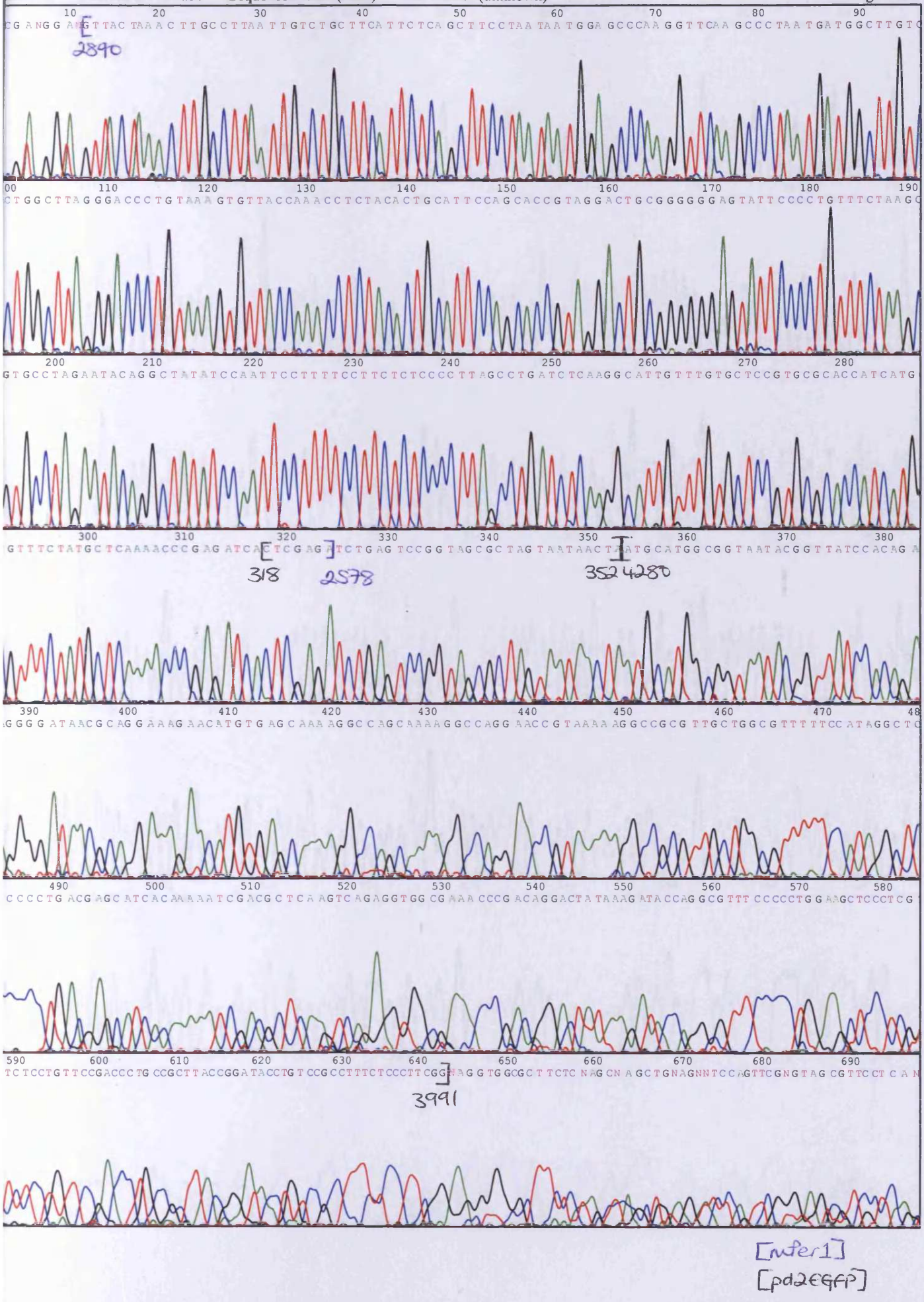
pBS-KS (Stratagene)



See http://www.stratagene.com/manuals/212205.pdf

APPENDIX D1

CHROMAS SEQUENCE FILES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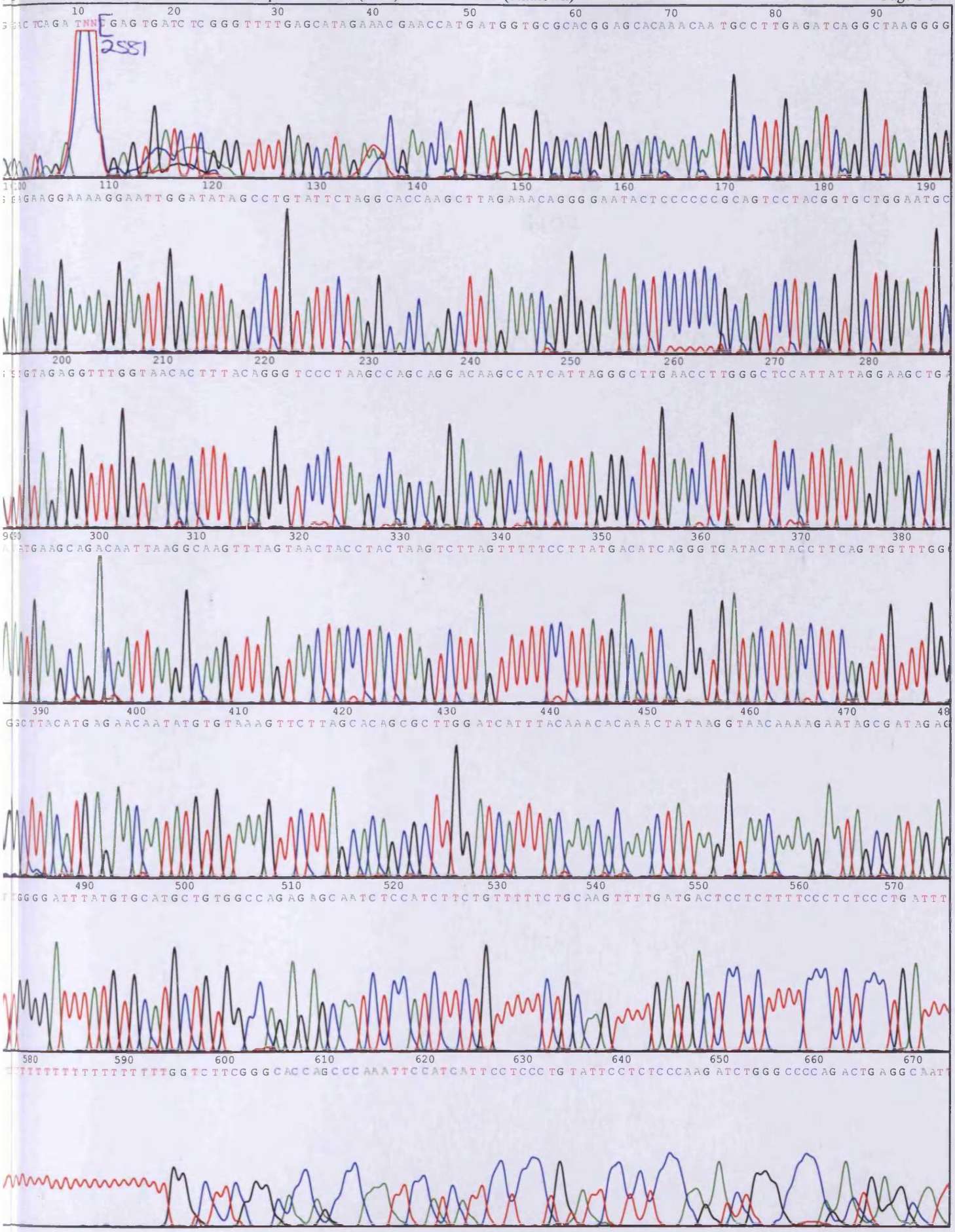
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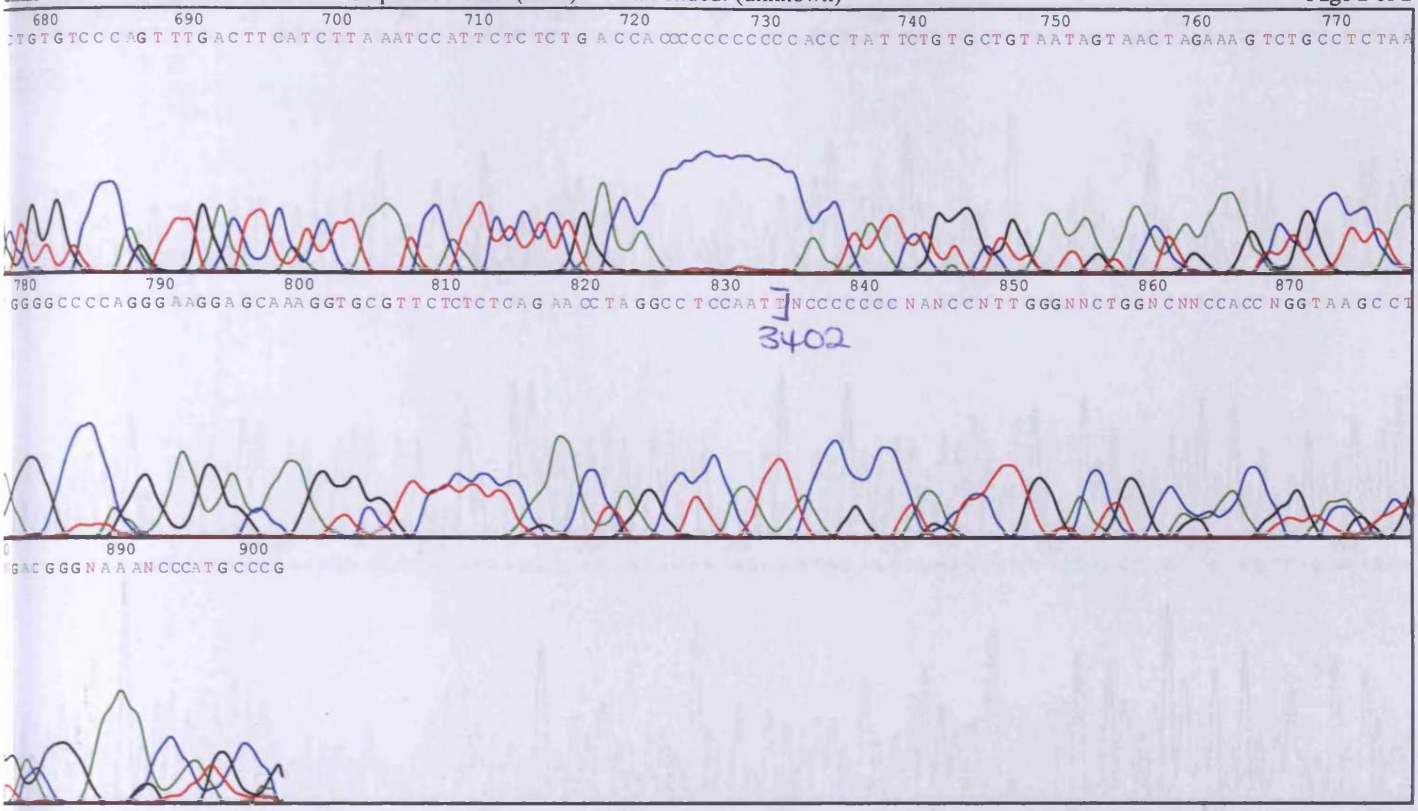
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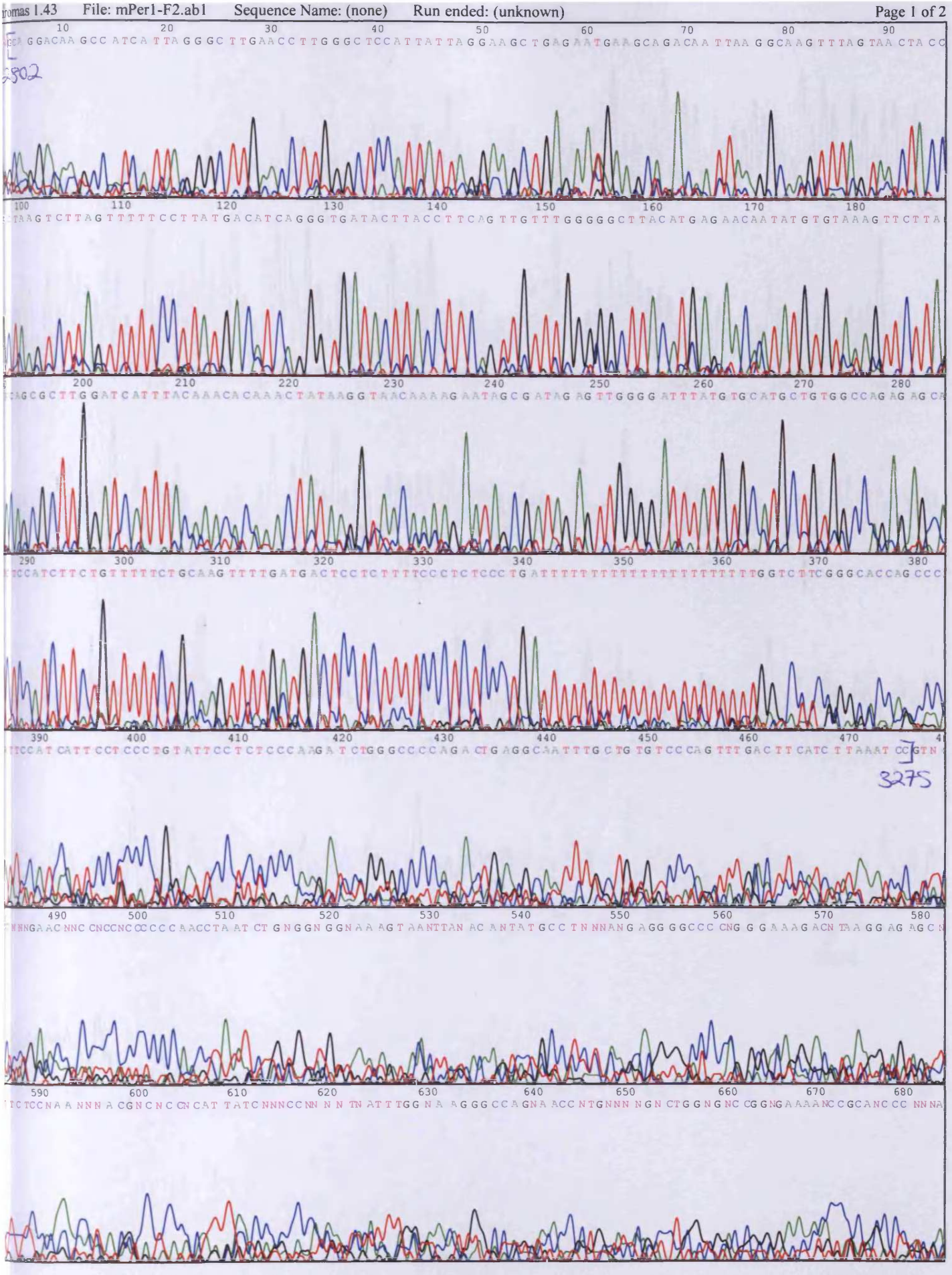
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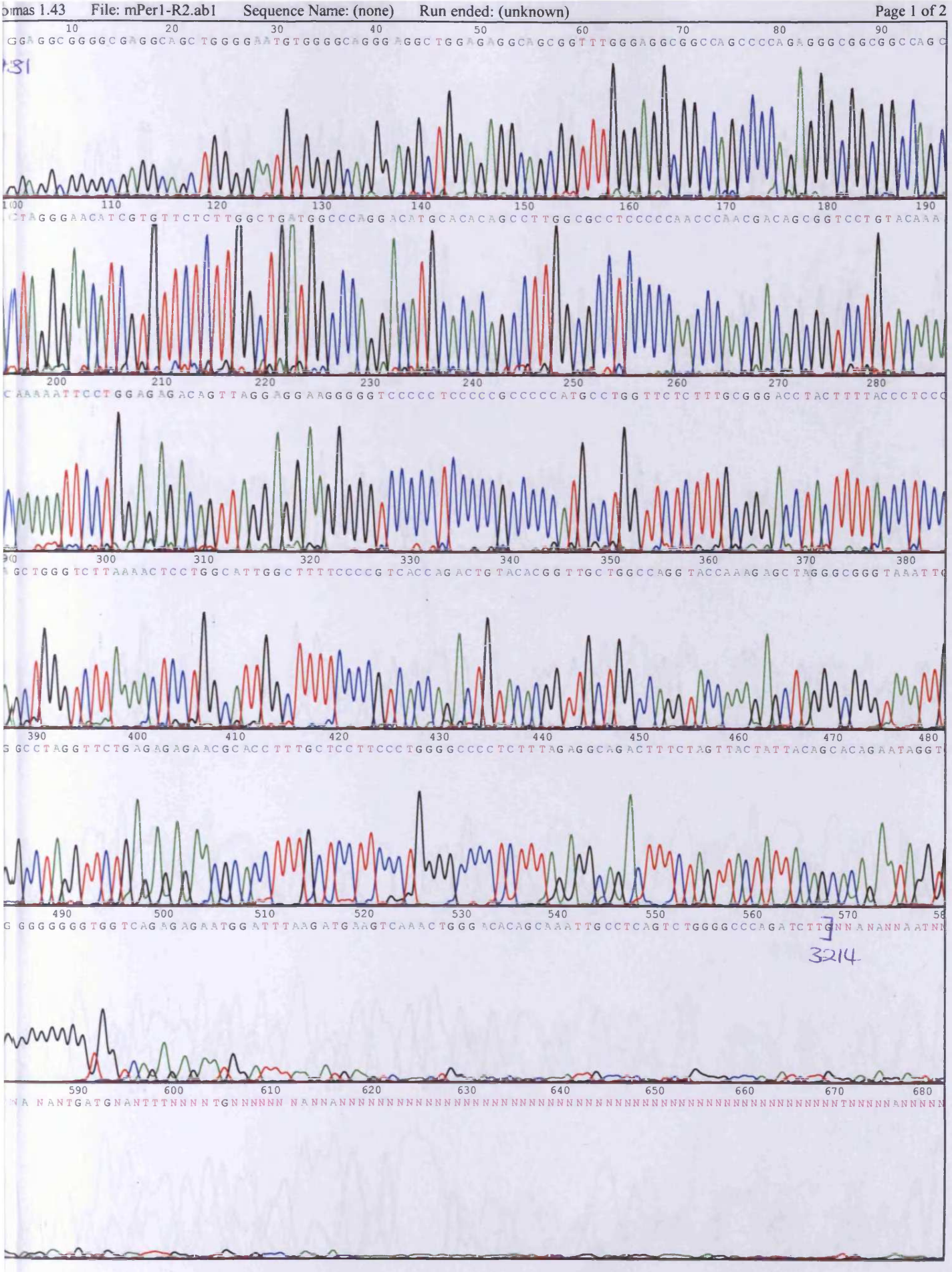
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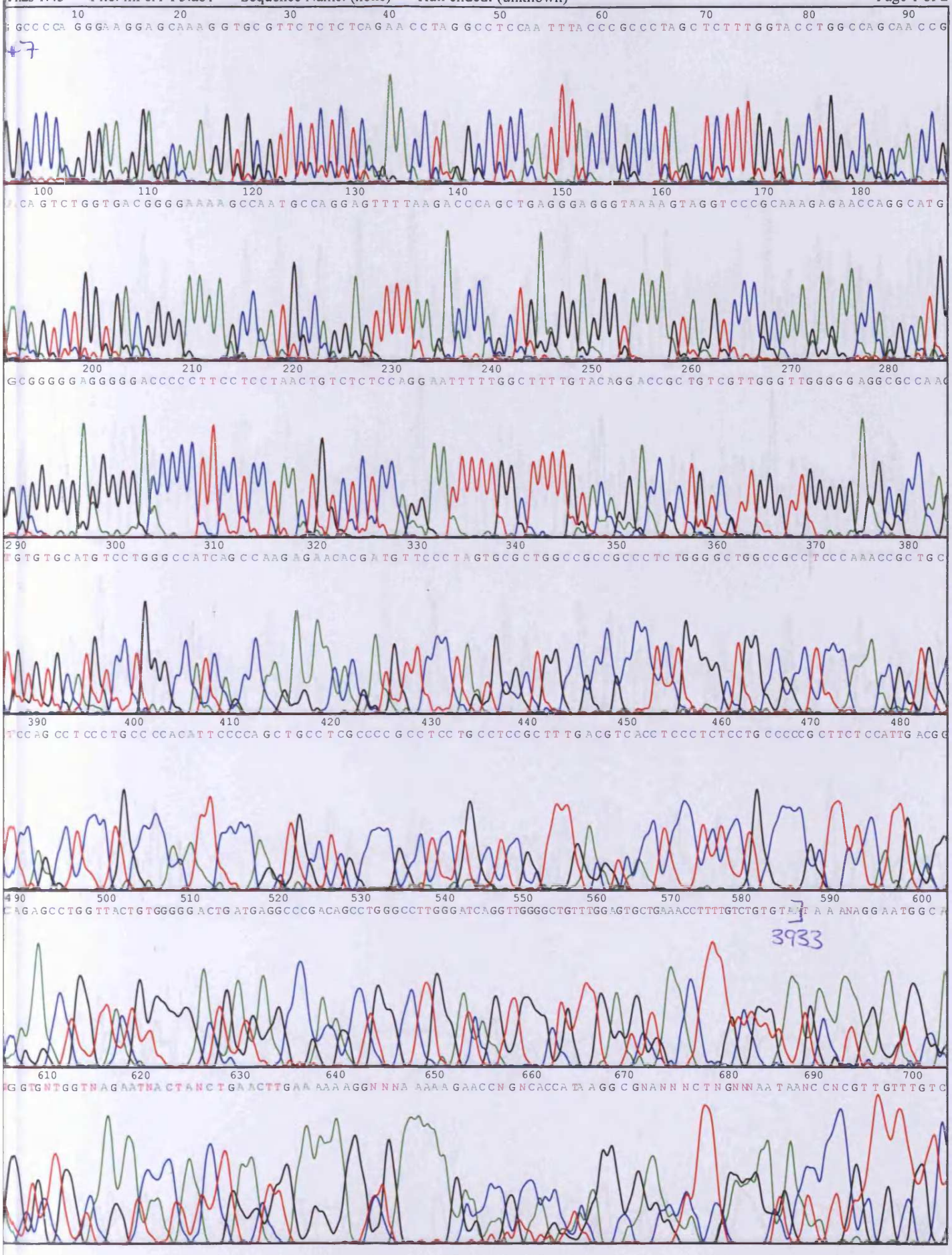
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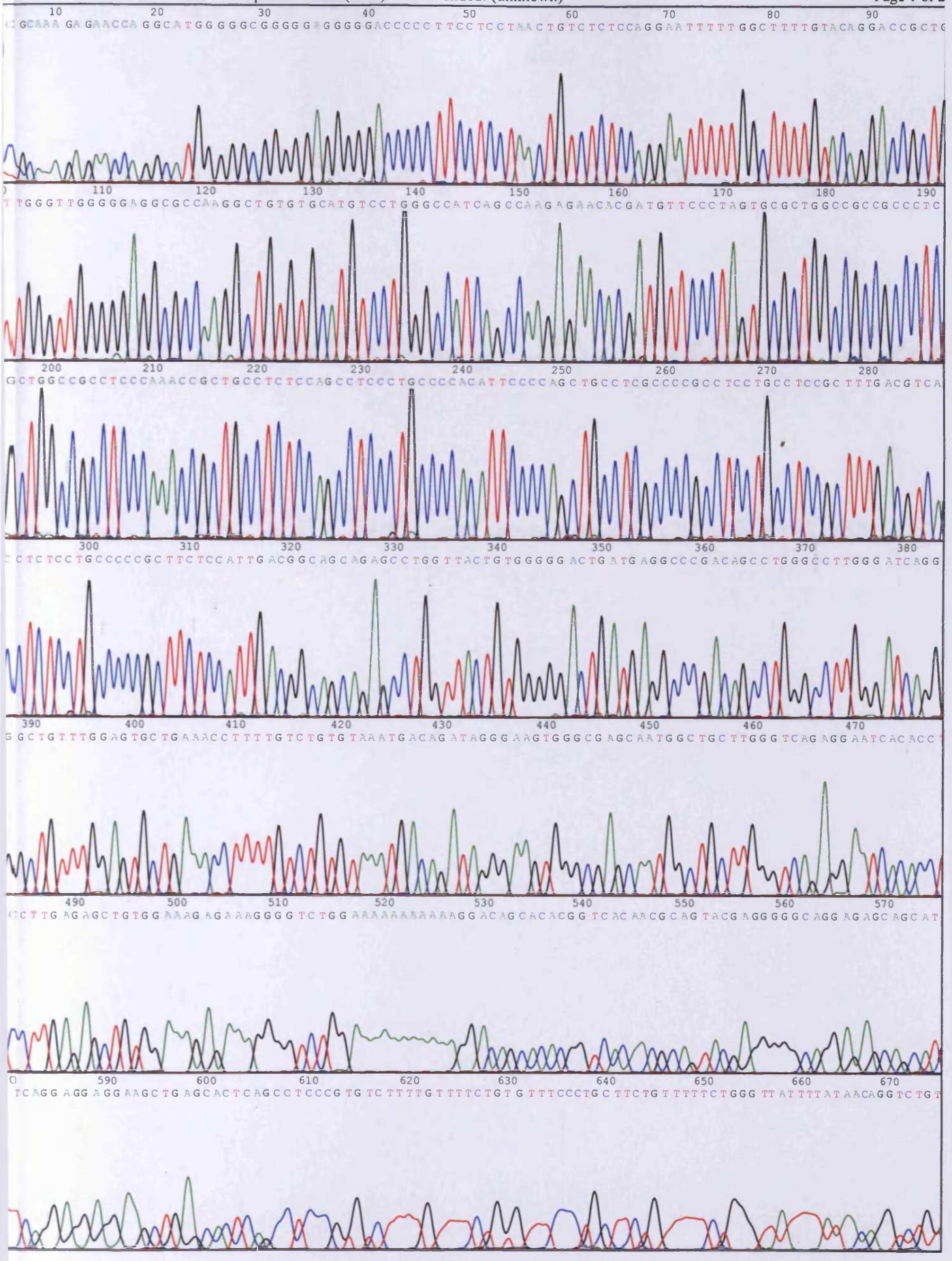

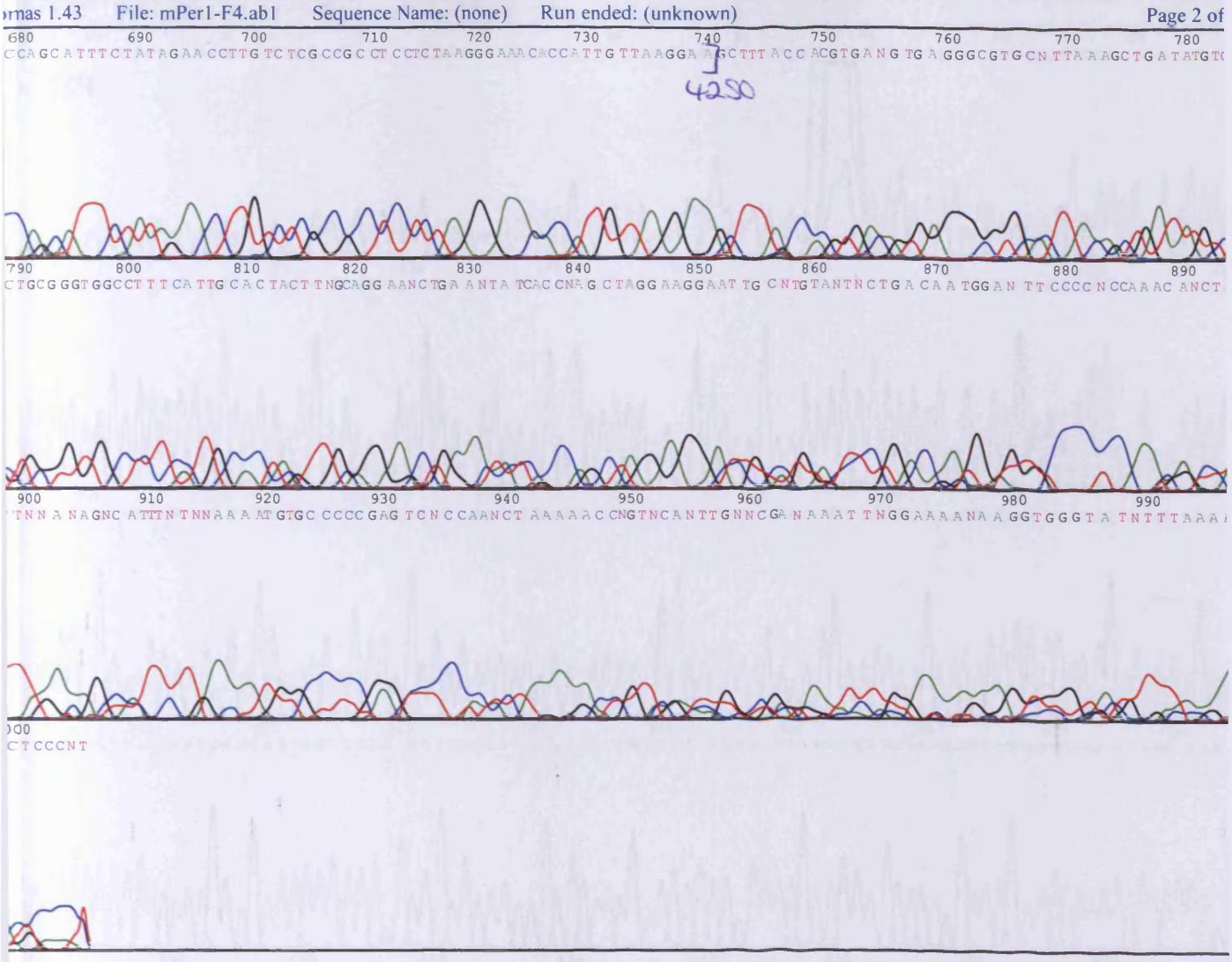
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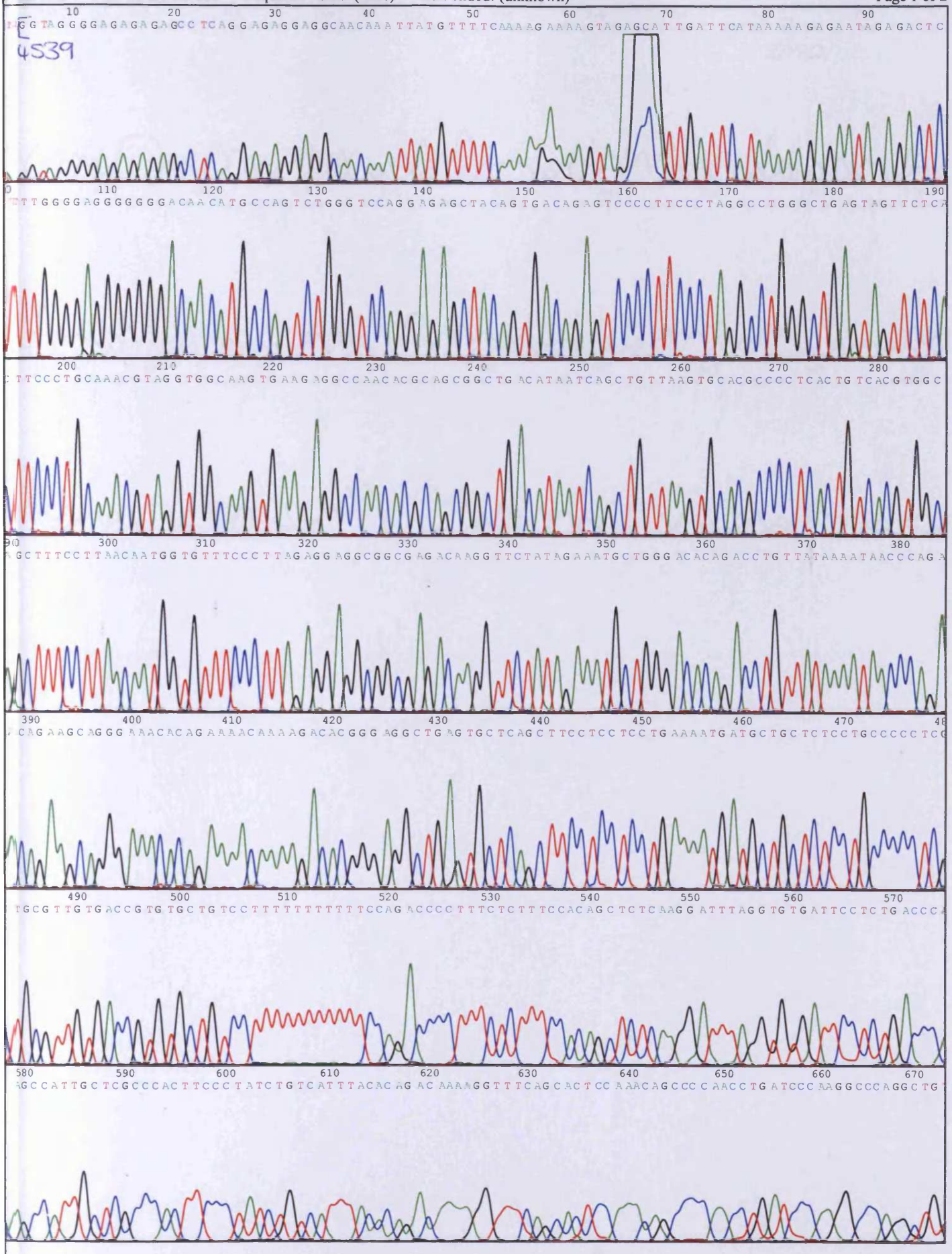
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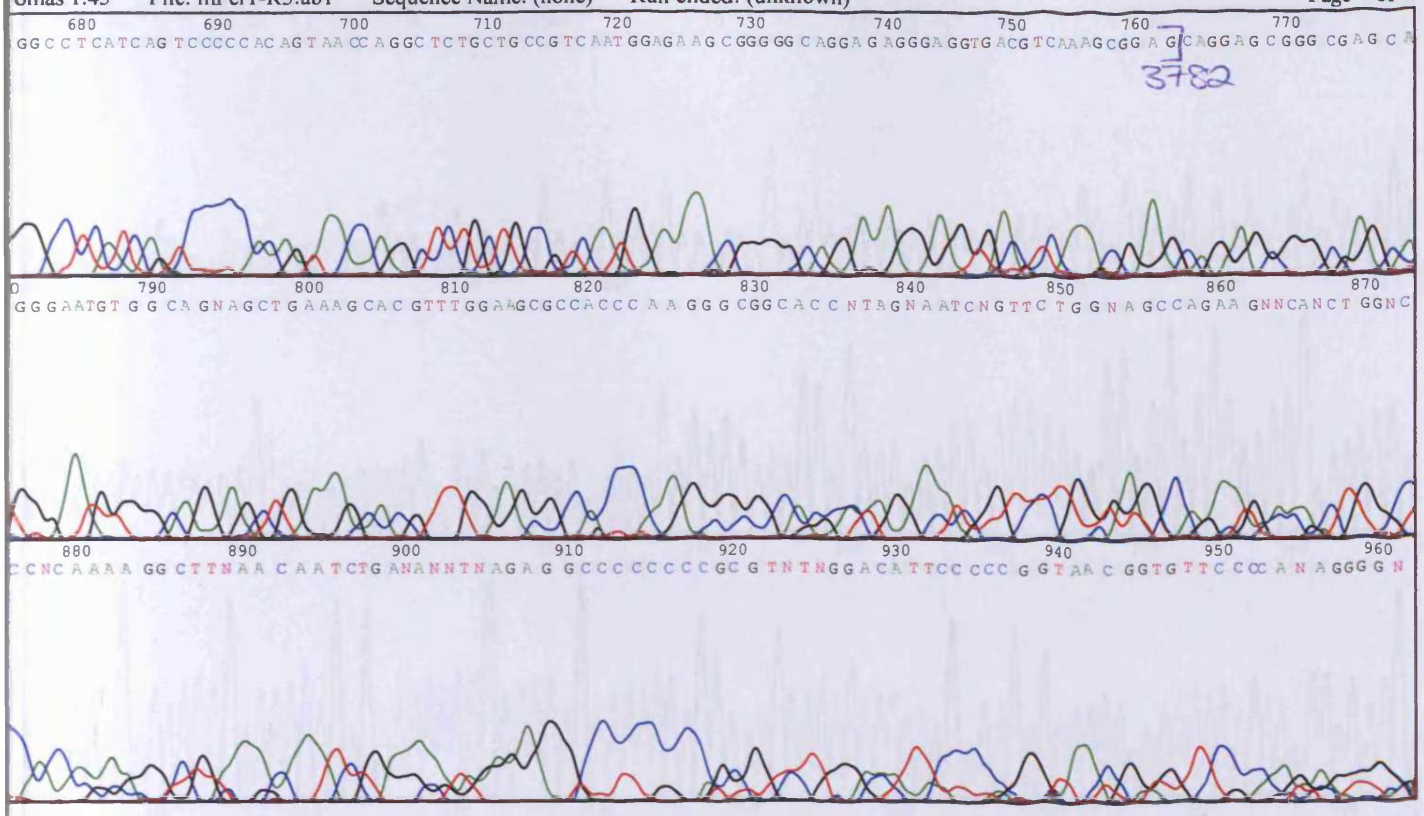
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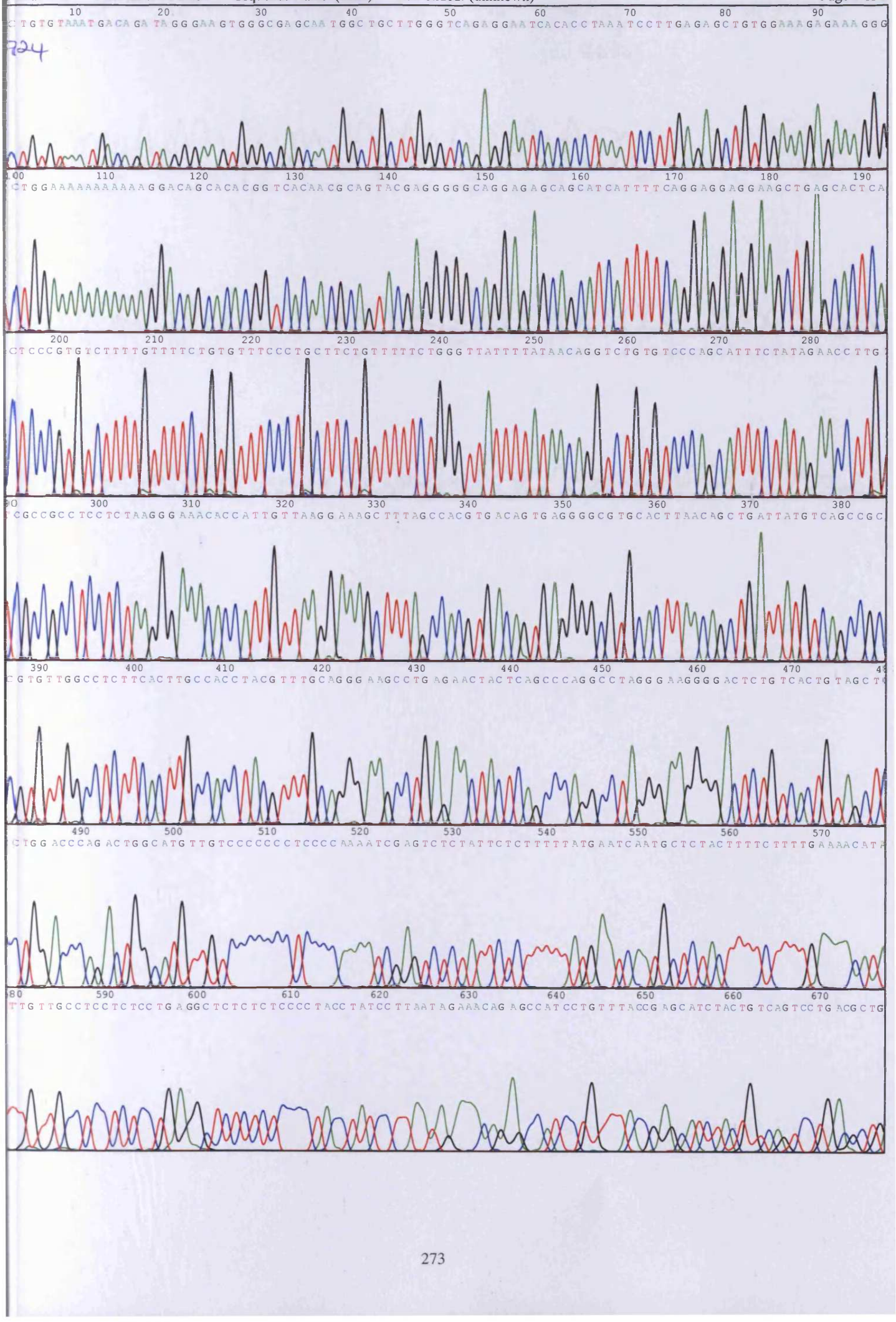
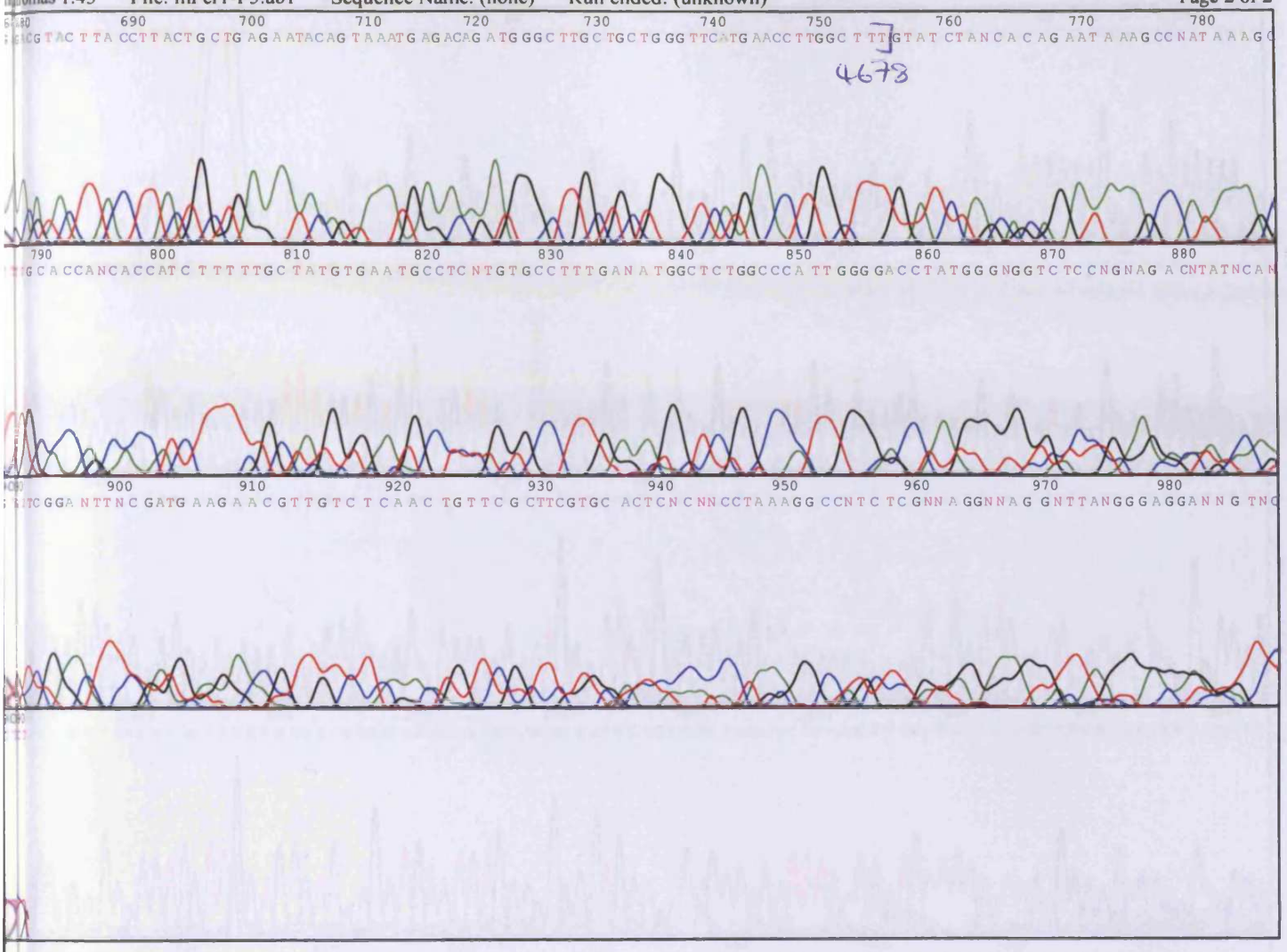
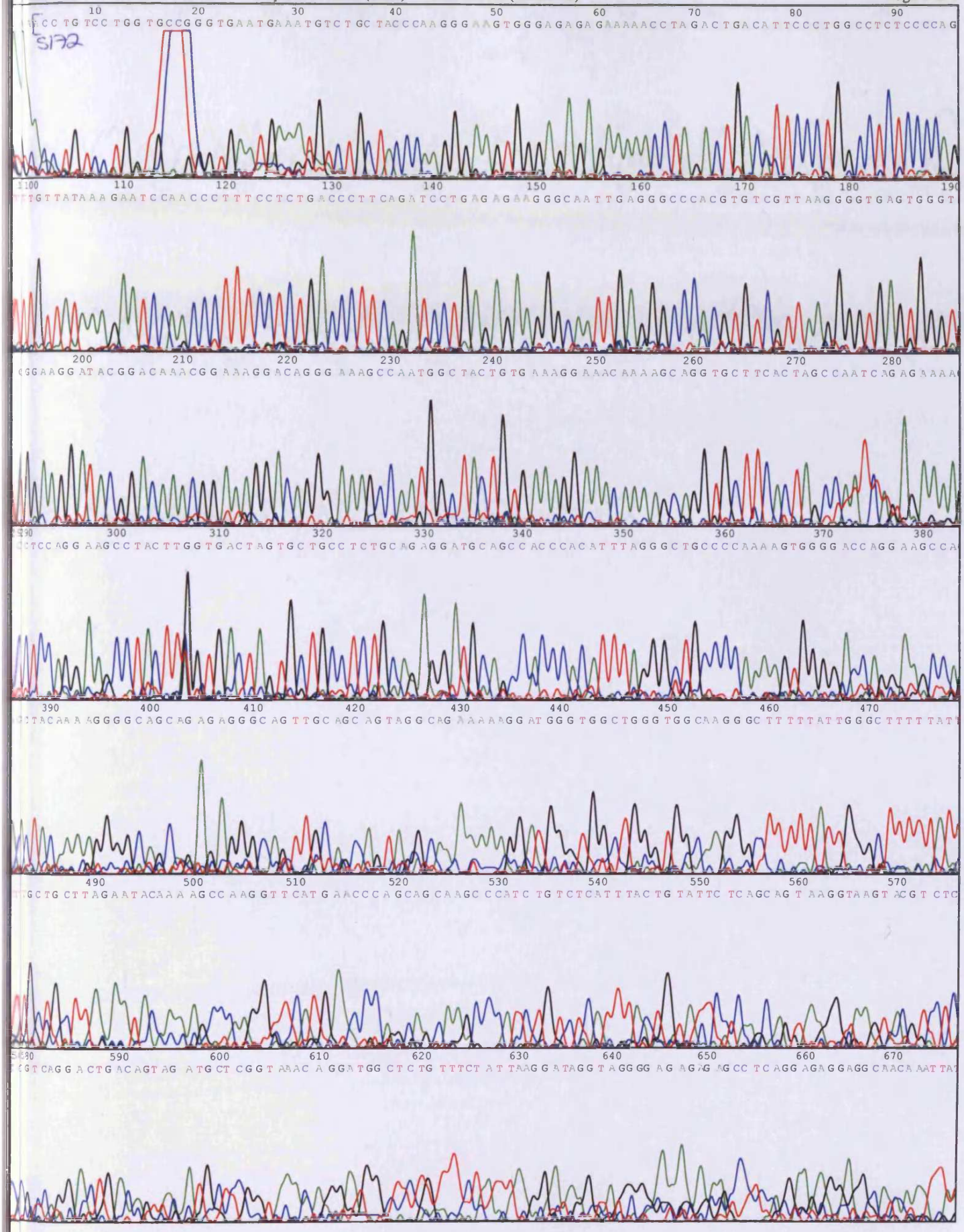
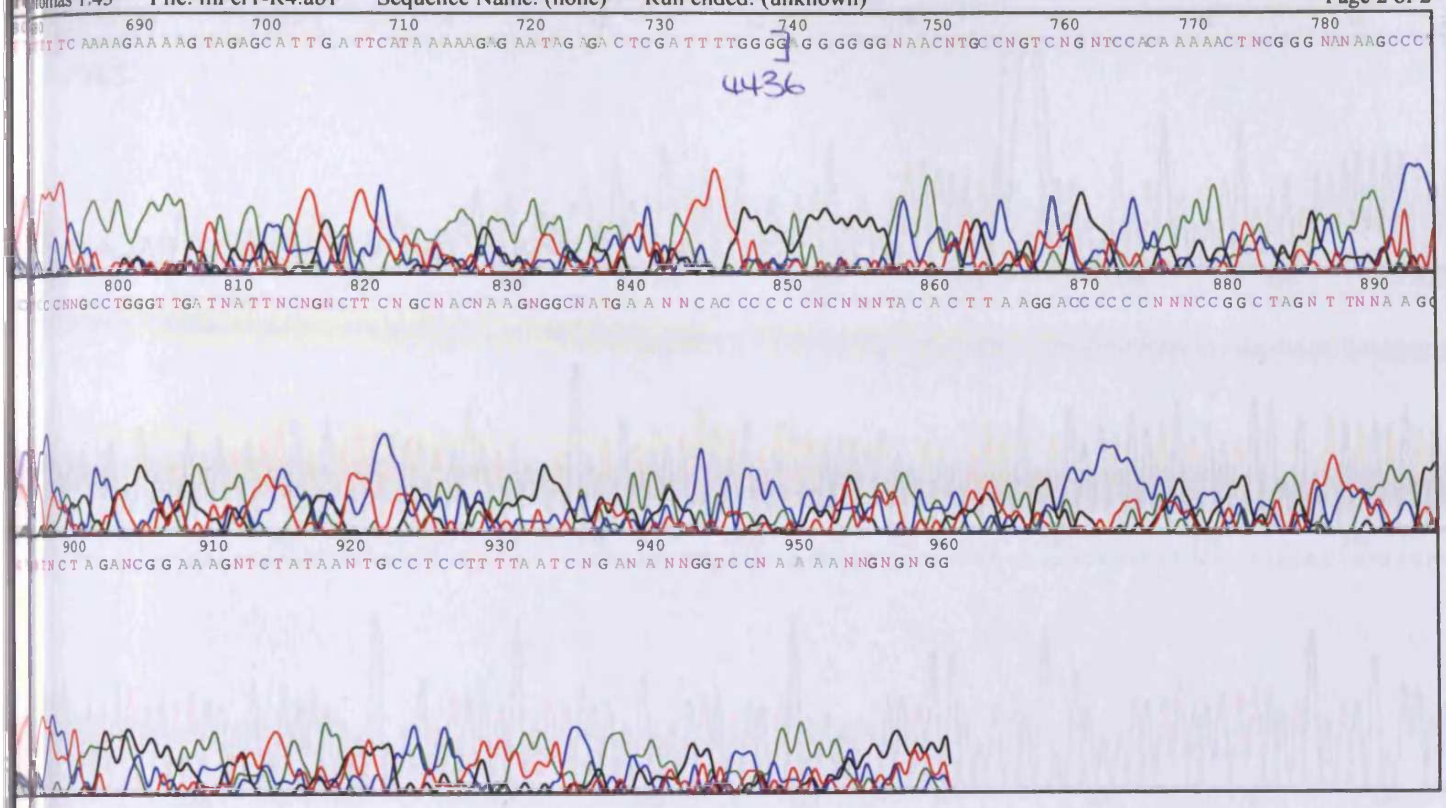
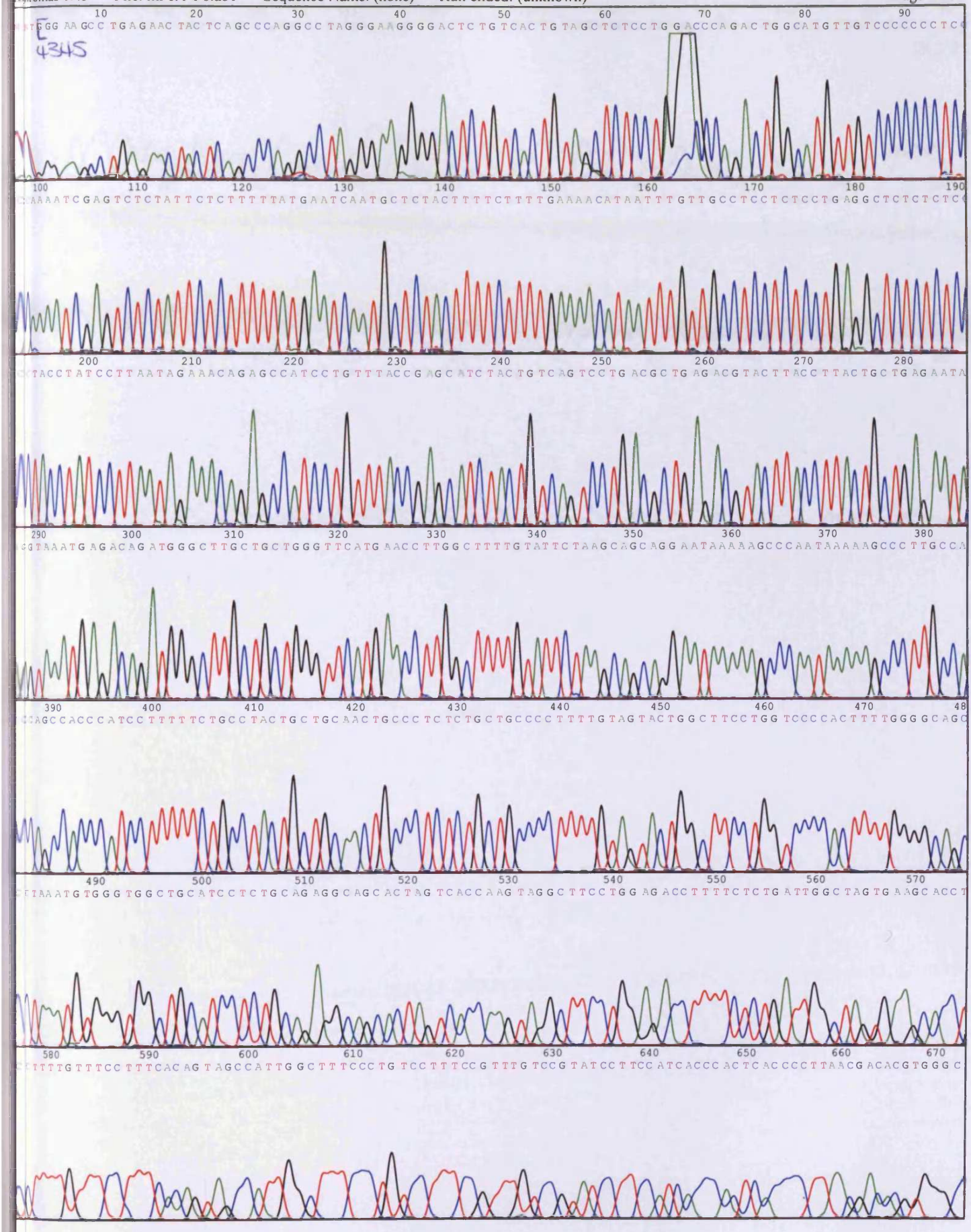


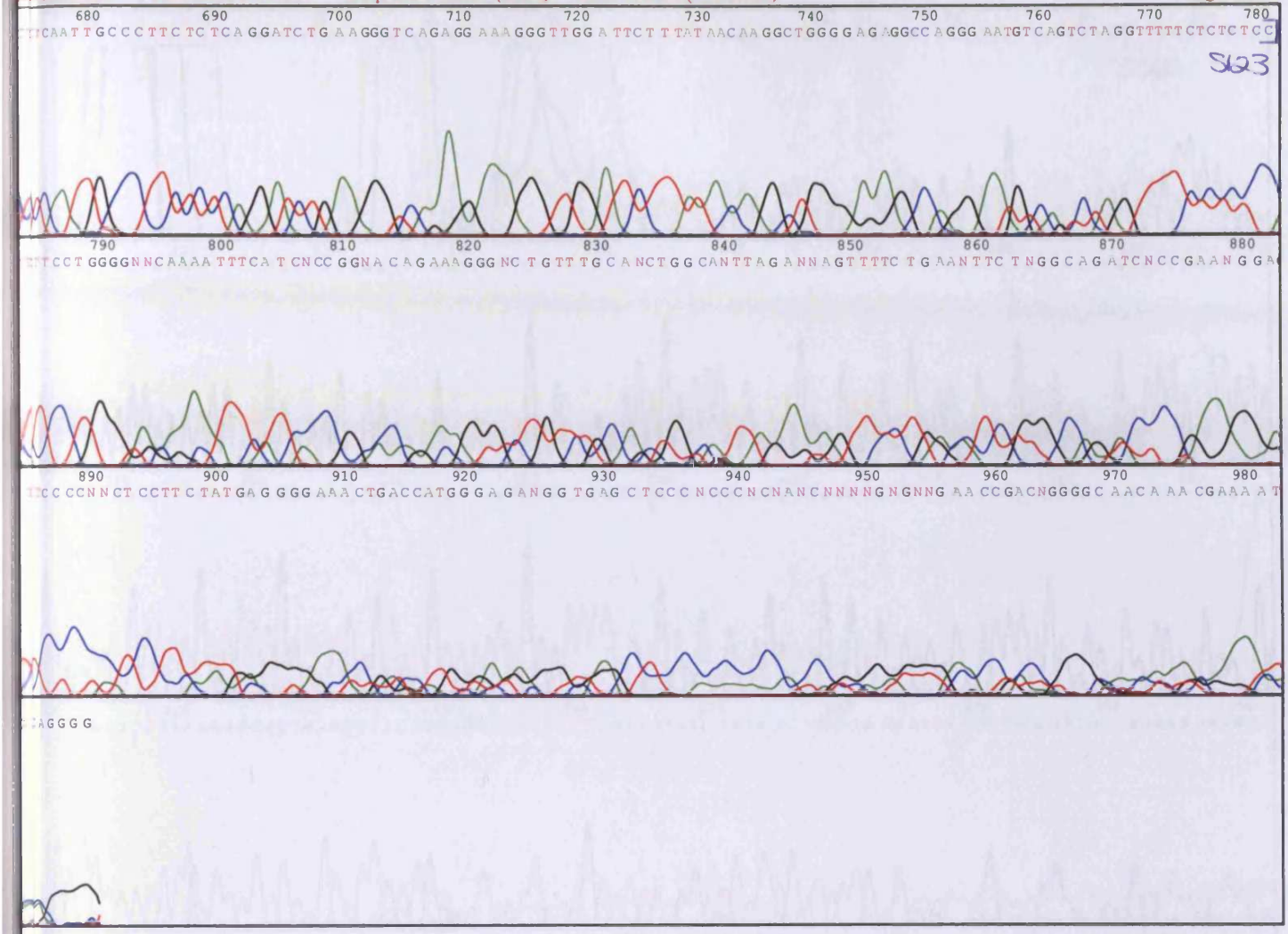

4678

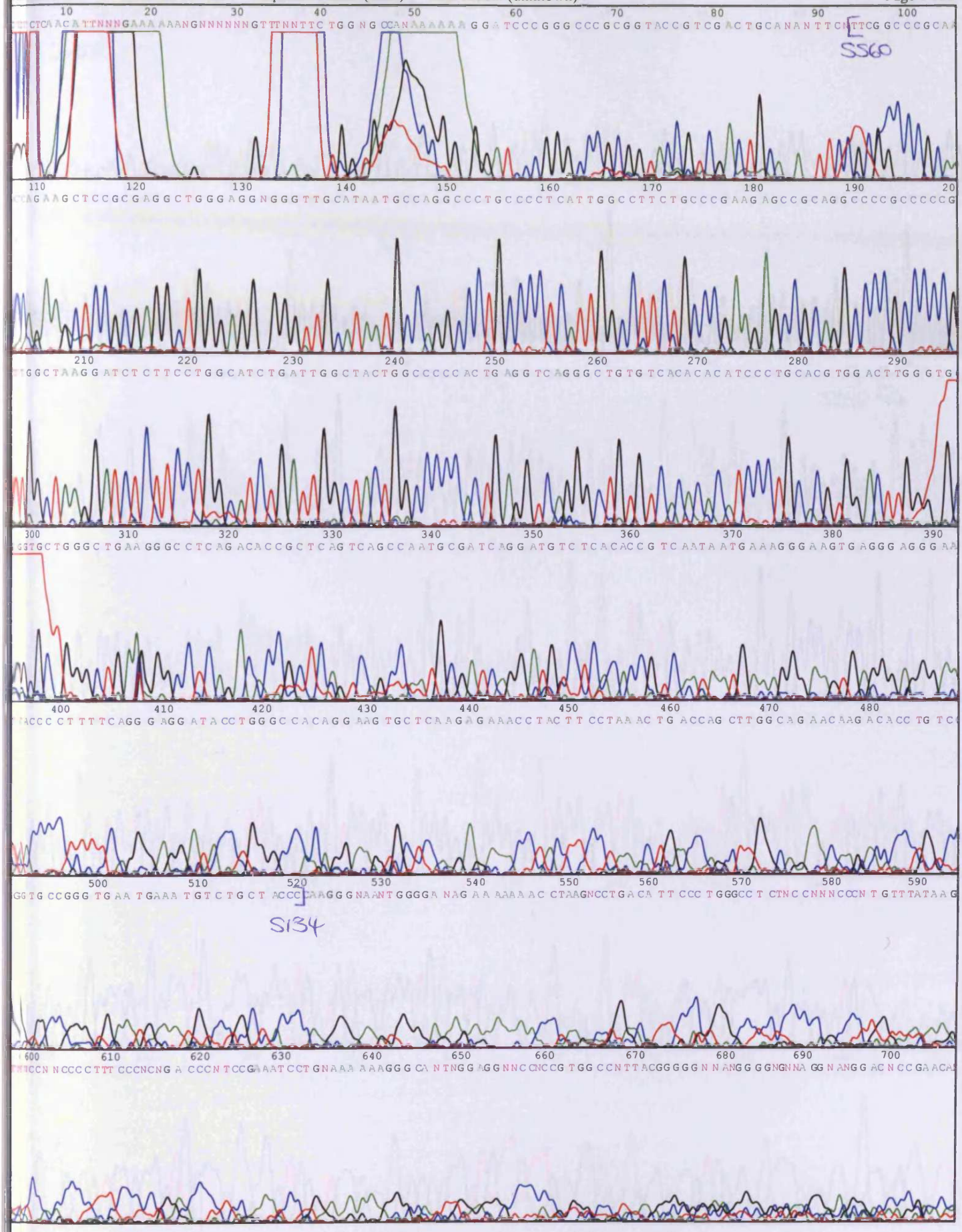

chromas 1.43 File: mPer1-R4.ab1 Sequence Name: (none) Run ended: (unknown)
S172

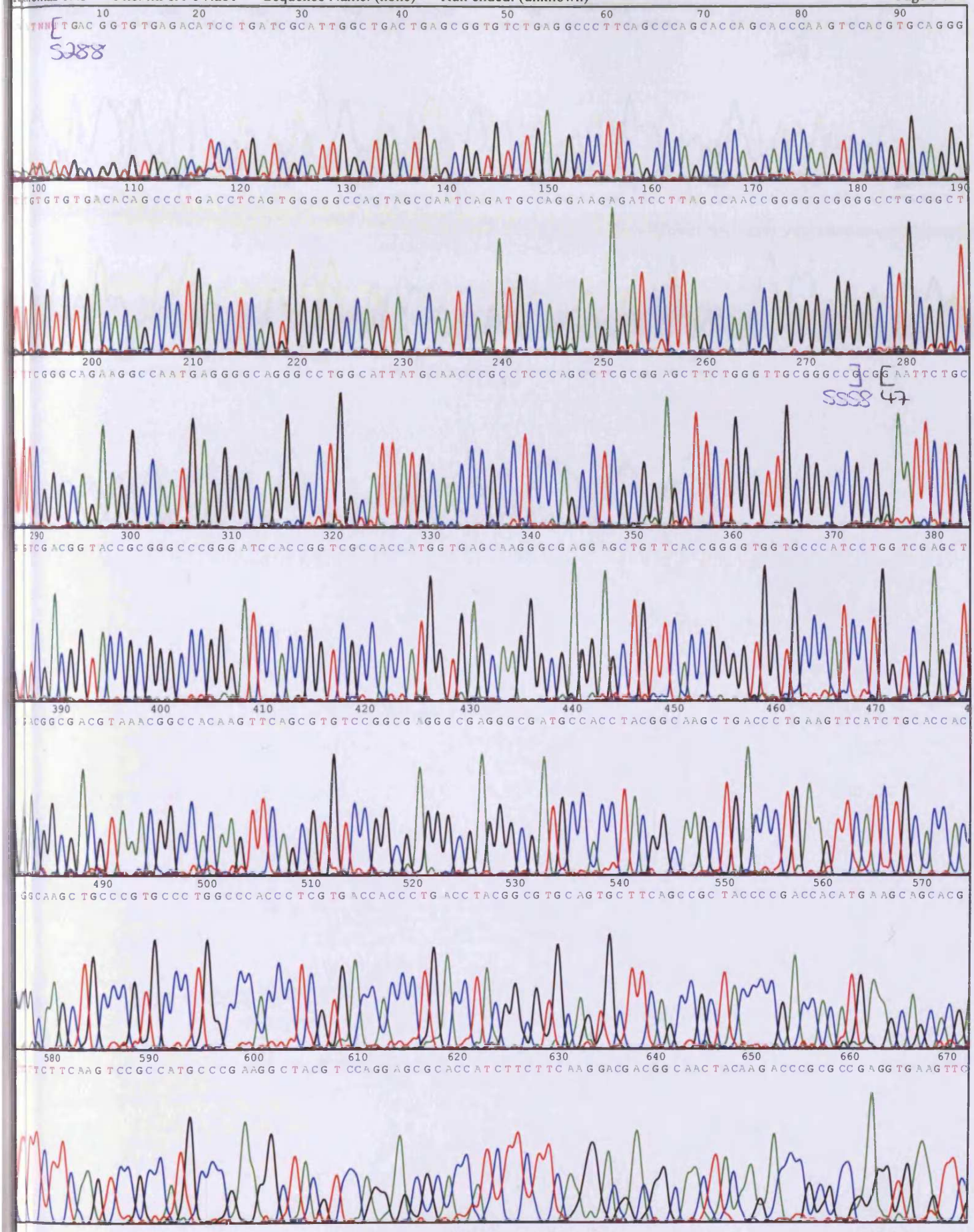
Chromas 1.43 File: mPer1-R4.ab1 Sequence Name: (none) Run ended: (unknown)

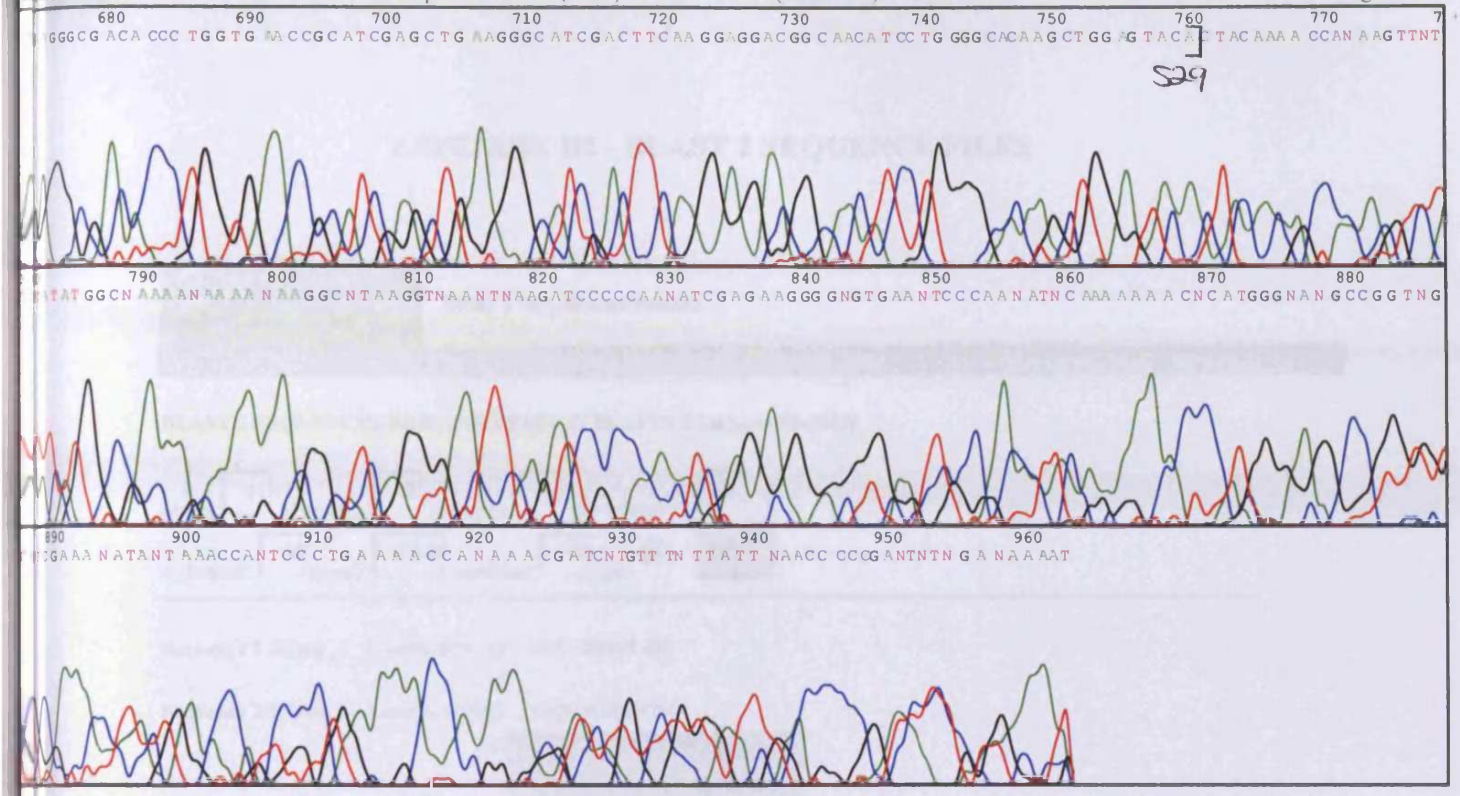



E
4345


680 690 700 710 720 730 740 750 760 770 780
S623
790 800 810 820 830 840 850 860 870 880
890 900 910 920 930 940 950 960 970 980


S560
S134


S288
SSSS 47


680 690 700 710 720 730 740 750 760 770
S29
790 800 810 820 830 840 850 860 870 880
890 900 910 920 930 940 950 960

APPENDIX D2 - BLAST 2 SEQUENCE FILES

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

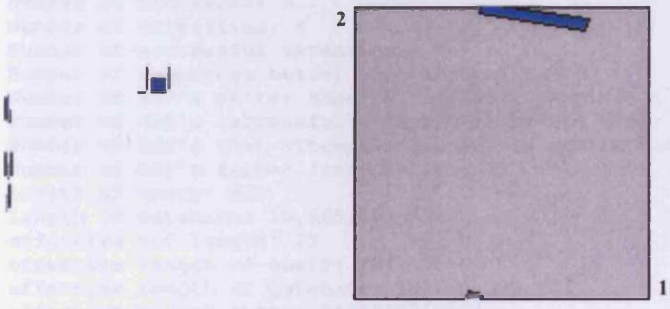
BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.6 [Apr-09-2003]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 50 expect: 10.0 wordsize: 11 Filter ☑ Align

Sequence 1 lcl|seq_1 **Length** 819 (1 .. 819) **mPer1-R1**

Sequence 2 lcl|seq_2 **Length** 4280 (1 .. 4280) **d2EGFP-1**



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score =  558 bits (290), Expect = e-156
Identities = 290/290 (100%)
 Strand = Plus / Minus
```

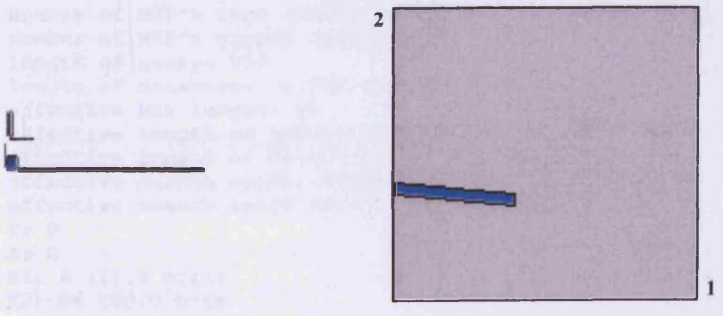
```
Query: 354  atgcatggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtg 413
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4280 atgcatggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtg 4221


Query: 414  agcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttcca 473
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4220 agcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttcca 4161


Query: 474  taggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaa 533
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4160 taggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaa 4101


Query: 534  cccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcc 593
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4100 cccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcc 4041


Query: 594  tgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgg 643
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4040 tgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgg 3991
```

```
Score = 69.9 bits (36), Expect = 8e-09
Identities = 36/36 (100%)
 Strand = Plus / Minus
```

```
Query: 318 ctcgagatctgagtccggtagcgctagtaataacta 353
           ||||||||||||||||||||||||||||||||||||
Sbjct: 36  ctcgagatctgagtccggtagcgctagtaataacta 1
CPU time:     0.02 user secs.            0.02 sys. secs     0.04 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 6
Number of Sequences: 0
Number of extensions: 6
Number of successful extensions: 4
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 819
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 794
effective length of database: 10,665,298,881
effective search space: 8468247311514
effective search space used: 8468247311514
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-R1** **Length** 819 (1 .. 819)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

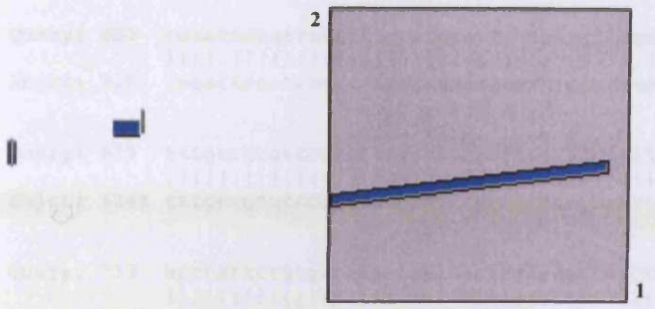
NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score =  602 bits (313), Expect = e-169
Identities = 313/313 (100%)
 Strand = Plus / Minus
```

```
Query: 12   gttactaaacttgccttaattgtctgcttcattctcagcttcctaataatggagcccaag 71
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2890 gttactaaacttgccttaattgtctgcttcattctcagcttcctaataatggagcccaag 2831


Query: 72   gttcaagccctaatgatggcttgtcctgctggcttagggaccctgtaaagtgttaccaaa 131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2830 gttcaagccctaatgatggcttgtcctgctggcttagggaccctgtaaagtgttaccaaa 2771


Query: 132  cctctacactgcattccagcaccgtaggactgcgggggggagtattcccctgtttctaagc 191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2770 cctctacactgcattccagcaccgtaggactgcgggggggagtattcccctgtttctaagc 2711


Query: 192  ttggtgcctagaatacaggctatatccaattccttttccttctctcccccttagcctgatc 251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct: 2710 ttggtgcctagaatacaggctatatccaattccttttccttctctcccccttagcctgatc 2651


Query: 252  tcaaggcattgtttgtgctccgtgcgcaccatcatggttcgtttctatgctcaaaacccg 311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2650 tcaaggcattgtttgtgctccgtgcgcaccatcatggttcgtttctatgctcaaaacccg 2591


Query: 312  agatcactcgaga 324
            ||||||||||||
Sbjct: 2590 agatcactcgaga 2578
CPU time:     0.01 user secs.            0.01 sys. secs     0.02 total secs.

Lambda      K      H
    1.33    0.621     1.12

Gapped
Lambda      K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 3
Number of Sequences: 0
Number of extensions: 3
Number of successful extensions: 1
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 819
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 794
effective length of database: 10,665,298,881
effective search space: 8468247311514
effective search space used: 8468247311514
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-F1** **Length** 902 (1 .. 902)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

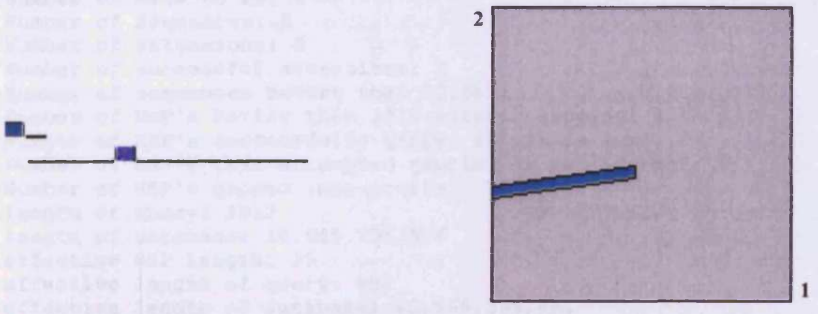
```
Score = 1454 bits (756), Expect = 0.0
Identities = 789/822 (95%)
 Strand = Plus / Plus
```

```
Query: 13   cgagtgatctcgggttttgagcatagaaacgaaccatgatggtgcgcacggagcacaaac 72
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2581 cgagtgatctcgggttttgagcatagaaacgaaccatgatggtgcgcacggagcacaaac 2640

Query: 73   aatgccttgagatcaggctaaggggagagaaggaaaaggaattggatatagcctgtattc 132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2641 aatgccttgagatcaggctaaggggagagaaggaaaaggaattggatatagcctgtattc 2700

Query: 133  taggcaccaagcttagaaacaggggaatactccccccgcagtcctacggtgctggaatgc 192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2701 taggcaccaagcttagaaacaggggaatactccccccgcagtcctacggtgctggaatgc 2760

Query: 193  agtgtagaggtttggtaacactttacagggtccctaagccagcaggacaagccatcatta 252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2761 agtgtagaggtttggtaacactttacagggtccctaagccagcaggacaagccatcatta 2820

Query: 253  gggcttgaaccttgggctccattattaggaagctgagaatgaagcagacaattaaggcaa 312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2821 gggcttgaaccttgggctccattattaggaagctgagaatgaagcagacaattaaggcaa 2880

Query: 313  gtttagtaactacctactaagtcttagtttttccttatgacatcagggtgatacttacct 372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2881 gtttagtaactacctactaagtcttagtttttccttatgacatcagggtgatacttacct 2940

Query: 373  tcagttgtttgggggcttacatgagaacaatatgtgtaaagttcttagcacagcgcttgg 432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2941 tcagttgtttgggggcttacatgagaacaatatgtgtaaagttcttagcacagcgcttgg 3000

Query: 433  atcatttacaaacacaaactataaggtaacaaaagaatagcgatagagttggggatttat 492
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3001 atcatttacaaacacaaactataaggtaacaaaagaatagcgatagagttggggatttat 3060

Query: 493  gtgcatgctgtggccagagagcaatctccatcttctgtttttctgcaagttttgatgact 552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3061 gtgcatgctgtggccagagagcaatctccatcttctgtttttctgcaagttttgatgact 3120

Query: 553  cctcttttccctctccctgannnnnnnnnnnnnnnnnnnnnnggtcttcgggcaccagcc 612
            ||||||||||||||||||||                      ||||||||||||||||||
Sbjct: 3121 cctcttttccctctccctgattttttttttttttttttttttggtcttcgggcaccagcc 3180
```

```
Query: 613  caaattccatcattcctccctgtattcctctcccaagatctgggccccagactgaggcaa 672
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3181 caaattccatcattcctccctgtattcctctcccaagatctgggccccagactgaggcaa 3240


Query: 673  tttgctgtgtcccagtttgacttcatcttaaatccattctctctgaccannnnnnnnnnn 732
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3241 tttgctgtgtcccagtttgacttcatcttaaatccattctctctgaccacccccccccc 3300


Query: 733  acctattctgtgctgtaatagtaactagaaagtctgcctctaaagaggggccccagggaa 792
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3301 acctattctgtgctgtaatagtaactagaaagtctgcctctaaagaggggccccagggaa 3360


Query: 793  ggagcaaaggtgcgttctctctcagaacctaggcctccaatt 834
            |||||||||||||||||||||||||||||||||||||||||
Sbjct: 3361 ggagcaaaggtgcgttctctctcagaacctaggcctccaatt 3402
CPU time:     0.04 user secs.            0.01 sys. secs     0.05 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 7
Number of Sequences: 0
Number of extensions: 7
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 902
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 877
effective length of database: 10,665,298,881
effective search space: 9353467118637
effective search space used: 9353467118637
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-F2** **Length** 1012 (1 .. 1012)

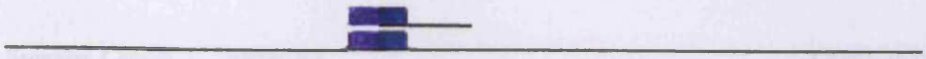
Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score =  829 bits (431), Expect = 0.0
Identities = 456/480 (95%)
 Strand = Plus / Plus
```

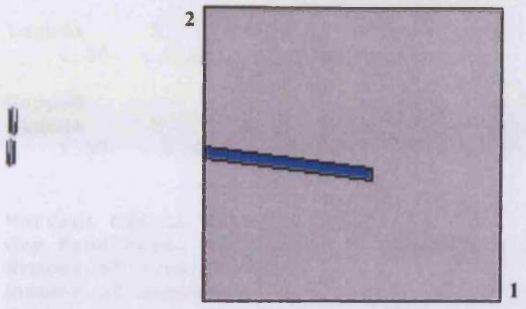


```
Query: 3    gcaggacaagccatcattagggcttgaaccttgggctccattattaggaagctgagaatg 62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2802 gcaggacaagccatcattagggcttgaaccttgggctccattattaggaagctgagaatg 2861


Query: 63   aagcagacaattaaggcaagtttagtaactacctactaagtcttagtttttccttatgac 122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2862 aagcagacaattaaggcaagtttagtaactacctactaagtcttagtttttccttatgac 2921


Query: 123  atcagggtgatacttaccttcagttgtttgggggcttacatgagaacaatatgtgtaaag 182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2922 atcagggtgatacttaccttcagttgtttgggggcttacatgagaacaatatgtgtaaag 2981


Query: 183  ttcttagcacagcgcttggatcatttacaaacacaaactataaggtaacaaaagaatagc 242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2982 ttcttagcacagcgcttggatcatttacaaacacaaactataaggtaacaaaagaatagc 3041


Query: 243  gatagagttggggatttatgtgcatgctgtggccagagagcaatctccatcttctgtttt 302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3042 gatagagttggggatttatgtgcatgctgtggccagagagcaatctccatcttctgtttt 3101


Query: 303  tctgcaagttttgatgactcctcttttccctctccctgannnnnnnnnnnnnnnnnnnn 362
            ||||||||||||||||||||||||||||||||||||||
Sbjct: 3102 tctgcaagttttgatgactcctcttttccctctccctgatttttttttttttttttttt 3161


Query: 363  nggtcttcgggcaccagcccaaattccatcattcctccctgtattcctctcccaagatct 422
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3162 tggtcttcgggcaccagcccaaattccatcattcctccctgtattcctctcccaagatct 3221


Query: 423  gggccccagactgaggcaatttgctgtgtcccagtttgacttcatcttaaatccgtnctc 482
            |||||||||||||||||||||||||||||||||||||||||||||||||||| | |||
Sbjct: 3222 gggccccagactgaggcaatttgctgtgtcccagtttgacttcatcttaaatccattctc 3281
CPU time:     0.01 user secs.             0.01 sys. secs     0.02 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 6
Number of Sequences: 0
Number of extensions: 6
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 1012
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 987
effective length of database: 10,665,298,881
effective search space: 10526649995547
effective search space used: 10526649995547
T: 0
A: 0
```

X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)

Sequence 1 lcl|seq_1 **mPer1-R2** **Length** 1022 (1 .. 1022)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

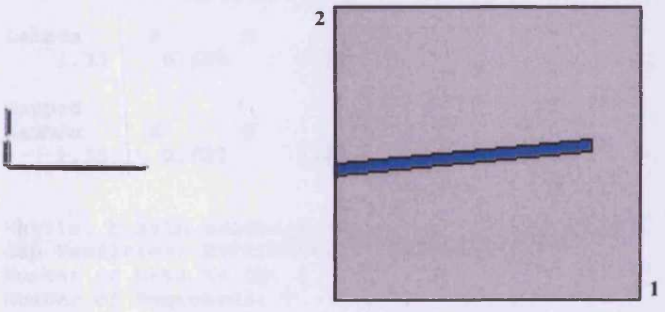
Score = 985 bits (512), Expect = 0.0
Identities = 540/568 (95%)
Strand = Plus / Minus

```
Query: 1    gcaggaggcggggcgaggcagctggggaatgtggggcagggaggctggagaggcagcggt 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3781 gcaggaggcggggcgaggcagctggggaatgtggggcagggaggctggagaggcagcggt 3722

Query: 61   ttgggaggcggccagccccagagggcggcggccagcgcactagggaacatcgtgttctct 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3721 ttgggaggcggccagccccagagggcggcggccagcgcactagggaacatcgtgttctct 3662

Query: 121  tggctgatggcccaggacatgcacacagccttggcgcctcccccaacccaacgacagcgg 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3661 tggctgatggcccaggacatgcacacagccttggcgcctcccccaacccaacgacagcgg 3602

Query: 181  tcctgtacaaaagccaaaaattcctggagagacagttaggaggaaggggtnnnnnnnnn 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3601 tcctgtacaaaagccaaaaattcctggagagacagttaggaggaaggggtcccccctccc 3542

Query: 241  nnnnnnnnatgcctggttctctttgcgggacctacttttaccctccctcagctgggtctt 300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3541 ccgcccccatgcctggttctctttgcgggacctacttttaccctccctcagctgggtctt 3482

Query: 301  aaaactcctggcattggcttttccccgtcaccagactgtacacggttgctggccaggtac 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3481 aaaactcctggcattggcttttccccgtcaccagactgtacacggttgctggccaggtac 3422

Query: 361  caaagagctagggcgggtaaattggaggcctaggttctgagagagaacgcacctttgctc 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3421 caaagagctagggcgggtaaattggaggcctaggttctgagagagaacgcacctttgctc 3362

Query: 421  cttccctggggcccctctttagaggcagactttctagttactattacagcacagaatagg 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3361 cttccctggggcccctctttagaggcagactttctagttactattacagcacagaatagg 3302
```

```
Query: 481  tnnnnnnnnnnntggtcagagagaatggatttaagatgaagtcaaactgggacacagcaa 540
            |           ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3301 tgggggggggggtggtcagagagaatggatttaagatgaagtcaaactgggacacagcaa 3242


Query: 541  attgcctcagtctggggcccagatcttg 568
            ||||||||||||||||||||||||||||
Sbjct: 3241 attgcctcagtctggggcccagatcttg 3214
CPU time:     0.03 user secs.            0.00 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 5
Number of Sequences: 0
Number of extensions: 5
Number of successful extensions: 2
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 1022
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 997
effective length of database: 10,665,298,881
effective search space: 10633302984357
effective search space used: 10633302984357
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-F3** **Length** 707 (1 .. 707)

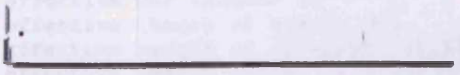
Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score = 1063 bits (553), Expect = 0.0
Identities = 570/587 (97%)
 Strand = Plus / Plus
```

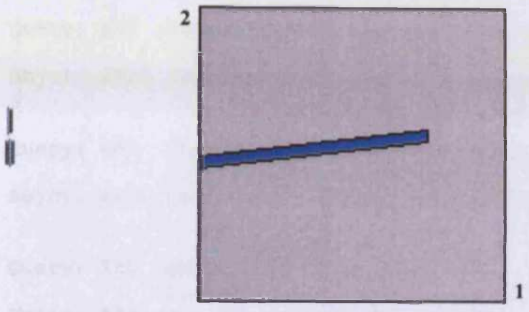


```
Query: 1    ggggccccagggaaggagcaaaggtgcgttctctctcagaacctaggcctccaatttacc 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3347 ggggccccagggaaggagcaaaggtgcgttctctctcagaacctaggcctccaatttacc 3406


Query: 61   cgccctagctctttggtacctggccagcaaccgtgtacagtctggtgacggggaaaagcc 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3407 cgccctagctctttggtacctggccagcaaccgtgtacagtctggtgacggggaaaagcc 3466


Query: 121  aatgccaggagttttaagacccagctgagggagggtaaaagtaggtcccgcaaagagaac 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3467 aatgccaggagttttaagacccagctgagggagggtaaaagtaggtcccgcaaagagaac 3526


Query: 181  caggcatnnnnnnnnnnnnnnnnnacccccttectcctaactgtctctccaggaattttt 240
             |||||||                 ||||||||||||||||||||||||||||||||||
Sbjct: 3527 caggcatgggggcgggggaggggggacccccttcctcctaactgtctctccaggaattttt 3586


Query: 241  ggcttttgtacaggaccgctgtcgttgggttgggggaggcgccaaggctgtgtgcatgtc 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3587 ggcttttgtacaggaccgctgtcgttgggttgggggaggcgccaaggctgtgtgcatgtc 3646


Query: 301  ctgggccatcagccaagagaacacgatgttccctagtgcgctggccgccgccctctgggg 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3647 ctgggccatcagccaagagaacacgatgttccctagtgcgctggccgccgccctctgggg 3706


Query: 361  ctggccgcctcccaaaccgctgcctctccagcctccctgccccacattccccagctgcct 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3707 ctggccgcctcccaaaccgctgcctctccagcctccctgccccacattccccagctgcct 3766


Query: 421  cgccccgcctcctgcctccgctttgacgtcacctccctctcctgccccgcttctccatt 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3767 cgccccgcctcctgcctccgctttgacgtcacctccctctcctgccccgcttctccatt 3826


Query: 481  gacggcagcagagcctggttactgtgggggactgatgaggcccgacagcctgggccttgg 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3827 gacggcagcagagcctggttactgtgggggactgatgaggcccgacagcctgggccttgg 3886


Query: 541  gatcaggttggggctgtttggagtgctgaaacctttgtctgtgtaa 587
             ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3887 gatcaggttggggctgtttggagtgctgaaacctttgtctgtgtaa 3933
CPU time:     0.02 user secs.            0.01 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 6
Number of Sequences: 0
Number of extensions: 6
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 707
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 682
effective length of database: 10,665,298,881
effective search space: 7273733836842
effective search space used: 7273733836842
T: 0
```

```
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-F4** **Length** 1006 (1 .. 1006)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

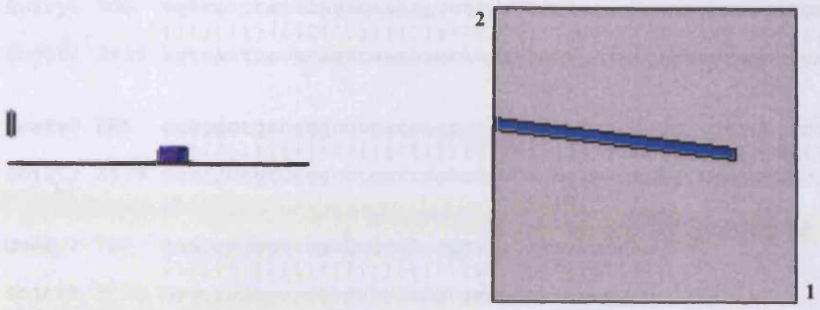
```
Score = 1315 bits (684), Expect = 0.0
Identities = 712/740 (96%)
 Strand = Plus / Plus
```

```
Query: 1    gtcccgcaaagagaaccaggcatnnnnnnnnnnnnnnnnnnacccccttcctcctaactgt 60
            ||||||||||||||||||||||                    ||||||||||||||||||||
Sbjct: 3511 gtcccgcaaagagaaccaggcatgggggcgggggagggggacccccttcctcctaactgt 3570

Query: 61   ctctccaggaatttttggcttttgtacaggaccgctgtcgttgggttgggggaggcgcca 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3571 ctctccaggaatttttggcttttgtacaggaccgctgtcgttgggttgggggaggcgcca 3630

Query: 121  aggctgtgtgcatgtcctgggccatcagccaagagaacacgatgttccctagtgcgctgg 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3631 aggctgtgtgcatgtcctgggccatcagccaagagaacacgatgttccctagtgcgctgg 3690

Query: 181  ccgccgccctctggggctggccgcctcccaaaccgctgcctctccagcctccctgcccca 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3691 ccgccgccctctggggctggccgcctcccaaaccgctgcctctccagcctccctgcccca 3750

Query: 241  cattccccagctgcctcgccccgcctcctgcctccgctttgacgtcacctccctctcctg 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3751 cattccccagctgcctcgccccgcctcctgcctccgctttgacgtcacctccctctcctg 3810

Query: 301  cccccgcttctccattgacggcagcagagcctggttactgtgggggactgatgaggcccg 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3811 cccccgcttctccattgacggcagcagagcctggttactgtgggggactgatgaggcccg 3870

Query: 361  acagcctgggccttgggatcaggttggggctgtttggagtgctgaaacctttgtctgtg 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3871 acagcctgggccttgggatcaggttggggctgtttggagtgctgaaacctttgtctgtg 3930

Query: 421  taaatgacagatagggaagtgggcgagcaatggctgcttgggtcagaggaatcacaccta 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3931 taaatgacagatagggaagtgggcgagcaatggctgcttgggtcagaggaatcacaccta 3990
```

```
Query: 481  aatccttgagagctgtggaaagagaaaggggtctggnnnnnnnnnnnnggacagcacacgg 540
            ||||||||||||||||||||||||||||||||||          ||||||||||||
Sbjct: 3991 aatccttgagagctgtggaaagagaaaggggtctggaaaaaaaaaaaaggacagcacacgg 4050


Query: 541  tcacaacgcagtacgaggggcaggagagcagcatcattttcaggaggaggaagctgagc 600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4051 tcacaacgcagtacgaggggcaggagagcagcatcattttcaggaggaggaagctgagc 4110


Query: 601  actcagcctcccgtgtcttttgttttctgtgtttccctgcttctgtttttctgggttatt 660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4111 actcagcctcccgtgtcttttgttttctgtgtttccctgcttctgtttttctgggttatt 4170


Query: 661  ttataacaggtctgtgtcccagcatttctatagaaccttgtctcgccgcctcctctaagg 720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4171 ttataacaggtctgtgtcccagcatttctatagaaccttgtctcgccgcctcctctaagg 4230


Query: 721  gaaacaccattgttaaggaa 740
            ||||||||||||||||||||
Sbjct: 4231 gaaacaccattgttaaggaa 4250
CPU time:     0.03 user secs.            0.00 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 11
Number of Sequences: 0
Number of extensions: 11
Number of successful extensions: 5
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 4
length of query: 1006
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 981
effective length of database: 10,665,298,881
effective search space: 10462658202261
effective search space used: 10462658202261
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-R3** **Length** 961 (1 .. 961)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

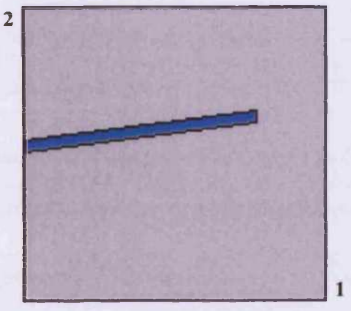
```
Score = 1388 bits (722), Expect = 0.0
Identities = 740/758 (97%)
 Strand = Plus / Minus
```

```
Query: 5    ggtaggggagagagagcctcaggagaggaggcaacaaattatgttttcaaaagaaaagta 64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4539 ggtaggggagagagagcctcaggagaggaggcaacaaattatgttttcaaaagaaaagta 4480

Query: 65   gagcattgattcataaaaagagaatagagactcgattttggggannnnnnnacaacatgc 124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4479 gagcattgattcataaaaagagaatagagactcgattttggggagggggggacaacatgc 4420

Query: 125  cagtctgggtccaggagagctacagtgacagagtccccttccctaggcctgggctgagta 184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4419 cagtctgggtccaggagagctacagtgacagagtccccttccctaggcctgggctgagta 4360

Query: 185  gttctcaggcttccctgcaaacgtaggtggcaagtgaagaggccaacacgcagcggctga 244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4359 gttctcaggcttccctgcaaacgtaggtggcaagtgaagaggccaacacgcagcggctga 4300

Query: 245  cataatcagctgttaagtgcacgcccctcactgtcacgtggctaaagctttccttaacaa 304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4299 cataatcagctgttaagtgcacgcccctcactgtcacgtggctaaagctttccttaacaa 4240

Query: 305  tggtgtttcccttagaggaggcggcgagacaaggttctatagaaatgctgggacacagac 364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4239 tggtgtttcccttagaggaggcggcgagacaaggttctatagaaatgctgggacacagac 4180

Query: 365  ctgttataaaataacccagaaaaacagaagcagggaaacacagaaaacaaaagacacggg 424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4179 ctgttataaaataacccagaaaaacagaagcagggaaacacagaaaacaaaagacacggg 4120

Query: 425  aggctgagtgctcagcttcctcctcctgaaaatgatgctgctctcctgccccctcgtact 484
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4119 aggctgagtgctcagcttcctcctcctgaaaatgatgctgctctcctgccccctcgtact 4060

Query: 485  gcgttgtgaccgtgtgctgtccnnnnnnnnnnnnccagacccctttctctttccacagctc 544
            ||||||||||||||||||||||          ||||||||||||||||||||||||||||
Sbjct: 4059 gcgttgtgaccgtgtgctgtccttttttttttttccagacccctttctctttccacagctc 4000

Query: 545  tcaaggatttaggtgtgattcctctgacccaagcagccattgctcgcccacttccctatc 604
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3999 tcaaggatttaggtgtgattcctctgacccaagcagccattgctcgcccacttccctatc 3940
```

```
Query: 605  tgtcatttacacagacaaaaggtttcagcactccaaacagccccaacctgatcccaaggc 664
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3939 tgtcatttacacagacaaaaggtttcagcactccaaacagccccaacctgatcccaaggc 3880


Query: 665  ccaggctgtcgggcctcatcagtcccccacagtaaccaggctctgctgccgtcaatggag 724
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3879 ccaggctgtcgggcctcatcagtcccccacagtaaccaggctctgctgccgtcaatggag 3820


Query: 725  aagcgggggcaggagagggaggtgacgtcaaagcggag 762
            |||||||||||||||||||||||||||||||||||||
Sbjct: 3819 aagcgggggcaggagagggaggtgacgtcaaagcggag 3782
CPU time:     0.03 user secs.            0.00 sys. secs     0.03 total secs.

Lambda      K       H
    1.33    0.621     1.12

Gapped
Lambda      K       H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 11
Number of Sequences: 0
Number of extensions: 11
Number of successful extensions: 4
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 4
length of query: 961
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 936
effective length of database: 10,665,298,881
effective search space: 9982719752616
effective search space used: 9982719752616
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer2-F5** **Length** 991 (1 .. 991)

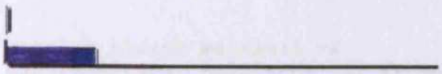
Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score = 1383 bits (719), Expect = 0.0
Identities = 737/755 (97%)
 Strand = Plus / Plus
```

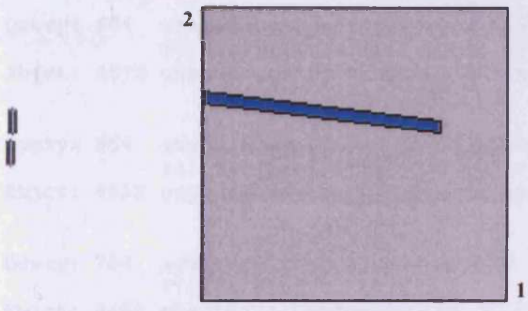


```
Query: 1    gtctgtgtaaatgacagatagggaagtgggcgagcaatggctgcttgggtcagaggaatc 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3924 gtctgtgtaaatgacagatagggaagtgggcgagcaatggctgcttgggtcagaggaatc 3983

Query: 61   acacctaaatccttgagagctgtggaaagagaaaaggggtctggnnnnnnnnnnnnggacag 120
             ||||||||||||||||||||||||||||||||||||||||||||          ||||||
Sbjct: 3984 acacctaaatccttgagagctgtggaaagagaaaaggggtctggaaaaaaaaaaaaggacag 4043

Query: 121  cacacggtcacaacgcagtacgaggggggcaggagagcagcatcattttcaggaggaggaa 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4044 cacacggtcacaacgcagtacgaggggggcaggagagcagcatcattttcaggaggaggaa 4103

Query: 181  gctgagcactcagcctcccgtgtcttttgttttctgtgtttccctgcttctgtttttctg 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4104 gctgagcactcagcctcccgtgtcttttgttttctgtgtttccctgcttctgtttttctg 4163

Query: 241  ggttattttataacaggtctgtgtcccagcatttctatagaaccttgtctcgccgcctcc 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4164 ggttattttataacaggtctgtgtcccagcatttctatagaaccttgtctcgccgcctcc 4223

Query: 301  tctaagggaaacaccattgttaaggaaagctttagccacgtgacagtgaggggcgtgcac 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4224 tctaagggaaacaccattgttaaggaaagctttagccacgtgacagtgaggggcgtgcac 4283

Query: 361  ttaacagctgattatgtcagccgctgcgtgttggcctcttcacttgccacctacgtttgc 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4284 ttaacagctgattatgtcagccgctgcgtgttggcctcttcacttgccacctacgtttgc 4343

Query: 421  agggaagcctgagaactactcagcccaggcctagggaaggggactctgtcactgtagctc 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4344 agggaagcctgagaactactcagcccaggcctagggaaggggactctgtcactgtagctc 4403

Query: 481  tcctggacccagactggcatgttgtnnnnnnntccccaaaatcgagtctctattctcttt 540
             ||||||||||||||||||||||||       ||||||||||||||||||||||||||||
Sbjct: 4404 tcctggacccagactggcatgttgtccccccctccccaaaatcgagtctctattctcttt 4463

Query: 541  ttatgaatcaatgctctacttttctttttgaaaacataatttgttgcctcctctcctgagg 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4464 ttatgaatcaatgctctacttttctttttgaaaacataatttgttgcctcctctcctgagg 4523

Query: 601  ctctctctcccctacctatccttaatagaaacagagccatcctgtttaccgagcatctac 660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4524 ctctctctcccctacctatccttaatagaaacagagccatcctgtttaccgagcatctac 4583

Query: 661  tgtcagtcctgacgctgagacgtacttaccttactgctgagaatacagtaaatgagacag 720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4584 tgtcagtcctgacgctgagacgtacttaccttactgctgagaatacagtaaatgagacag 4643

Query: 721  atgggcttgctgctgggttcatgaaccttggcttt 755
             |||||||||||||||||||||||||||||||||
Sbjct: 4644 atgggcttgctgctgggttcatgaaccttggcttt 4678
CPU time:     0.00 user secs.          0.02 sys. secs     0.02 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12
```

```
Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 10
Number of Sequences: 0
Number of extensions: 10
Number of successful extensions: 3
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 3
length of query: 991
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 966
effective length of database: 10,665,298,881
effective search space: 10302678719046
effective search space used: 10302678719046
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-R4** **Length** 960 (1 .. 960)

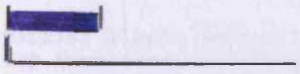
Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score = 1429 bits (743), Expect = 0.0
Identities = 743/743 (100%)
 Strand = Plus / Minus
```



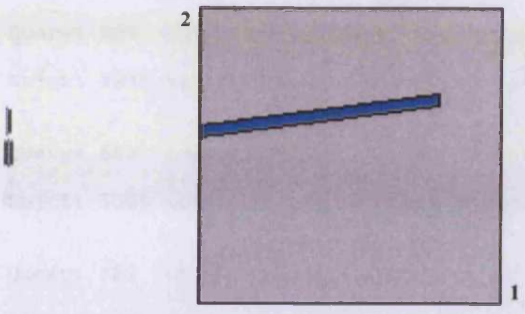
```
Query: 4    acctgtcctggtgccgggtgaatgaaatgtctgctacccaagggaagtgggagagagaaa 63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5172 acctgtcctggtgccgggtgaatgaaatgtctgctacccaagggaagtgggagagagaaa 5113

Query: 64   aacctagactgacattccctggcctctccccagccttgttataaagaatccaaccctttc 123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5112 aacctagactgacattccctggcctctccccagccttgttataaagaatccaaccctttc 5053

Query: 124  ctctgaccccttcagatcctgagagaagggcaattgagggcccacgtgtcgttaaggggtg 183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5052 ctctgaccccttcagatcctgagagaagggcaattgagggcccacgtgtcgttaaggggtg 4993

Query: 184  agtgggtgatggaaggatacggacaaacggaaaggacagggaaagccaatggctactgtg 243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4992 agtgggtgatggaaggatacggacaaacggaaaggacagggaaagccaatggctactgtg 4933
```

```
Query: 244  aaaggaaacaaaagcaggtgcttcactagccaatcagagaaaaggtctccaggaagccta 303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4932 aaaggaaacaaaagcaggtgcttcactagccaatcagagaaaaggtctccaggaagccta 4873


Query: 304  cttggtgactagtgctgcctctgcagaggatgcagccacccacatttagggctgccccaa 363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4872 cttggtgactagtgctgcctctgcagaggatgcagccacccacatttagggctgccccaa 4813


Query: 364  aagtggggaccaggaagccagtactacaaaaggggcagcagagagggcagttgcagcagt 423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4812 aagtggggaccaggaagccagtactacaaaaggggcagcagagagggcagttgcagcagt 4753


Query: 424  aggcagaaaaaggatgggtggctgggtggcaagggctttttattgggctttttattcctg 483
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4752 aggcagaaaaaggatgggtggctgggtggcaagggctttttattgggctttttattcctg 4693


Query: 484  ctgcttagaatacaaaagccaaggttcatgaacccagcagcaagcccatctgtctcattt 543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4692 ctgcttagaatacaaaagccaaggttcatgaacccagcagcaagcccatctgtctcattt 4633


Query: 544  actgtattctcagcagtaaggtaagtacgtctcagcgtcaggactgacagtagatgctcg 603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4632 actgtattctcagcagtaaggtaagtacgtctcagcgtcaggactgacagtagatgctcg 4573


Query: 604  gtaaacaggatggctctgtttctattaaggataggtaggggagagagagcctcaggagag 663
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4572 gtaaacaggatggctctgtttctattaaggataggtaggggagagagagcctcaggagag 4513


Query: 664  gaggcaacaaattatgttttcaaaagaaaagtagagcattgattcataaaaagagaatag 723
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4512 gaggcaacaaattatgttttcaaaagaaaagtagagcattgattcataaaaagagaatag 4453


Query: 724  agactcgattttggggaggggg 746
            |||||||||||||||||||||
Sbjct: 4452 agactcgattttggggaggggg 4430
CPU time:     0.02 user secs.            0.01 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 10
Number of Sequences: 0
Number of extensions: 10
Number of successful extensions: 1
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 960
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 935
effective length of database: 10,665,298,881
effective search space: 9972054453735
effective search space used: 9972054453735
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
```

S1: 12 (23.8 bits)
S2: 21 (41.1 bits)

Sequence 1 lcl|seq_1 **mPer1-F6** **Length** 989 (1 .. 989)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

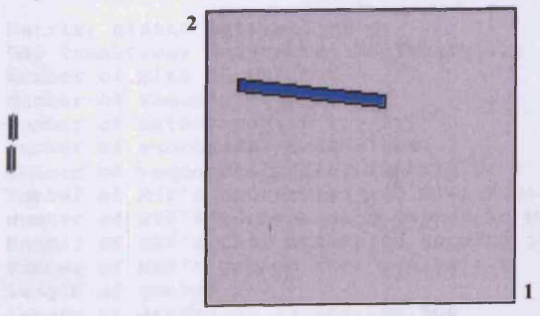
Score = 1471 bits (765), Expect = 0.0
Identities = 772/779 (99%)
Strand = Plus / Plus

```
Query: 3    gggaagcctgagaactactcagcccaggcctagggaaggggactctgtcactgtagctct 62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4345 gggaagcctgagaactactcagcccaggcctagggaaggggactctgtcactgtagctct 4404

Query: 63   cctggacccagactggcatgttgtnnnnnnntccccaaaatcgagtctctattctctttt 122
            |||||||||||||||||||||||       |||||||||||||||||||||||||||||
Sbjct: 4405 cctggacccagactggcatgttgtcccccccctccccaaaatcgagtctctattctctttt 4464

Query: 123  tatgaatcaatgctctacttttcttttgaaaacataatttgttgcctcctctcctgaggc 182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4465 tatgaatcaatgctctacttttcttttgaaaacataatttgttgcctcctctcctgaggc 4524

Query: 183  tctctctcccctacctatccttaatagaaacagagccatcctgtttaccgagcatctact 242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4525 tctctctcccctacctatccttaatagaaacagagccatcctgtttaccgagcatctact 4584

Query: 243  gtcagtcctgacgctgagacgtacttaccttactgctgagaatacagtaaatgagacaga 302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4585 gtcagtcctgacgctgagacgtacttaccttactgctgagaatacagtaaatgagacaga 4644

Query: 303  tgggcttgctgctgggttcatgaaccttggcttttgtattctaagcagcaggaataaaaa 362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4645 tgggcttgctgctgggttcatgaaccttggcttttgtattctaagcagcaggaataaaaa 4704

Query: 363  gcccaataaaaagcccttgccacccagccacccatccttttctgcctactgctgcaact 422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4705 gcccaataaaaagcccttgccacccagccacccatccttttctgcctactgctgcaact 4764

Query: 423  gccctctctgctgccccttttgtagtactggcttcctggtccccacttttggggcagccc 482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4765 gccctctctgctgccccttttgtagtactggcttcctggtccccacttttggggcagccc 4824

Query: 483  taaatgtgggtggctgcatcctctgcagaggcagcactagtcaccaagtaggcttcctgg 542
```

```
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4825 taaatgtgggtggctgcatcctctgcagaggcagcactagtcaccaagtaggcttcctgg 4884


Query: 543  agacctttctctgattggctagtgaagcacctgcttttgtttcctttcacagtagccat 602
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4885 agacctttctctgattggctagtgaagcacctgcttttgtttcctttcacagtagccat 4944


Query: 603  tggctttccctgtcctttccgtttgtccgtatccttccatcacccactcacccccttaacg 662
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 4945 tggctttccctgtcctttccgtttgtccgtatccttccatcacccactcacccccttaacg 5004


Query: 663  acacgtgggccctcaattgcccttctctcaggatctgaagggtcagaggaaagggttgga 722
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5005 acacgtgggccctcaattgcccttctctcaggatctgaagggtcagaggaaagggttgga 5064


Query: 723  ttctttataacaaggctggggagaggccagggaatgtcagtctaggtttttctctctcc 781
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5065 ttctttataacaaggctggggagaggccagggaatgtcagtctaggtttttctctctcc 5123
CPU time:     0.01 user secs.            0.02 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 8
Number of Sequences: 0
Number of extensions: 8
Number of successful extensions: 1
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 989
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 964
effective length of database: 10,665,298,881
effective search space: 10281348121284
effective search space used: 10281348121284
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-R5** **Length** 914 (1 .. 914)

Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence

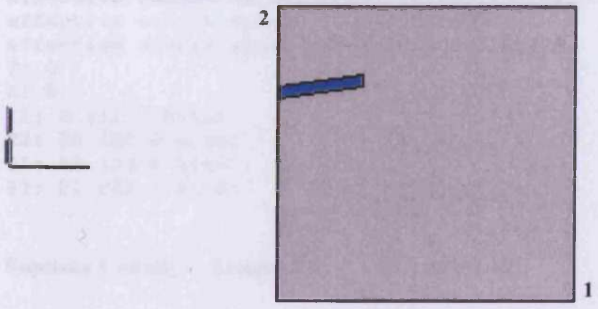
```
Score =  817 bits (425), Expect = 0.0
Identities = 426/427 (99%)
 Strand = Plus / Minus
```

```
Query: 94   ttcggcccgcaacccagaagctccgcgaggctgggaggnggggttgcataatgccaggccc 153
            ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 5560 ttcggcccgcaacccagaagctccgcgaggctgggaggcgggttgcataatgccaggccc 5501


Query: 154  tgcccctcattggccttctgcccgaagagccgcaggccccgcccccggttggctaaggat 213
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5500 tgcccctcattggccttctgcccgaagagccgcaggccccgcccccggttggctaaggat 5441


Query: 214  ctcttcctggcatctgattggctactggcccccactgaggtcagggctgtgtcacacaca 273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5440 ctcttcctggcatctgattggctactggcccccactgaggtcagggctgtgtcacacaca 5381


Query: 274  tccctgcacgtggacttgggtgctggtgctgggctgaagggcctcagacaccgctcagtc 333
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5380 tccctgcacgtggacttgggtgctggtgctgggctgaagggcctcagacaccgctcagtc 5321


Query: 334  agccaatgcgatcaggatgtctcacaccgtcaataatgaaagggaagtgagggagggaaa 393
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5320 agccaatgcgatcaggatgtctcacaccgtcaataatgaaagggaagtgagggagggaaa 5261


Query: 394  ctacccctttcagggaggatacctgggccacaggaagtgctcaagagaaacctacttcc 453
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5260 ctacccctttcagggaggatacctgggccacaggaagtgctcaagagaaacctacttcc 5201


Query: 454  taaactgaccagcttggcagaacaagacacctgtcctggtgccgggtgaatgaaatgtct 513
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5200 taaactgaccagcttggcagaacaagacacctgtcctggtgccgggtgaatgaaatgtct 5141


Query: 514  gctaccc 520
            |||||||
Sbjct: 5140 gctaccc 5134
CPU time:     0.02 user secs.              0.01 sys. secs     0.03 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 6
Number of Sequences: 0
Number of extensions: 6
Number of successful extensions: 2
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 2
length of query: 914
length of database: 10,665,298,906
effective HSP length: 25
```

```
effective length of query: 889
effective length of database: 10,665,298,881
effective search space: 9481450705209
effective search space used: 9481450705209
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

Sequence 1 lcl|seq_1 **mPer1-F7** **Length** 963 (1 .. 963)

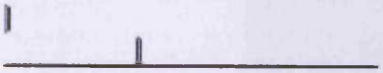
Sequence 2 gi 9437540 Mus musculus PER1 (Per1) gene, partial cds, alternatively spliced **Length** 7502 (1 .. 7502)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score =  521 bits (271), Expect = e-145
Identities = 271/271 (100%)
 Strand = Plus / Plus
```



```
Query: 5    ttgacggtgtgagacatcctgatcgcattggctgactgagcggtgtctgaggcccttcag 64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5288 ttgacggtgtgagacatcctgatcgcattggctgactgagcggtgtctgaggcccttcag 5347


Query: 65   cccagcaccagcacccaagtccacgtgcagggatgtgtgtgacacagccctgacctcagt 124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5348 cccagcaccagcacccaagtccacgtgcagggatgtgtgtgacacagccctgacctcagt 5407


Query: 125  gggggccagtagccaatcagatgccaggaagagatccttagccaaccgggggcggggcct 184
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Query: 185  gcggctcttcgggcagaaggccaatgaggggcagggcctggcattatgcaacccgcctcc 244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5468 gcggctcttcgggcagaaggccaatgaggggcagggcctggcattatgcaacccgcctcc 5527


Query: 245  cagcctcgcggagcttctgggttgcgggccg 275
            ||||||||||||||||||||||||||||||
Sbjct: 5528 cagcctcgcggagcttctgggttgcgggccg 5558
CPU time:     0.01 user secs.            0.02 sys. secs     0.03 total secs.

Lambda     K      H
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Gapped
Lambda     K      H
    1.33    0.621     1.12
```

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Matrix: blastn matrix:1 -2
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Number of extensions: 5
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Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 963
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 938
effective length of database: 10,665,298,881
effective search space: 10004050350378
effective search space used: 10004050350378
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
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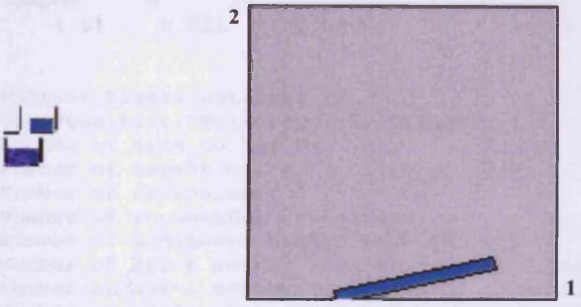
Sequence 1 lcl|seq_1 **Length** 963 (1 .. 963) **mPer1-F7**

Sequence 2 lcl|seq_2 **Length** 4280 (1 .. 4280) **pdEGFP-1**



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

```
Score =  929 bits (483), Expect = 0.0
Identities = 483/483 (100%)
 Strand = Plus / Plus
```

```
Query: 278 gaattctgcagtcgacggtaccgcgggcccgggatccaccggtcgccaccatggtgagca 337
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 47  gaattctgcagtcgacggtaccgcgggcccgggatccaccggtcgccaccatggtgagca 106


Query: 338 agggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaa 397
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 107 agggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaa 166


Query: 398 acggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctga 457
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 167 acggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctga 226


Query: 458 ccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgacca 517
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 227 ccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgacca 286
```

```
Query: 518 ccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgact 577
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Query: 638 acggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgca 697
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Sbjct: 407 acggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgca 466


Query: 698 tcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagt 757
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 467 tcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagt 526


Query: 758 aca 760
           |||
Sbjct: 527 aca 529
CPU time:     0.01 user secs.            0.01 sys. secs     0.02 total secs.

Lambda     K      H
    1.33    0.621     1.12

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 5
Number of Sequences: 0
Number of extensions: 5
Number of successful extensions: 1
Number of sequences better than 10.0: 1
Number of HSP's better than 10.0 without gapping: 1
Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in prelim test: 0
Number of HSP's gapped (non-prelim): 1
length of query: 963
length of database: 10,665,298,906
effective HSP length: 25
effective length of query: 938
effective length of database: 10,665,298,881
effective search space: 10004050350378
effective search space used: 10004050350378
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 21 (41.1 bits)
```

APPENDIX D3 – Raw Data for *mPer1*/d2EGFP Transgenic Project

Superovulation				Number of Eggs								
Session # (Transgene concentration ng/μl)	**Number of animals**	**Average age of animals (days)**	**Average weight of animals (g)**	**Total isolated**	**Dead**	**Non-injectable**	**Un-injected**	**Injected**	**Died after MI**	**Survived after MI**	**Lost (B-before MI; A-after MI)**	**Available for OVT**
1 (4)	14	42	139	197	0	90	0	106	19	87	B – 1; A – 1	**86**
2 (4)	12	40	127	263	3	98	0	162	28	134	0	**134**
3 (4)	8	39	123	233	10	82	9	132	29	103	A – 9	**94**
4 (3)	8	39	124	141	0	56	0	85	14	71	A – 33	**38**
5 (3)	8	42	136	196	0	46	0	147	37	110	B – 3; A – 45	**65**
6 (3)	10	41	130	60	0	18	0	42	3	39	0	**39**
7 (3)	8	30	120	110	0	44	0	66	13	53	0	**53**
8 (3)	10	41	132	77	3	20	0	54	13	41	0	**41**
9 (3)	10	40	128	107	2	41	0	62	6	56	B – 2; A – 2	**54**
10 (3)	8	38	125	50	0	16	0	34	4	30	A – 5	**25**
11 (3)	7	35	121	40	0	13	0	27	2	25	0	**25**
12 (3)	4	40	127	41	0	15	0	26	5	21	1	**20**
13 (2)	8	42	148	99	7	26	0	63	11	52	B – 3; A – 0	**52**
TOTAL	**115**	**40**	**130**	**1614** (average of 14 per animal)	**25**	**565**	**9**	**1006**	**184**	**822**	**B – 9; A – 96**	**726**
TOTAL % value	**N/A**	**N/A**	**N/A**	**100**	**1.5**	**35**	**0.5**	**62.5**	**11.5**	**51**	**B – 0.5; A – 6**	**45**
% total MI eggs								**100**	**18**	**82**	**9.5**	**72.5**

The table above contains the complete superovulation / micro-injection (MI) data set for all 13 transgenic sessions performed.

APPENDIX D4 – Raw Data for *mPer1*/d2EGFP Transgenic Project

Session # (Transgene concentration ng/μl)	OVT ♀ rat reference #	Age on transfer day (months)	Weight of OVT ♀ rat (g)	Number of eggs in left OVT	Number of eggs in right OVT	Total number of pups born	Number of still-born or savaged pups	Number of surviving pups	Number of trans-genics
1 (4)	PMR/YR 1	4	273.7	21	21	5	0	5	0
	PMR/YR 2	3	210.7	21	23	0	0	0	0
2 (4)	PMR/YR 3	5	265.5	20	20	0	0	0	0
	PMR/YR 4	**3**	**221.4**	**20**	**21**	**3**	**0**	**3**	**2**
3 (4)	PMR/YR 5	4	237.5	20	20	0	0	0	0
	PMR/YR 6	4	268.0	26	0	1	1	0	0
	PMR/YR 7	3	191.3	28	0	0	0	0	0
4 (3)	PMR/YR 8	4	279.1	31	7	1	1	0	0
5 (3)	**PMR/YR 9**	**6**	**295.0**	**20**	**20**	**3**	**0**	**3**	**1**
	PMR/YR 10	5	256.0	25	0	0	0	0	0
6 (3)	PMR/YR 11	5	299.0	20	19	0	0	0	0
7 (3)	PMR/YR 12	6	243.0	24	0	1	1	0	0
	PMR/YR 13	3	163.0	0	29	2	0	2	0
8 (3)	PMR/YR 14	4	272.0	20	0	0	0	0	0
	PMR/YR 15	4	270.0	0	21	0	0	0	0
9 (3)	PMR/YR 16	4	283.0	27	0	5	5	0	0
	PMR/YR 17	3	180.0	0	27	1	0	1	0
10 (3)	PMR/YR 18	3	268.0	0	25	0	0	0	0
11 (3)	PMR/YR 19	3	275.0	0	25	0	0	0	0
12 (3)	PMR/YR 20	4	260.0	20	0	0	0	0	0
13 (2)	PMR/YR 21	4	298.0	0	22	0	0	0	0
	PMR/YR 22	**3**	**193.0**	**0**	**30**	**4**	**0**	**4**	**1**

The table above contains the complete oviduct transfer (OVT) data set for all 13 transgenic sessions performed.

APPENDIX D5 – Raw Data for *mPer1*/d2EGFP Transgenic Project

OVT transfer - Raw data

U - Unilateral B – Bilateral	**Day 0** **U-Left OVT**	**Day 0** **U-Right OVT**	**Day 0** **U-OVT totals**	**Day 0** **B-OVT**	**Total**
Number of eggs used for OVT	170	179	349	324	**673**
Number of OVTs	7	7	14	(2x) 8	(8+) **22**
Average number of eggs per OVT	24	25.5	25	(2x) 20	**23**
Number of OVTs with live births	3	3	6	3	**9**
Number of live pups	7	7	14	12	**26**
Average number of pups per OVT	2.3	2.3	2.3	(2x) 2	**2.2**
Number of surviving pups	0	7	7	11	**18**
Number of transgenics generated	0	1	1	3	**4**

OVT transfer data- % values

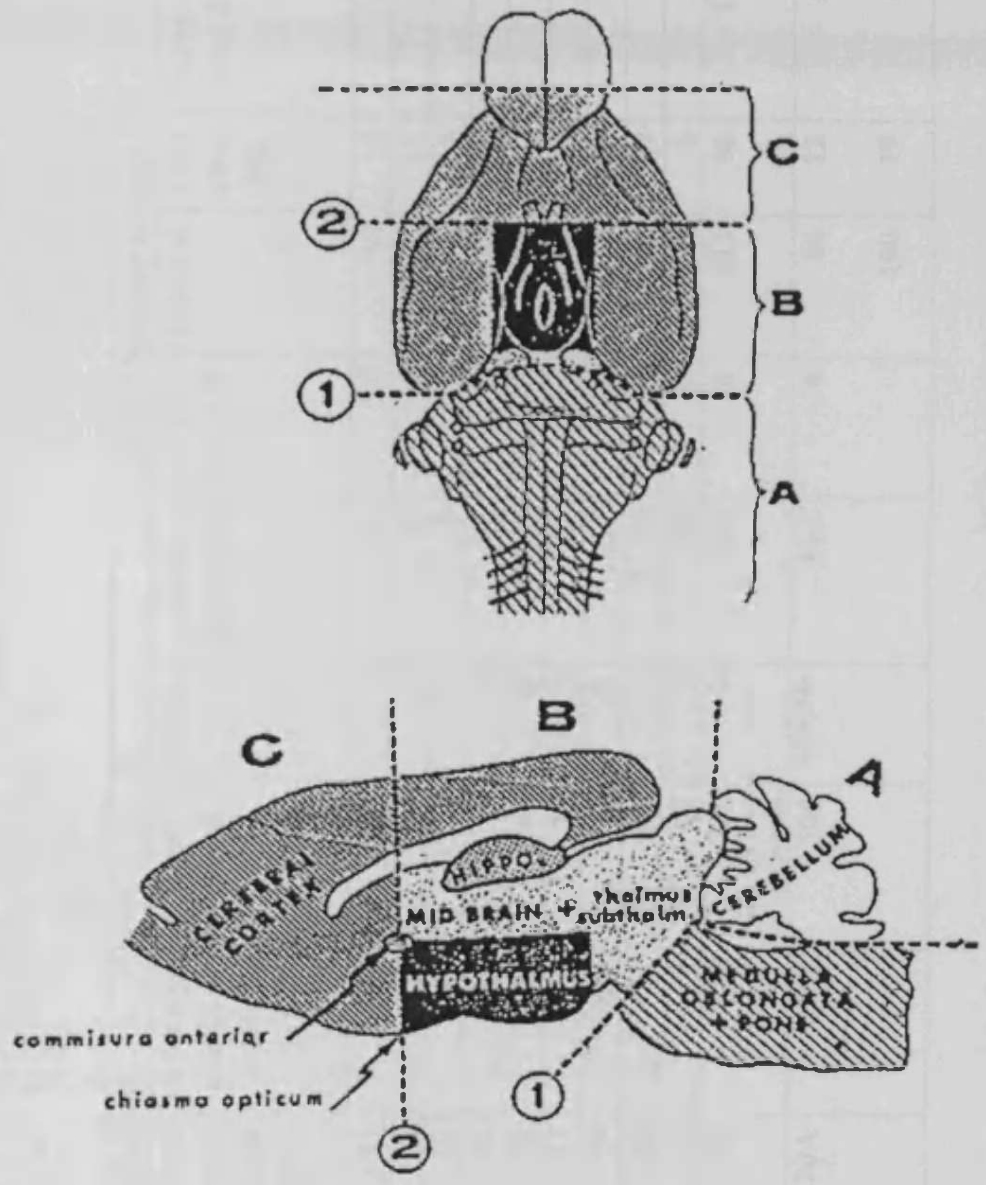
%	**Day 0** **U-OVT**	**Day 0** **B-OVT**	**%**
Number of eggs used for OVT	48	45	**93**
OVTs with live births	43	38	**41**
Birth efficiency (% of transferred eggs generating live births)	4	3.7	**3.9**
Frequency of transgenic births	7	25	**16**
Transgenic efficiency (% of transferred eggs generating transgenic offspring)	0.3	0.9	**0.6**

Overall transgenesis efficiency = 4 transgenics/13 sessions
≡ 31% efficient

The table above contains a summary of the oviduct transfer (OVT) data set for all 13 transgenic sessions performed.

APPENDIX E

DISSECTION GUIDE



Brain maps used to guide dissections of rat brain tissues for further analysis. Dotted lines labelled 1 and 2 indicate positions of dissection, resulting in 3 regions: **A** striatum and cerebral cortex; **B** midbrain, hypothalamus and hippocampus; and **C** medulla oblongata and cerebellum, for further dissection of desired brain tissues. Adapted from Glowinski and Iversen (1966).

APPENDIX F1 – Raw Data for *Egr-1*/d2EGFP Transgenic Project

Superovulation				Number of Eggs								
Session # (Transgene concentration ng/μl)	**Number of animals**	**Average age of animals (days)**	**Average weight of animals (g)**	**Total isolated**	**Dead**	**Non-injectable**	**Un-injected**	**Injected**	**Died after MI**	**Survived after MI**	**Lost (B-before MI; A-after MI)**	**Available for OVT**
1 (5)	10	41	138	129	0	61	0	68	12	56	A – 1	**55**
2 (5)	5	40	132	96	0	31	0	65	11	54	0	**54**
3 (5)	5	39	128	46	1	16	0	24	3	21	B – 5	**21**
4 (5)	5	42	136	75	0	23	0	52	10	42	0	**42**
5 (5)	5	38	129	45	0	13	0	32	4	28	0	**28**
6 (5)	5	37	125	75	0	23	0	52	6	46	0	**46**
7 (5)	11	39	128	117	0	27	0	90	15	75	A – 5	**70**
8 (2)	8	40	132	136	0	37	0	99	29	70	0	**70**
9 (2)	9	32	121	59	0	30	0	29	6	23	A – 1	**22**
TOTAL	**63**	**39**	**130**	**778** (average of 12 per animal)	**1**	**261**	**0**	**511**	**96**	**415**	**B – 5; A – 7**	**408**
TOTAL	**N/A**	**N/A**	**N/A**	**100**	**negligible**	**33.5**	**0**	**66**	**12**	**53**	**B – 0.5; A – 1**	**52**
% value								**100**	**19**	**81**	**1**	**80**
% total MI eggs												

The table above contains the complete superovulation / micro-injection (MI) data set for all 9 transgenic sessions performed.

APPENDIX F2 – Raw Data for *Egr-1*/d2EGFP Transgenic Project

Session # (Transgene concentration ng/μl)	OVT ♀ rat reference #	Age on transfer day (months)	Weight of OVT ♀ rat (g)	Number of eggs in left OVT	Number of eggs in right OVT	Total number of pups born	Number of still-born or savaged pups	Number of surviving pups	Number of trans-genics
1 (5)	PMR/ZR 1	5	263	0	25	0	0	0	0
	PMR/ZR 2	3	197	0	30	0	0	0	0
2 (5)	PMR/ZR 3	5	327	0	27	8	0	8	0
	PMR/ZR 4	3	267	0	27	8	0	8	3
3 (5)	PMR/ZR 5	3	205	0	21	3	0	3	0
4 (5)	PMR/ZR 6	3	207	0	21	2	0	2	0
	PMR/ZR 7	3	214	0	21	2	0	2	1
5 (5)	PMR/ZR 8	4	255	0	28	10	0	10	2
6 (5)	PMR/ZR 9	5	295	0	23	0	0	0	0
	PMR/ZR 10	4	290	0	23	0	0	0	0
7 (5)	PMR/ZR 11	3	185	35	35	0	0	0	0
8 (2)	PMR/ZR 12	4	246	0	24	6	0	6	0
	PMR/ZR 13	3	255	0	24	8	0	8	0
	PMR/ZR 14	3	208	0	22	1	0	1	0
9 (2)	PMR/ZR 15	3	191	0	22	8	0	8	0

The table above contains the complete oviduct transfer (OVT) data set for all 9 transgenic sessions performed.

APPENDIX F3 – Raw Data for *Egr-1*/d2EGFP Transgenic Project

OVT transfer - Raw data **OVT transfer data- % values**

U - Unilateral B – Bilateral	**Day 0** **U-Left OVT**	**Day 0** **U-Right OVT**	**Day 0** **U-OVT totals**	**Day 0** **B-OVT**	**Total**	**%**			**Day 0** **U-OVT**	**Day 0** **B-OVT**	**Day 0** **OVTs**
	Number of eggs used for OVT			0	338	338	70	**408**			
	Number of OVTs			0	14	14	(2x) 1	(1+) **15**			
Average number of eggs per OVT	0	24	24	(2x) 35	**27**	Number of eggs used for OVT			83	17	**100**
Number of OVTs with live births	0	10	10	0	**10**	OVTs with live births			71	0	**67**
Number of live pups	0	56	56	0	**56**	Birth efficiency (% of transferred eggs generating live births)			17	0	**14**
	Average number of pups per OVT			0	4	4	0	**4**			
	Number of surviving pups			0	56	56	0	**56**			
Number of transgenics generated	0	6	6	0	**6**	Frequency of transgenic births			11	0	**11**
		Transgenic efficiency (% of transferred eggs generating transgenic offspring)			1.8	0	**1.5**				

Overall transgenesis efficiency = 6 transgenics/9 sessions
≡ 67% efficient

The table above contains a summary of the oviduct transfer (OVT) data set for all 9 transgenic sessions performed.

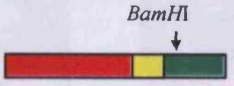
APPENDIX G

SUPPLEMENTARY DATA

TRANSGENE INTEGRATION EVENTS

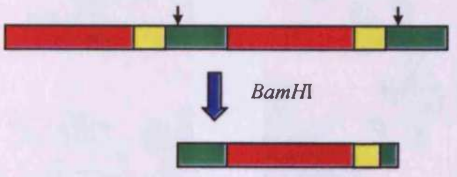
KEY
- *mPer-1* promoter
- *mPer-1* exon
- d2EGFP reporter

A. Organisation of Transgene



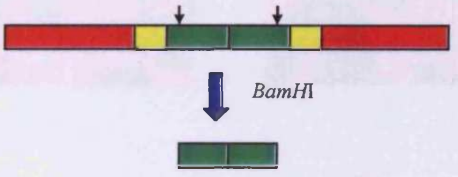
B. Transgene Integration Events

1. Head – Tail integration



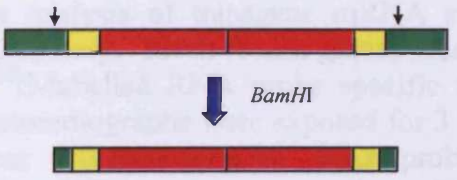
4.1 kb

2. Tail – Tail integration



~ 2 kb

3. Head – Head integration



~ 6 kb

Small black arrows indicate location of *BamHI* cleavage sites.

APPENDIX H

SUPPLEMENTARY DATA: PUBLISHED DATA

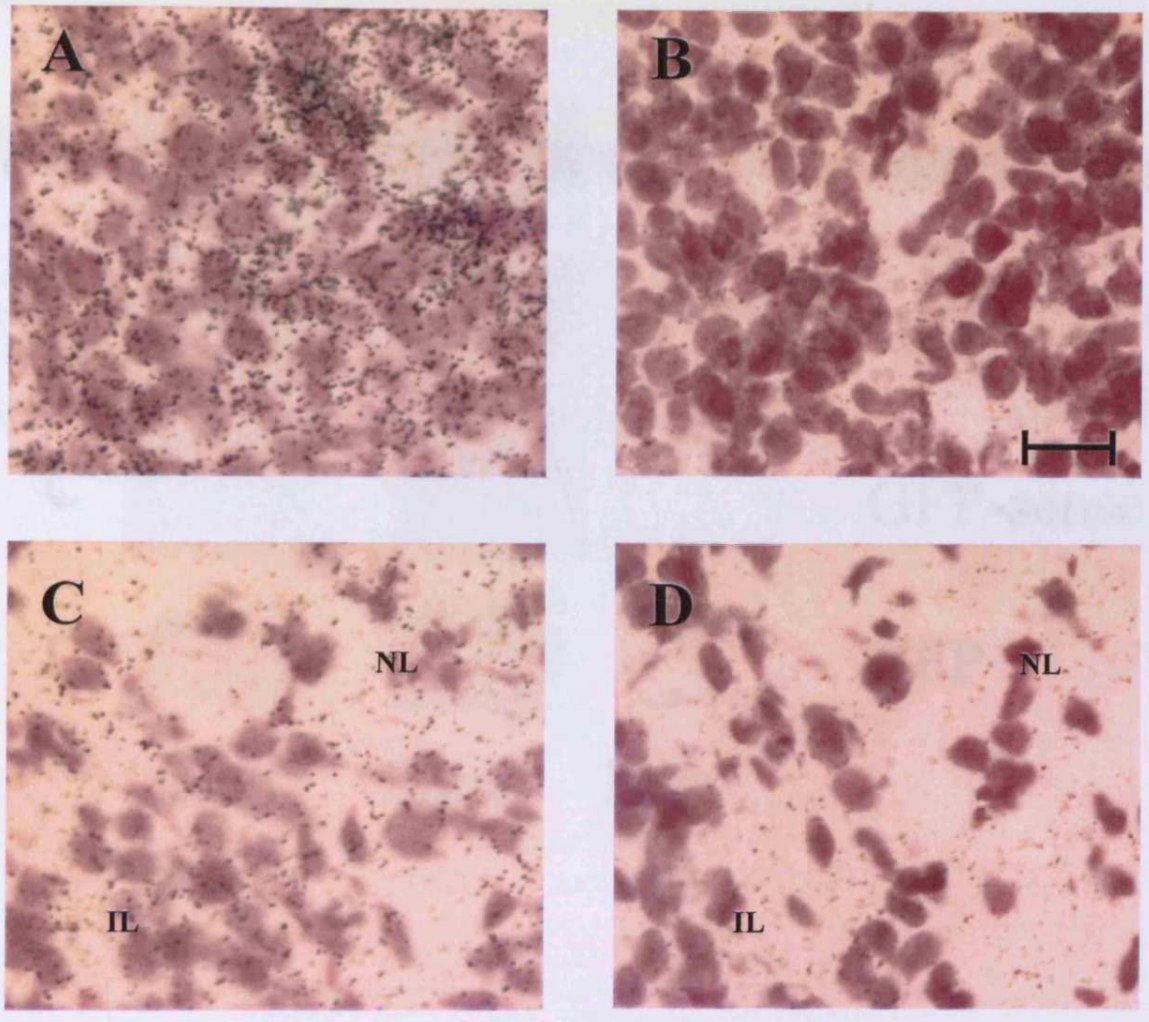


Figure 4.21 (Slade, 2001).

In situ hybridization analysis of transgene mRNA expression in female transgenic (57C) rat pituitary. Fresh frozen glands were sectioned (12 μm) and probed with a ^{35}S-labelled RNA probe specific for transgene (GFP) mRNA. Emulsion autoradiographs were exposed for 3 weeks. (**A**) Anterior lobe - antisense probe; (**B**) Anterior lobe - sense probe; (**C**) Neural (NL) and intermediate (IL) lobes - antisense probe; (**D**) Neural (NL) and intermediate (IL) lobes - sense probe. Silver grains are observed over a specific sub-population of cells in the anterior pituitary gland only, indicating a cell-specific expression pattern. Scale bar = 15 μm.
Experiment performed by Miss P. Man.

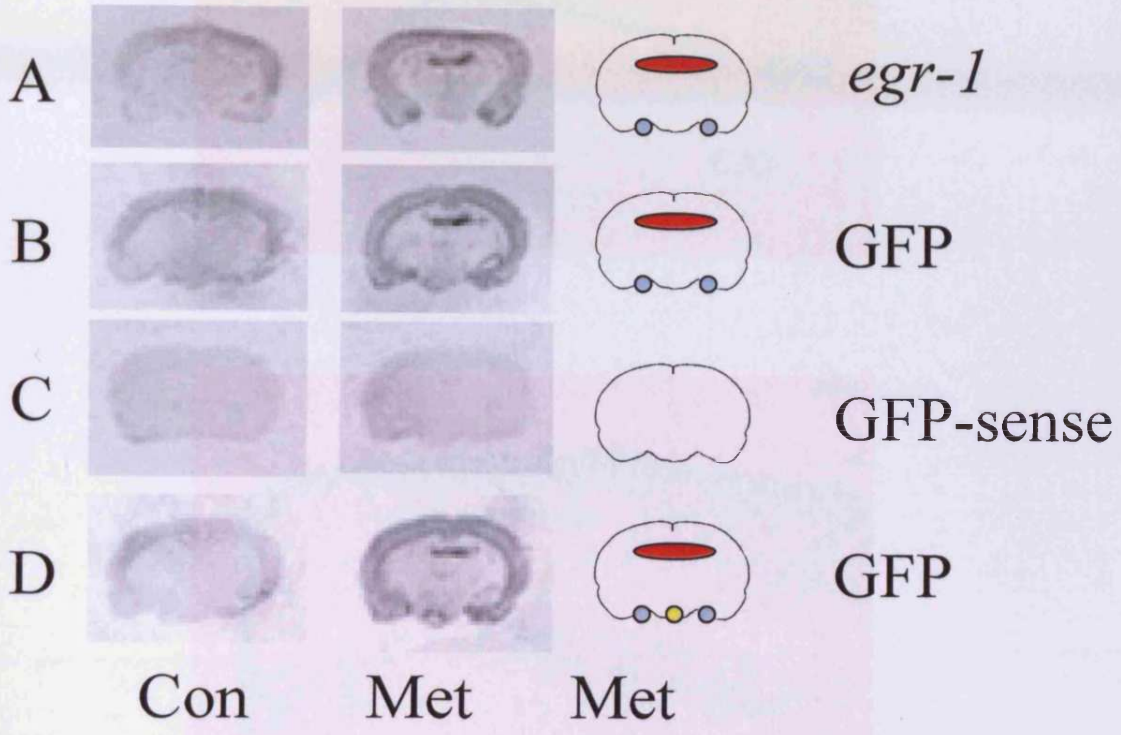
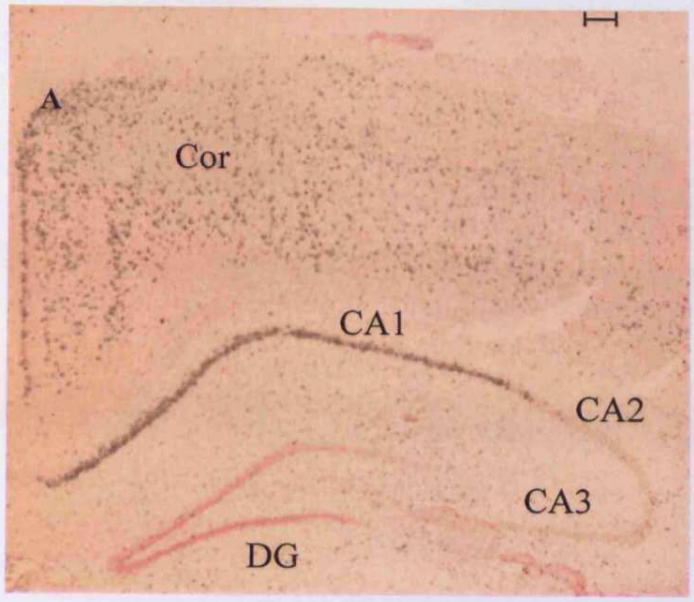


Figure 4.22 (Slade, 2001).

Localization of Egr-1/d4EGFP transgene transcript expression following pharmacological induction. *In situ* hybridization analysis of transgene mRNA expression compared with endogenous egr-1 mRNA in brain of either control (Con) or metrazole-treated (Met) transgenic (57C) rats. Fresh frozen rat brains were sectioned (12 μm) and probed with ^{35}S-labelled RNA probes specific for either transgene (GFP) or egr-1 mRNA (**A**). Similar neuronal hybridization patterns are seen in GFP- (**B** and **D**) and transgene-probed sections, with an absence of hybridization associated with the sense GFP probe (**C**). Magnification: ~ x1.
Experiment performed by Miss P. Man.



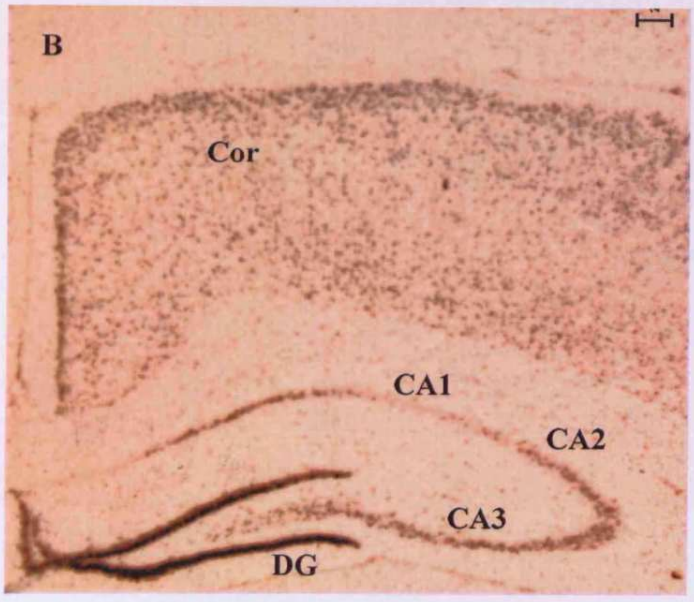


Figure 4.23 (Slade, 2001).

Pharmacological upregulation of Egr-1/d4EGFP transgene mRNA in rat brain. *In situ* hybridization analysis of transgene mRNA expression in brain of (**A**) control and (**B**) metrazole-treated transgenic (57C) rat. Fresh frozen rat brains were sectioned (12 μm) and probed with a ^{35}S-labelled RNA probe specific for transgene (GFP) mRNA. Emulsion autoradiographs were exposed for 3 weeks. Basal expression of transgene in observed in cerebral cortex (cor), and the CA1 area of the hippocampus. There is an absence of expression in both the CA2 and CA3 hippocampal areas and the dentate gyrus (DG). Minor induction of transgene in seen in cerebral cortex (cor), and the CA2 and CA3 areas of the hippocampus. Major up-regulation of transgene expression in observed in the dentate gyrus (DG).
Scale bars = 200μm. Experiment performed by Miss P. Man.

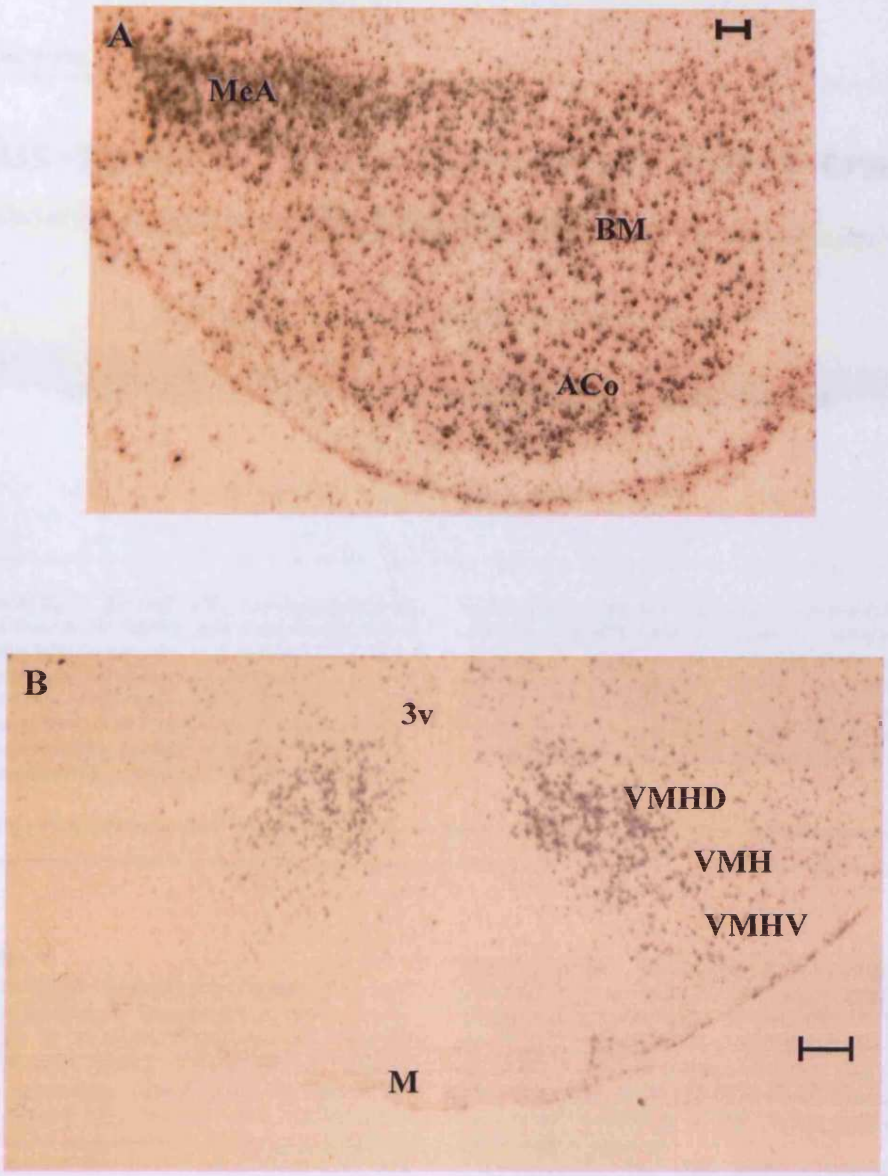


Figure 4.24 (Slade, 2001).

Cell-specific up-regulation of Egr-1/d4EGFP transgene mRNA expression in (**A**) amygdaloid complex and (**B**) ventromedial nucleus (VMN). *In situ* hybridization analysis of transgene transcript expression in brain of metrazole-treated transgenic (57C) rat. Fresh frozen rat brains were sectioned (12 μm) and probed with a ^{35}S-labelled RNA probe specific for transgene (GFP) mRNA. Emulsion autoradiographs were exposed for 3 weeks. Within the amygdaloid complex induction of transgene is observed in anterodorsal medial amygdaloid nucleus (MeAD), anterior basomedial amygdaloid nucleus (BMA) and in the anterior cortical amygdaloid nucleus (Aco). Scale bar = 100 μm. Within the VMN induction of transgene is predominantly distinguished in the dorsomedial region (VMHDM) compared with the central (VMHC) and ventrolateral (VMHVL) divisions. ME = median eminence, 3v = third ventricle. Scale bar = 200μm. Experiment performed by Miss P. Man.

Stimulus-specific induction of an *Egr-1* transgene in rat brain

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Regulated expression of Egr-1 (Zif268/NGFIA) in a variety of brain networks suggests a diversity of roles in neuronal plasticity. Here, we aimed to determine whether an *egr-1* transgene would mediate transcriptional responses to different pharmacological and physiological stimuli in the brain of transgenic rats. Constitutive transgene expression was observed in the cortex, CA1 hippocampal area and pituitary, recapitulating expression of *egr-1*. Transgene induction was stimulus-specific. Metrazole induced widespread expression in the dentate gyrus, CA2 and CA3 areas, amygdala, and ventromedial nucleus. In contrast, induction following a light stimulus was restricted to the hypothalamic suprachiasmatic and periventricular nuclei. Our studies have provided novel insights into the differential regulation of *egr-1*, and facilitated approaches to the genetic manipulation of *Egr-1*-regulated neuronal systems. *NeuroReport* 13:1–5 © 2002 Lippincott Williams & Wilkins.

Key words: Hippocampus; Light; Periventricular nucleus; Suprachiasmatic nucleus; Transgenic; Zif268; NGFIA; Ventromedial nucleus

INTRODUCTION

Egr-1 is a C_2H_2 zinc-finger transcription factor that was originally isolated in molecular screens for growth factor-inducible genes [1,2]. It is alternatively named NGFIA, Zif268, Krox24, Tis8 and ZENK depending on origin, although Egr-1 is becoming established as the most appropriate nomenclature [3]. Studies with null-mutant mice initially identified a requirement for Egr-1 only in the maintenance of fertility [3], but more recent work has now demonstrated an essential requirement for this factor in synaptic plasticity within the hippocampus [4]. However, the *egr-1* gene is induced in multiple different neuronal systems suggesting roles across the neural axis [5–8]. Functional investigation of Egr-1 within individual brain networks will require cell-specific genetic approaches, and to this end we have now investigated the induction of an egr-1 transgene in novel lines of transgenic rats. This species was selected because of the use of rats in many previous investigations [5–8], and also in our studies of *egr-1* induction within the neuroendocrine system [9–12].

MATERIALS AND METHODS

Generation and analysis of transgenic rats: A rat *egr-1/NGFIA* genomic clone (pJDM290) containing 1.58 kb. 5′ flanking sequence was extended [13] to include the egr-1 intron through the addition of 0.9 kb PCR-generated rat genomic sequence (bases 1060–1987 of Accession number J04154). A 1.1 kb fragment of pd4EGFP (Clontech, Palo Alto, CA, USA) containing enhanced GFP, destabilizing ornithine decarboxylase, and SV40 polyA sequences was then inserted downstream of the egr-1 sequence to give the transgene construct pEgr-1/d4EGFP (Fig. 1a). The full length transgene fragment was isolated with a combination of *BstXI* and *AflII* restriction enzymes, and purified for oocyte microinjection as described [14]. All animal procedures were conducted according to UK Home Office regulations, and local ethical review.

Sprague–Dawley rats were maintained in approved laboratory conditions on a 14:10 h light:dark cycle. Transgenic rats were generated as described [14], and identified by Southern analysis of *BglII*-cut tail tip genomic DNA. For transgene expression analysis, animals were killed by decapitation at appropriate times relative to experimental stimuli: 1 h after an injection of either metrazole (pentylenetetrazole 50 mg/kg, i.p.; Sigma,-Aldrich, Poole, Dorset, UK) or sterile water, or 1 h after exposure to a light pulse (24.00–01.00 h) during the dark phase.

Transgene expression analysis: Northern analysis was performed as described [15]. Blots were sequentially probed with ^{32}1P-labelled cDNA probes specific for EGFP (*NotI/SalI* fragment of pEGFP-N1), *egr-1* (*EcoRI/DraI* fragment of the rat NGFI-A cDNA [2], and 18S ribosomal RNA (DecaTemplate, Ambion, Austin, TX, USA), washed, and exposed to either a storage Phosphor screen (Kodak-K; EGFP and *egr-1*) or X-ray film (18S). *In situ* hybridization (ISH) histochemical analysis of transcript expression was performed essentially as described previously [16] using

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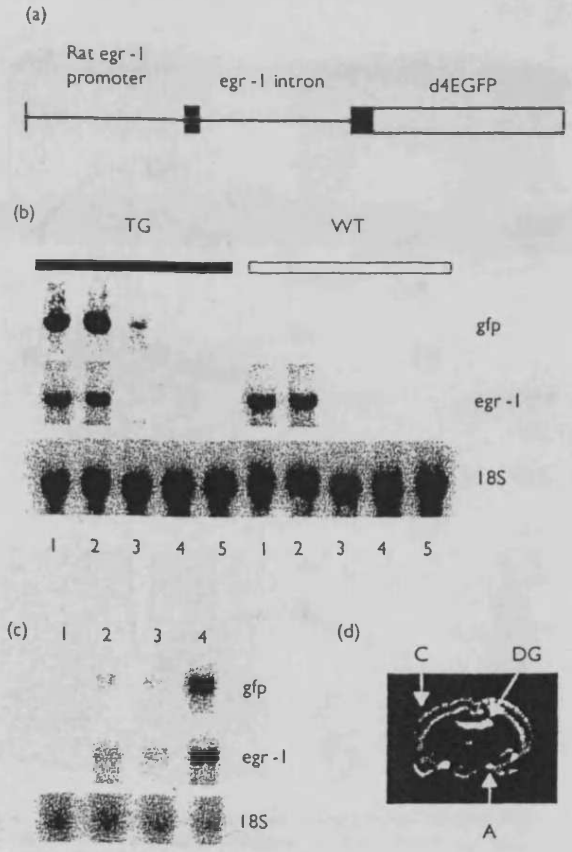

Fig. 1. Transgene construct and expression in 57C line. (**a**) The *egr-1/d4EGFP* transgene (not to scale). Black boxes represent the (truncated) *egr-1* exons. (**b**) Northern blot analysis of basal transgene and egr-1 mRNA in transgenic (TG) and wild-type (WT) rats. Total cellular RNA (10 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (GFP, 3 days and *egr-1*, 2 days) or X-ray film (18S, 4 h). 1. cerebral cortex; 2. anterior pituitary ; 3. liver; 4. kidney; 5. heart. (**c**) Northern blot analysis of metrazole-regulated transgene and egr-1 expression in transgenic rats. Total cellular RNA (15 μg, adrenal; 10 μg, hippocampus) was probed as above. Blots were exposed to either a Phosphor screen (GFP and *egr-1*, 2 days) or X-ray film (18S, 1 h). 1. whole adrenal gland control; 2. whole adrenal gland, metrazole -treated; 3. hippocampus, control; 4. hippocampus, metrazole-treated. (D) ISH analysis (as Fig. 2) of transgene expression in metrazole-treated 57C rat brain showing neuron-specific expression in dentate gyrus (DG), cerebral cortex (C), and amygdala (A). Also note expression in ventral CA3 hippocampal sub-field (detected only on right-hand side in this section) Inverted phosphor screen image (3 day exposure). Approx. magnification: × 1.

antisense and sense ^{35}S-labelled RNA probes transcribed using the Riboprobe system (Promega, Madison, WI, USA). Sequences used were, for transgene (EGFP) mRNA, a 426 bp fragment (bases 971–1396) of pd4EGFP (Clontech), and for *egr-1*, a 726 bp fragment (bases 228–953) of the NGFI-A cDNA [2]. Initial ISH images were obtained by Phosphor screen exposure. Subsequently developed emulsion autoradiographs were counter-stained, viewed with a Leica DM-RD microscope, and images were captured using a Spot camera (1.30) and Spot Advanced Image software (Spot software 2.2; Diagnostic Instruments, Sterling Heights, MI, USA), imported into Adobe Photoshop (4.0) and rendered gray-scale as appropriate. Unless otherwise stated, all determinations of transgene expression were confirmed with duplicate experimental animals.

RESULTS

The rat *egr-1* transgene was incorporated into the genome of five founder rats. Four transgenic lines derived from these founders exhibited abundant, tissue-specific transgene expression (1.6 kb transcript) in the cerebral cortex and anterior pituitary gland (Fig. 1b), recapitulating expression of the endogenous gene (Fig. 1b). Low abundance expression was also observed in the liver (Fig. 1b). A single transgenic line did not exhibit (Northern blot) detectable expression, presumably reflecting transgene integration into a repressive area of the genome. One of the expressing lines, designated 57C, was selected for further analysis. ISH analysis of constitutive transgene expression in 57C brains revealed (antisense probe-specific) expression in both the retrosplenial and parietal cortex with an abundance of hybridizing neurons in layers II, IV and VI (Fig. 2a). Transgene expression was also observed in the CA1, but not other subfields of the hippocampus (Fig. 2a). Other areas of the brain did not exhibit constitutive transgene expression, apart from minor expression in cells associated with superficial blood vessels (not shown). A similar pattern of expression was observed for the endogenous egr-1 gene (not shown).

The capacity of the *egr-1* transgene sequences to mediate transcriptional up-regulation in response to a (generalized, pharmacological) neural stimulus was confirmed using the convulsant metrazole, which was found to increase transgene (and *egr-1*) expression in both the hippocampus and adrenal gland (Fig. 1c). Despite the presence of readily detectable transgene transcripts in the 57C line, fluorescence microscopy of brain sections did not reveal GFP fluorescence. Cell-type specific upregulation of the transgene in brain was therefore investigated with ISH (Fig. 1d, Fig. 2). Following metrazole treatment, the most prominent increase in transgene expression, compared with control animals, was observed in the dentate gyrus (Fig. 1d, Fig. 2b), but increased expression was also found in the CA2 and CA3 subfields of the hippocampus (Fig. 2b), in retrosplenial, parietal (Figs. 1d, Fig. 2b) and perirhinal cortex (Fig. 1d), amygdala (Fig. 1d, Fig. 2c), and ventromedial (VMN, Fig. 1d Fig. 2d), but not other hypothalamic nuclei. In the latter two areas, expression was particularly prominent in the anterodorsal medial amygdaloid nucleus (Fig. 2c), and in the dorsomedial region of the VMN (Fig. 2d). In general, ISH analysis of transgene expression revealed localized, high-level expression in specific groups of neurons with an absence of expression in many brain areas (Fig. 1d). Similar observations were made for *egr-1* mRNA (not shown).

In a second series of experiments, the capacity of *egr-1* transgene sequences to mediate transcriptional up-regulation in response to a physiological stimulus was investigated using an established circadian, light-pulse paradigm. Following this stimulus, a discrete induction of transgene

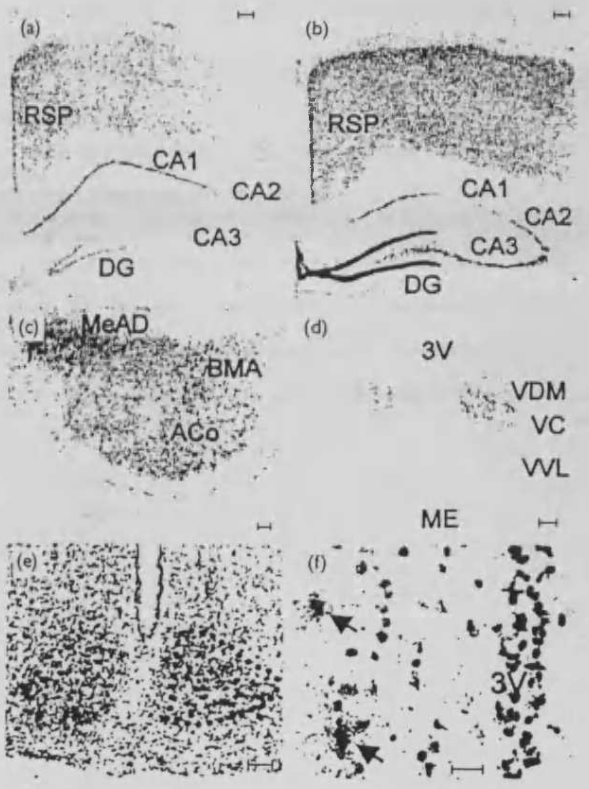

Fig. 2. Cell-specific basal expression, and up-regulation of transgene in 57C rat brain. ISH analysis of transgene in either control (**a**), metrazole-treated (**b–d**) or light-pulse stimulated (**e,f**) rats. Brains were sectioned (12 μM) and probed with a ^{35}S-labelled RNA probe specific for transgene mRNA. Emulsion autoradiographs were exposed for 3 weeks. Following development, sections were photographed under bright field, and rendered gray-scale (a–d only). (a) Basal expression of transgene in both cerebral cortex (including retrosplenial, RSP), and CA1 hippocampal area. Note absence of expression in both CA2 and CA3, and the dentate gyrus (DG). (b) Metrazole-induced expression in hippocampus and cortex. Note relatively minor induction of transgene in both RSP, and CA2-3 areas of the hippocampus compared with major up-regulation in DG. (c) Metrazole-induced expression of transgene in amygdaloid nuclei. Note induction of transgene in anterodorsal medial amygdaloid nucleus (MeAD), anterior basomedial amygdaloid nucleus (BMA) and in the anterior cortical amygdaloid nucleus (Aco). (d) Metrazole-induced expression in ventromedial nucleus of the hypothalamus. Note induction of transgene principally in the dorsomedial region (VDM) compared with the central (VC) and ventrolateral (VVL) divisions. 3V, third ventricle; ME, median eminence. (e) Light-induced transgene expression in suprachiasmatic nucleus. Note that mRNA is detected throughout the SCN (demarkated with dashed line). (f) Light-induced transgene expression in individual periventricular neurons (arrowed). Bars = 200 μM (a,b,d), 100 μM (c,e), 20 μM (f).

was observed throughout the suprachiasmatic nucleus (SCN; Fig. 2e), and in a subset of neurons within the hypothalamic periventricular nucleus (PeN; Fig. 2f). Both of these observations mirrored the light-induced expression of *egr-1* (not shown). The light stimulus was not associated with transgene induction in other areas of the neural axis including the hippocampus and cortex (not shown), and SCN/PeN induction was not observed in non-stimulated animals sampled at 01.00 h.

DISCUSSION

Our novel transgenic rat model has firstly demonstrated that sequences within the proximal 5′-flanking and intronic regions of the rat egr-1 gene are sufficient to mediate stimulus-induced expression within the brain. More importantly, the transcriptional up-regulation that is inferred from these observations has been shown to be stimulus-specific; discrete SCN and PeN induction by a nocturnal light pulse contrasts with the cortical, forebrain and VMN activation of a pharmacological stimulus. In further studies, it will be interesting to observe whether transgene expression in the latter collection of brain areas can also be selectively induced by different specific stimuli. For example, the novel pattern of VMN induction observed here, which is not mirrored by *egr-1* during stress [5] may be selectively regulated during mating stimuli [7]. Amygdala-specific induction during fear-memory retrieval is another possibility [6]. In addition to providing correlative evidence of Egr-1 function within specific areas, the demarcation of regulation within discrete cell groups also provides for conditional transgenesis models in which transgene promoter-linked ORFs (e.g. an Egr-1 dominant-negative) [17] may be expressed in a stimulus-specific manner. In the paradigm demonstrated here, a non-invasive light stimulus is shown to have the potential for driving functional transgene expression in the SCN and PeN. The regulation and role of Egr-1 is of particular interest with respect to the PeN in which Egr-1 is co-localized with somatostatin within hypophysiotrophic neurons [8,12].

Our findings have furthermore confirmed and extended a previous study in mice [18] which showed that proximal regions of the egr-1 gene confer appropriate constitutive expression in transgenic animals. An apparent exception to this finding, namely the minor transgene expression in liver, in fact reflects a variable and regulated expression of egr-1 in hepatocytes [18]. In the brain, the fidelity of transgene-regulated constitutive expression is particularly well documented here in the hippocampal formation in which restriction of a high level of basal expression of transgene transcripts selectively to the CA1 subfield reflects a similar pattern of constitutive *egr-1* expression (present study; [5]). The relative importance of intronic egr-1 sequences (absent in the mouse transgene) [18] in confering both basal and regulated transgene expression [16] must await further analysis. Similarly, the absence of detectable GFP fluorescence here despite robust transcription of the transgene requires additional investigation, including a full analysis of transgene activity *in vitro*, before this deficit can be explained.

CONCLUSIONS

Our demonstration of stimulus-specific neuronal induction of an *egr-1* transgene is timely with respect to the recent definition of a role for *egr-1* in neuronal plasticity [4]. Our findings facilitate the development of functional transgenes that can be used to address the role of specific substrates of

adaptive plasticity in different neurophysiological and behavioural paradigms.

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Oestrogenic regulation of an *egr-1* transgene in rat anterior pituitary

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Abstract

The C_2H_2 zinc-finger transcription factor Egr-1 has previously been shown to play an essential role within the endocrine system as a molecular determinant of LH β-subunit synthesis in anterior pituitary gonadotrophs. The extent to which Egr-1 may be a dynamic mediator of changes in gonadotroph function during the oestrous cycle is currently unclear. We have recently produced a novel rat transgenic model of *egr-1* gene function in which proximal regions of the rat *egr-1* gene drive expression of a reporter gene. In the present study, we have investigated the expression and physiological regulation of our *egr-1/d4* enhanced green fluorescent protein (EGFP) transgene in the female rat pituitary gland. *In situ* hybridization analysis has revealed anterior pituitary-specific expression that is limited to a sub-population of cells that includes immunohistochemically defined gonadotrophs. Expression of the transgene is up-regulated 5-fold following ovariectomy. The transgene also exhibits regulated expression during the oestrous cycle, mRNA levels being significantly raised on pro-oestrus. Using an explant culture system, we have also demonstrated a direct stimulatory effect of 17β-oestradiol on anterior pituitary transgene and *egr-1* expression. The acute response of *egr-1* to an oestrogenic stimulus is attenuated by the MEK (MAPK kinase) inhibitor U0126, and is accompanied by increased levels of phospho-p44/42 MAPK protein, suggesting regulation of *egr-1* through a MAPK-linked pathway in the pituitary. These findings provide further evidence of cyclical endocrine regulation of *egr-1* in the rat, demonstrate that proximal sequences of the *egr-1* gene mediate endocrine-regulated expression, and indicate a novel pathway through which pituitary transcriptional responses to oestrogen may be mediated.

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Introduction

Early growth response gene-1 (*egr-1*; also termed *NGFI-A*, *zif268*, *krox-24*) is one member of a transcription factor gene family that includes three related genes (*egr-2, 3* and *4*; see O'Donovan *et al.* 1999). Extensive analysis of the roles of the DNA-binding protein Egr-1 has revealed important, and unique, roles in fertility (Lee *et al.* 1996, Topilko *et al.* 1998), memory (Jones *et al.* 2001), and the ischaemic stress response (Yan *et al.* 2000). The first of these roles is of major relevance to endocrinology, because fertility is Egr-1-dependent due to transcriptional regulatory activity of Egr-1 on the pituitary hormone gene that codes for the luteinizing hormone (LH) β-subunit (Lee *et al.* 1996, Topilko *et al.* 1998; see also Dorn *et al.* 1999, Tremblay & Drouin 1999, Wolfe & Call 1999, Sevetson *et al.* 2000, Duan *et al.* 2002). Recent studies have shown that Egr-1 may also affect fertility through regulation of LH receptor gene expression (Topilko *et al.* 1998, Yoshino *et al.* 2002).

The functional association between Egr-1 and the reproductive axis has been explored mainly at the developmental level using null-mutant mice (Lee *et al.* 1996, Topilko *et al.* 1998), but our recent studies have provided evidence of an additional association, at a physiological level. Thus, we have described cyclical changes in anterior pituitary Egr-1 activity that correlate with cyclical changes in the reproductive axis in adult female rats (Slade & Carter 2000). In other studies (Knight *et al.* 2000), we have shown that the nuclear 75 kDa form of Egr-1 protein is primarily co-localized with LH

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β-subunit-expressing cells in adult rat anterior pituitary. Taken together, these findings indicate that Egr-1 might play a role in mediating changes in gonadotroph function during the rat oestrous cycle, possibly contributing to the cyclical changes in LH release (Butcher *et al.* 1974, Fink 1979). This potential role is obscure, however, because gonadotroph function is regulated at multiple levels, and many different molecular factors have now been shown to be associated with this axis (see Brown & McNeilly 1999). Currently there is no direct evidence to link physiological changes in Egr-1 expression (Slade & Carter 2000) with changes in gonadotroph gene (LH β-subunit, or otherwise) expression because the null-mutant genetic models (Lee *et al.* 1996, Topilko *et al.* 1998) lack Egr-1 prior to the establishment of reproductive cycles, and adult-onset genetic models have yet to be developed. Consequently, further studies are required to address the physiological role of Egr-1 in the pituitary.

We have recently begun an investigation of *egr-1* promoter activity with the aim of developing new *in vivo* genetic models. Our transgenic model has demonstrated that 1·6 kb of the rat *egr-1* promoter, together with the *egr-1* intron, can direct cell-specific, and regulatable expression in the brain of rats (Slade *et al.* 2002). In the present study, we have used this model to investigate transgene expression in the anterior pituitary. The use of a promoter–reporter transgenic approach is justified because analysis of transgene expression will inform us of the extent to which the transgene can recapitulate expression of the endogenous *egr-1* gene. The consequent insights into the (*in vivo*) regulatory capacity of the promoter region used in the transgene may, furthermore, have practical applications in the design of secondary transgenic models in which the function of Egr-1 in the pituitary may be addressed through transgenic targeting. Additionally in this study, we have also investigated the physiological factors that determine both transgene and endogenous *egr-1* expression in the anterior pituitary.

Materials and methods

Animal procedures

All animal procedures were conducted according to UK Home Office regulations, and local ethical review. Sprague–Dawley rats were maintained in approved laboratory conditions on a 14 h light:10 h darkness cycle. Transgenic rats of the 57C line (Slade *et al.* 2002) were identified by Southern analysis of BglII-cut genomic DNA using an enhanced green fluorescent protein (EGFP) sequence-specific probe (1·4 kb HindIII fragment of pd4 EGFP; Slade *et al.* 2002). In one experiment, transgenic rats were either ovariectomized using standard surgical procedures and injected with vehicle (s.c. sesame oil, daily on days 8–10 after surgery), ovariectomized and injected with 17β-oestradiol (E_2) (Sigma) (20 μg/100 g body weight s.c.) or sham-ovariectomized. Following surgery, rats were maintained for 10 days prior to killing. All other experimental rats were killed on particular days of the oestrous cycle as determined by vaginal cytology (dioestrus day 2 for organ culture experiments; pro-oestrus for *in situ* hybridization (ISH) analysis). Animals were killed at 1200 h by decapitation. Anterior pituitary glands were rapidly dissected and rinsed in ice-cold saline before freezing on dry ice prior to subsequent RNA extraction. Alternatively whole pituitary glands were snap-frozen in isopentane cooled with dry ice pellets prior to ISH analysis. Unless otherwise stated, all determinations of gene and protein expression were confirmed with duplicate experimental animals or organ cultures.

Transgene expression analysis

Total RNA extraction and Northern analysis of transgene and *egr-1* transcript expression was performed as described (Slade *et al.* 2002). Blots were sequentially probed with ^{32}P-labelled cDNA probes specific for EGFP (NotI/SalI fragment of pEGFP-N1), *egr-1* (EcoRI/DraI fragment of the rat NGFI-A cDNA (Milbrandt 1987)) and 18S ribosomal RNA (DecaTemplate, Ambion, Austin, TX, USA), washed, and exposed to either a Phosphor screen (Kodak-K) (EGFP and *egr-1*) or X-ray film (18S). Densitometric analysis of mRNA levels between groups was performed using Imagequant software (Amersham Pharmacia Biotech).

ISH histochemical analysis of transgene transcript expression was performed as described (Slade *et al.* 2002) using antisense and sense ^{35}S-labelled RNA probes transcribed using the Riboprobe system (Promega). As reported previously (Slade

et al. 2002), ISH was used for transgene expression analysis because, despite robust transcription of the transgene, GFP fluorescence deriving from the transgene reporter is not readily detectable. The transgene-specific ISH probe was derived from a plasmid containing 426 bp of pd4 EGFP (bases 971–1396; Clontech, Palo Alto, CA, USA) in pGEM-11Z (Promega). Probe hybridization (1×10^7 c.p.m./ml) was performed overnight at 56 °C, prior to washing and exposure to a storage phosphor screen (Kodak-K) for 3 days, and subsequent coating in photographic emulsion (LM-1; Amersham Pharmacia Biotech). After 4 weeks, coated slides were developed, counterstained (Mayer's haematoxylin), and viewed with a Leica DM-RD microscope. Images were captured using a Spotcamera (1·30) and Spot Advanced Image software (Spot software 2·2; Diagnostic Instruments, Sterling Heights, MI, USA), imported into Adobe Photoshop (4·0).

Dual ISH/immunocytochemistry was performed using a previously described protocol (Allen *et al.* 1997, Morgan *et al.* 2000) adapted for gonadotrophs. All solutions used in the initial immunocytochemical step were prepared in diethylpyrocarbonate-treated water, and also contained 0·1 U/μl RNasin ribonuclease inhibitor (Promega). Sections (8 μm) of 57C rat pituitary were post-fixed in 4% paraformaldehyde in PBS for 5 min, and permeabilized in methanol (−20 °C, 2 min). LH β-subunit immunoreactivity was detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol, using 3,3′-diaminobenzidine as chromogen. The primary antisera to LH β-subunit (NIDDK-anti-rbetaLH-IC-2; AFP22238790 GPOLHB) was diluted 1:1000. Following development of the chromogen, slides were washed, dried and stored at −70 °C prior to ISH analysis (see above). Following development of the photographic emulsion, sections were counterstained with Mayer's haematoxylin.

Explant culture of rat anterior pituitary

Explant culture of rat anterior pituitary fragments was performed as described previously (Carter *et al.* 1993) with some modifications. The media used was phenol red-free DMEM (Life Technologies) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 °C for 24 h prior to experimental stimuli. Drugs were diluted into the medium to give appropriate final concentrations of 10 nM E_2 (Sigma) and 10 μM MEK (MAPK kinase) inhibitor U0126 (New England Biolabs, Beverly, MA, USA). Control cultures received similar amounts of drug vehicle (ethanol or DMSO).

Immunoblot analysis

Western analysis of p44/42, phospho-p44/42 (ERK1/2 (extracellular signal-regulated kinases)), and phospho-p38 MAPK was performed on whole cell anterior pituitary extracts using a protocol described by the manufacturer of the primary antisera (New England Biolabs). Protein concentrations were determined by a dye-binding method (Bradford 1976), and mass estimates were obtained with reference to Broad Range Protein Markers (New England Biolabs). Primary and secondary (anti-rabbit IgG, horseradish peroxidase-linked) antisera used were used at 1:1000 and 1:2000 dilutions respectively. Protein bands were detected by chemiluminescence (Luminol reagent; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative level of protein bands were compared using densitometric analysis (Imagequant, see above).

Results

ISH histochemical analysis demonstrated that the antisense GFP (transgene-specific) probe hybridized to a sub-population (approximately 10–15%) of anterior pituitary cells (Fig. 1A). The cells exhibiting hybridization were often associated into small groups (Fig. 1A). The aggregations of silver grains that define transgene mRNA expression were restricted to the anterior lobe of the pituitary gland (see Fig. 1C). In control experiments, using a sense GFP probe, there was no evidence of hybridization above background either in the anterior pituitary (Fig. 1B) or in other lobes of the gland (not shown). Immunocytochemical analysis of transgenic rat pituitary glands using a previously characterized (Knight *et al.* 2000) LH β-subunit antiserum revealed a sub-population of β-subunit-expressing cells in the anterior pituitary gland (Fig. 1D). Dual ISH/immunocytochemistry (Allen *et al.* 1997, Morgan *et al.* 2000) using the LH β-subunit

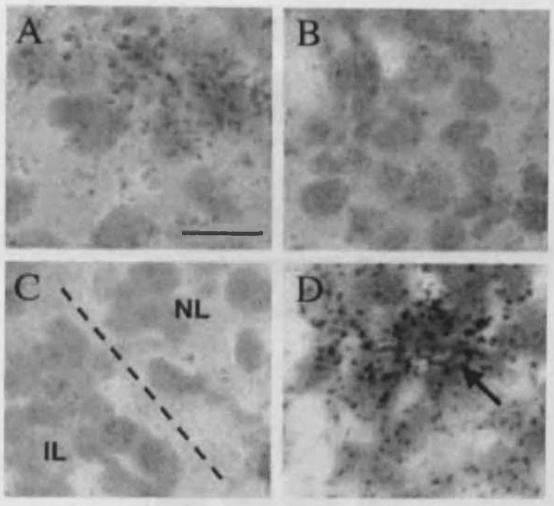

Figure 1 Anterior pituitary-specific expression of *egr-1/d4* EGFP transcripts in 57C transgenic rats. ISH histochemical analysis of transgene transcript expression in the pituitary of a female transgenic rat killed on the day of pro-oestrus. Fresh-frozen glands were sectioned (12 μm) and probed with a ^{35}S-labelled RNA probe specific for transgene (GFP) mRNA. Emulsion autoradiographs were exposed for 3 weeks. (A) Representative image of anterior lobe cells probed with antisense GFP probe. Note the presence of a group of cells associated with aggregated silver grains, and other cells associated with a background distribution of silver grains. (B) Representative image of anterior lobe cells probed with sense GFP probe. (C) Representative image of the neural (NL)–intermediate (IL) lobe boundary (dashed line) probed with the antisense probe. (D) Representative image of anterior lobe cells probed with both an LH β-subunit antisera and the antisense GFP probe. Arrow indicates the association of an aggregation of silver grains with cells exhibiting the orange-brown staining that is representative of LH β-subunit immunoreactivity. In this grey-scale image, the aggregated silver grains associated with the LHβ-subunit staining are apparent as blacker grains compared with grains not associated with LHβ-subunit staining. Scale bar=15 μm.

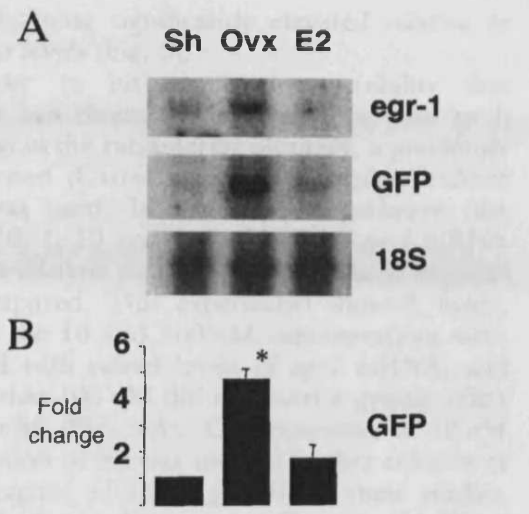

Figure 2 Regulation of *egr-1/d4* EGFP transgene, and *egr-1* transcript expression in 57C rat anterior pituitary gland following ovariectomy, and oestrogen replacement. (A) Representative Northern blot analysis of transgene, and endogenous *egr-1* mRNA in sham-operated (Sh), ovariectomized (Ovx) and ovariectomized, E_2-treated (E2) rats. Immobilized total cellular RNA extracted from individual glands (12 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed to a Phosphor screen (Kodak-K). Exposure times were 3 days (GFP and *egr-1*) and 10 min (18S). (B) Histogram showing densitometric analysis of multiple GFP Northern blots, as described in (A). Levels of transgene mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level for sham-operated animals (means±S.E.M.). *P<0·05 compared with other groups (ANOVA followed by Duncan's multiple range test). n=4 (Sh and Ovx) and 3 (E2) pituitary glands in each group.

antiserum in combination with the antisense GFP probe revealed co-localization of LH β-subunit immunoreactivity with transgene mRNA (Fig. 1D). The distribution and abundance of silver grain aggregations in the dual analysis sections was similar to that observed for single ISH analysis (Fig. 1A), indicating that the specificity of GFP probe hybridization was not compromised in the dual analysis procedure. Co-localization was observed in the majority of LH β-subunit-expressing cells; however, a sub-population (approximately 10%) of β-subunit-positive/GFP mRNA-negative cells was also observed. Conversely, a similar sub-population of GFP mRNA-positive/β-subunit-negative cells was also found, indicating, firstly, that the aggregations of silver grains are not non-specifically associated with immunopositive cells, and secondly, that the transgene is also expressed in a small population of cells other than gonadotrophs.

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to physiological (endocrine) stimuli was investigated by observing transgene mRNA levels in ovariectomized rats (Fig. 2), and in rats sampled during the four days of the oestrous cycle (Fig. 3). Following ovariectomy, we found a

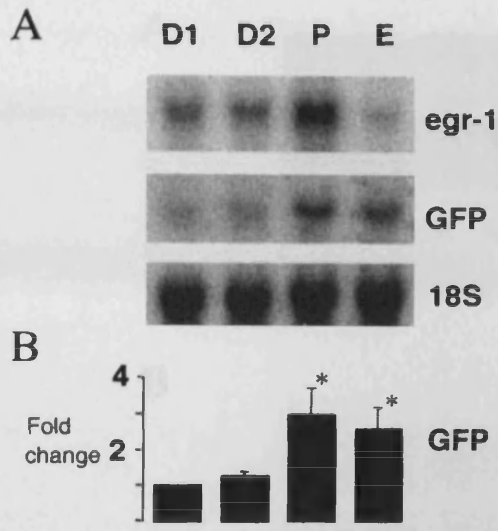

Figure 3 Regulation of *egr*-1/d4 EGFP transgene transcript expression in 57C rat anterior pituitary glands during the oestrous cycle. (A) Representative Northern blot analysis of transgene mRNA compared with endogenous *egr-1* mRNA. Immobilized total cellular RNA extracted from paired glands (12 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; GFP and *egr-1*) or X-ray film (18S). Exposure times were 2 days (GFP, *egr-1*) and 1 h (18S). Samples loaded were: D1, dioestrus day 1; D2, dioestrus day 2; P, pro-oestrus; E, oestrus. (B) Histogram showing densitometric analysis of multiple GFP Northern blots, as described in (A). Levels of transgene mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level for D1 (means±S.E.M.). *P<0·05 compared with D1 (ANOVA followed by Duncan's multiple range test). n=3 samples of paired pituitary glands in each group.

marked and significant (P<0·05) elevation of transgene mRNA levels in the anterior pituitary (Fig. 2); this response was reversed by treatment with E_2 (Fig. 2). These responses of the transgene were shown to recapitulate regulation of the endogenous *egr-1* mRNA (Fig. 2; Slade & Carter 2000). In intact rats also, we observed regulated expression of transgene mRNA; a significant (P<0·05) up-regulation of transgene mRNA levels was observed on pro-oestrus (Fig. 3), mirroring an endogenous *egr-1* mRNA response (Fig. 3). Transgene mRNA levels were generally observed to be reduced on the subsequent day of oestrus, but the reduction was not as great as that observed for endogenous *egr-1* transcripts, transgene mRNA levels remaining significantly elevated relative to dioestrous levels (Fig. 3).

In order to investigate the possibility that oestrogen can directly affect transgene (and *egr-1*) expression in the rat anterior pituitary, a previously characterized (Carter *et al.* 1993) explant culture model was used. In an initial experiment, the effects of 0, 1, 10 and 100 nM E_2 on *egr-1* mRNA levels in wild-type anterior pituitary gland explants were compared. This experiment showed, firstly, that only the 10 and 100 nM concentrations were associated with raised levels of *egr-1* mRNA, and secondly that 100 nM did not exert a greater effect than 10 nM (Fig. 4A). Consequently, a 10 nM concentration of E_2 was used in further cultures of 57C transgenic pituitary glands. In these studies, 10 nM E_2 was shown to effect a significant elevation of both *egr-1* (Fig. 4B) and transgene (Fig. 4C) mRNA. However, the time course of these effects differed in that the elevation of *egr-1* mRNA was transient, falling significantly at 60 min relative to the 30 min time point (Fig. 4B), whereas the levels of transgene mRNA remained significantly elevated at 60 min (Fig. 4C). The rapid, and transient effects of E_2 on *egr-1* expression are suggestive of signal transduction via the MAPK pathway (see Discussion).

Consequently, an additional series of *in vitro* experiments were conducted using a specific inhibitor of this pathway, and it was shown that the MEK inhibitor U0126 effected a significant inhibition of the stimulatory effect of E_2 on anterior pituitary *egr-1* mRNA levels (Fig. 5A and B). In a further study, the effect of oestrogens on anterior pituitary protein kinases was examined, and it was shown that E_2 treatment was associated with significantly raised levels of the phospho-p44/42 (ERK1/2) MAPK (Fig. 5C and D), but did not change levels of phospho-p38 MAPK (Fig. 5C).

Discussion

Previous analysis of our *egr-1* transgenic rat model (Slade *et al.* 2002) showed that the *egr-1*/d4 EGFP transgene confers both tissue-specific constitutive expression and inducible expression in the brain. These findings paralleled the results obtained with a recently generated transgenic mouse model (Tsai *et al.* 2000) in which 5′-flanking sequences of the murine *egr-1* gene were shown to direct both

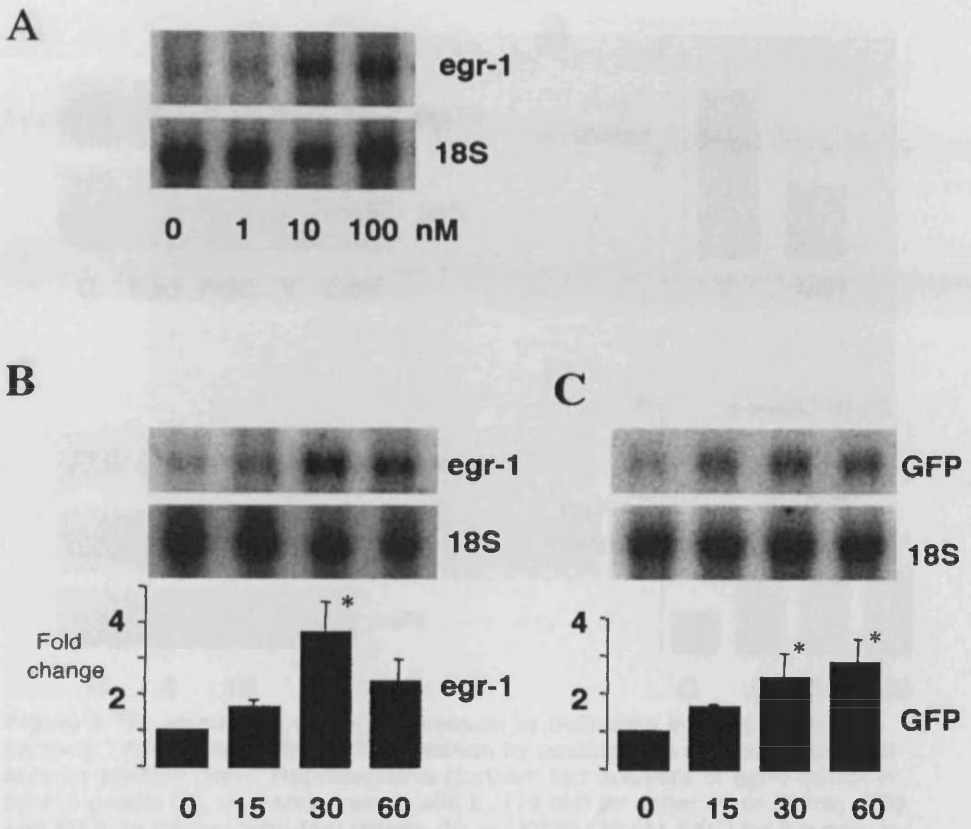

Figure 4 Regulation of *egr-1* and *egr-1*/d4 EGFP transgene expression by oestrogen in explant cultured 57C rat anterior pituitary gland. (A) Representative Northern blot analysis of *egr-1* mRNA in extracts of glands treated with E_2 (0, 1, 10 and 100 nM) for 30 min. Immobilized total cellular RNA extracted from paired glands (6 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; *egr-1*) or X-ray film (18S). Exposure times were 3 days (*egr-1*) and 1 h (18S). (B and C) Representative Northern blot analyses of (B) *egr-1* mRNA and (C) transgene mRNA in extracts of glands treated with E_2 (10 nM) for 0, 15, 30 or 60 min. Immobilized total cellular RNA extracted from paired glands (6 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed as in (A). Below each Northern blot are corresponding histograms that show densitometric analysis of multiple blots (n=3 individual explant cultures/group). Levels of mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level at time 0 (means±S.E.M.). *P<0·05 compared with time 0 (ANOVA followed by Duncan's multiple range test).

constitutive expression in brain, heart and liver, and hepatectomy-associated gene induction in liver. In the present study, we have now shown that sequences within the rat *egr-1*/d4 EGFP transgene confer region-specific, and physiologically regulated expression in the rat pituitary gland. Each of these aspects of transgene expression mirror expression of the endogenous *egr-1* gene (see Slade & Carter 2000, Tsai *et al.* 2000, Slade *et al.* 2002). Accordingly, the body of data that is emerging from these transgenic studies argues that proximal sequences within the *egr-1* gene are sufficient to recapitulate, at least broadly, expression of the endogenous *egr-1* gene in rodents. Taken together with extensive *in vitro* evidence of the regulatory capacity of the *egr-1* proximal 5′-flanking sequence (Changelian *et al.* 1989, Sakamoto *et al.* 1991, DeFranco *et al.* 1993, Cohen *et al.* 1996, Yan *et al.* 1999, Bernal-Mizrachi *et al.* 2000), it can be further argued that this region alone is sufficient

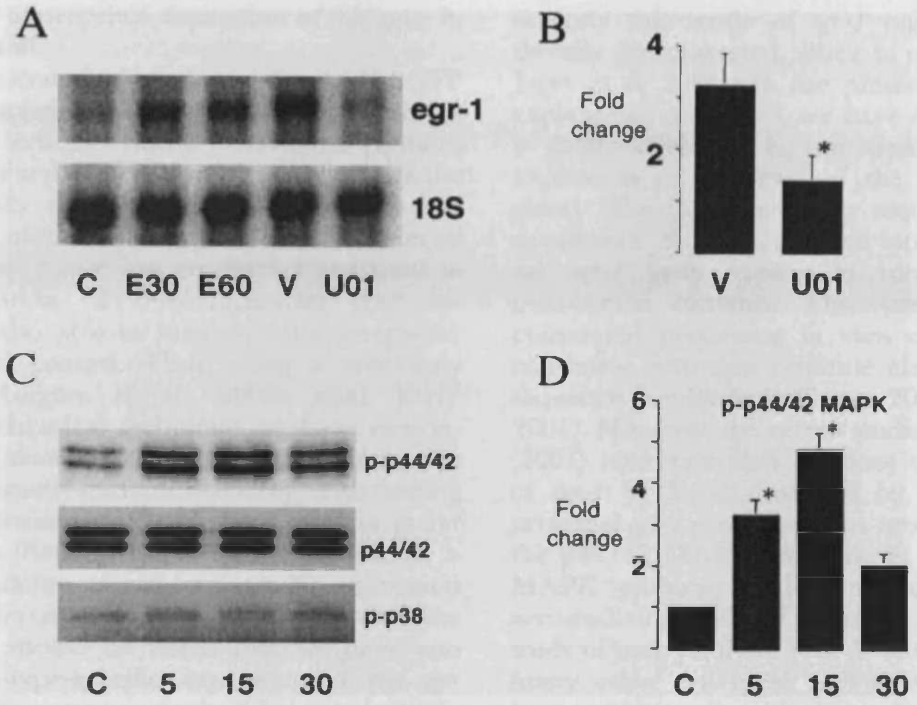

Figure 5 The stimulation of *egr-1* expression by oestrogen involves the MAPK pathway. (A) Regulation of *egr-1* expression by oestrogen in explant cultured rat anterior pituitary gland. Representative Northern blot analyses of *egr-1* mRNA in control glands (C), or glands treated with E_2 (10 nM) for either 30 or 60 min (E30 and E60), or treated with drug vehicle (V) or U0126 (10 μM, U01) for 2 h prior to treatment with E_2. Immobilized total cellular RNA extracted from individual explant cultures (6 μg/lane) was probed sequentially with ^{32}P-labelled cDNA probes specific for *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; GFP and *egr-1*) or X-ray film (18S). Exposure times were 3 days (*egr-1*) and 1 h (18S). (B) Histogram showing densitometric analysis of multiple blots as in (A) (n=3 individual explant cultures/group). Levels of mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level in control cultures (means±S.E.M.). *P<0·05: U0126 vs vehicle (ANOVA followed by Duncan's multiple range test).
(C) Regulation of phospho-p44/42 MAPK by oestrogen in explant cultured rat anterior pituitary gland. Representative Western blot analyses of whole cell protein extracts (15 μg/lane) derived from either control (C) glands, or glands treated with E_2 (10 nM) for either 5, 15 or 30 min. p-p44/42, phospho-p44/42 MAPK; p44/42, p44/42 MAPK; p-p38, phospho-p38 MAPK. (D) Histogram showing densitometric analysis of multiple blots as in (C) (n=3 individual explant cultures/group). Levels of phospho-p44/42 MAPK were corrected against the equivalent level of p44/42 MAPK, and expressed as fold difference over the level in control cultures (means±S.E.M.). *P<0·05 compared with controls (ANOVA followed by Duncan's multiple range test).

to confer an appropriate pattern of expression. However, the presence of consensus, albeit functionally unproven, *cis*-acting element sequences within the *egr-1* intron (incorporated in the rat, but not the mouse transgene) that include a core serum response element (SRE) at base 1444, and an AP-1-like element at base 1682 of the rat *egr-1* gene (Acc. No. J04154; Changelian *et al.* 1989) suggests that a more extensive analysis of the contribution of the *egr-1* intron sequences should be conducted. In this context, our transgenic studies of the melatonin-regulating *arylalkylamine N-acetyltransferase* gene (Burke *et al.* 1999) have demonstrated an important role of intronic

sequences for appropriate expression of this gene in the pineal gland.

The demonstrated restriction of Egr-1/d4 EGFP transgene expression to cells of the anterior lobe of the pituitary indicates that the transgene contains sufficient regulatory information to discriminate the embryologically distinct cells of the neural and intermediate pituitary lobes. Within the anterior lobe, transgene transcripts are further restricted to a sub-population of cells indicating that the transgene is also able to mediate cell-type-specific transcriptional control. Thus, using a previously validated (Morgan *et al.* 2000) dual ISH/immunohistochemical technique we have demonstrated co-localization of the transgene transcript with LH β-subunit immunoreactivity. This finding reflects our demonstration of Egr-1 protein in rat gonadotrophs (Knight *et al.* 2000), and indicates a remarkable fidelity of cell-type-specific expression that is conferred by *egr-1* sequences in the transgene. It should be noted that we have also observed cell-type-specific expression of the *egr-1*/d4 EGFP transgene in brain (Slade *et al.* 2002). The full extent of the cellular specificity of transgene expression in the anterior pituitary will, however, require considerable further analysis in which, firstly, the small population of transgene-positive/LH β-subunit-negative cell type(s) are characterized, and secondly the co-localization of transgene and *egr-1* transcripts is quantified. It is apparent from our results, however, that the former population of cells is limited in number and would not appear to constitute a major anterior pituitary cell type. A similar distribution of expression is observed for Egr-1 protein (see Knight *et al.* 2000).

Our finding that transgene mRNA levels are up-regulated following ovariectomy, in an oestrogen-dependent manner, is most likely explained by increased hypothalamic drive to the pituitary following the removal of the negative feedback effects of oestrogen. In this respect, LH-releasing hormone (LHRH)-stimulated *egr-1* expression has been well documented (Tremblay & Drouin 1999, Wolfe & Call 1999, Duan *et al.* 2002). However, our finding that the *egr-1*/d4 EGFP transgene is up-regulated prior to the increase in LHRH pulse amplitude that occurs during the night of pro-oestrus (Sarkar *et al.* 1976) is extremely interesting because our previous studies (Slade & Carter 2000) have implicated transcriptional regulation of the *egr-1* gene by oestrogen, and recently this mode of *egr-1* regulation has been directly demonstrated, albeit in other cell types (de Jager *et al.* 2001). In the present study, using an explant culture model, we have now demonstrated a direct effect of E_2 on transgene (and *egr-1*) expression at the level of the anterior pituitary gland. Therefore, *cis*-acting sequences within the proximal 5′-flanking, and first intronic region of the rat *egr-1* gene appear to mediate an *in vivo* oestrogenic stimulus. This conclusion may be considered perplexing in view of the absence of consensus oestrogen response elements within this sequence (see Slade & Carter 2000, de Jager *et al.* 2001). However, the recent studies of de Jager *et al.* (2001) have provided evidence that up-regulation of *egr-1* by E_2 is mediated by SREs within the proximal *egr-1* promoter via a mechanism involving the p44/42 MAPK pathway. E_2 signalling via the MAPK pathway is, in fact, consistent with an accumulating body of evidence derived from the study of both pituitary cells (Watters *et al.* 2000) and many other cell types and systems (see Kelly & Levin 2001). By blocking E_2-stimulated *egr-1* expression with a specific inhibitor of the MAPK pathway, and demonstrating an increased abundance of phospho-p44/42 MAPK in E_2-stimulated pituitary glands, we have now provided further evidence of E_2 acting via this pathway. Further studies are now required both to define the cell populations in which MAPK is activated and to establish the functional consequences of the $E_2 \rightarrow$MAPK$\rightarrow$*egr-1* pathway in the pituitary gland.

The *in vitro* evidence described above has indicated one mechanism through which E_2 may influence anterior pituitary gene expression, and consequently effect changes in pituitary cell function during the oestrous cycle. However, the transient up-regulation of pituitary *egr-1* mRNA demonstrated here *in vitro* would not appear to be reflective of a sustained, physiological, *egr-1* response to rising levels of E_2 over the dioestrous–pro-oestrous period, and therefore other factors may modulate the *egr-1* response *in vivo*. It is possible, for example, that *egr-1* mRNA half-life may be differentially regulated *in vivo* (see Cao *et al.* 1992). In this context, the differences observed between the dynamics of the *egr-1* and transgene mRNA responses are interesting, and most likely reflect the absence of destabilizing 3′ UTR sequences (Wilson & Treisman 1988) in the transgene transcript. The transient *egr-1* response

may be a phenomenon of the artificially acute E_2 stimulation used in the explant culture experiments. Consequently, although the *in vitro* paradigm has been useful in demonstrating a direct effect of E_2 on *egr-1*/transgene expression at the pituitary level, it may have little further application in probing *in vivo* mechanisms of *egr-1* gene regulation during the oestrous cycle.

In conclusion, we have used a transgenic approach to demonstrate that proximal regions of the *egr-1* gene can direct cell-specific and physiologically regulated expression in the anterior pituitary gland. Expression of the transgene, both anatomical and regulated, recapitulates expression of the endogenous *egr-1* gene, a finding that reflects observations in an *egr-1* transgenic mouse model (Tsai *et al.* 2000). Therefore, although we have used only a single transgenic line (57C) for these studies, it is considered unlikely that the observed expression patterns in the pituitary gland are artefactual. Accordingly, our transgene construct could be useful in future studies that are designed to direct expression of functional cDNAs to a subset of pituitary cells. Our novel evidence of a direct effect of oestrogen on transgene (and *egr-1*) expression at the level of the pituitary, together with previous evidence of *egr-1* gene regulation by LHRH, suggests that the molecular response to these two physiological factors may be integrated, possibly through a co-operative action, at the *egr-1* promoter.

Acknowledgements

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