Regulation and Function of POU domain transcription factors, Brn-3a and Brn-3b

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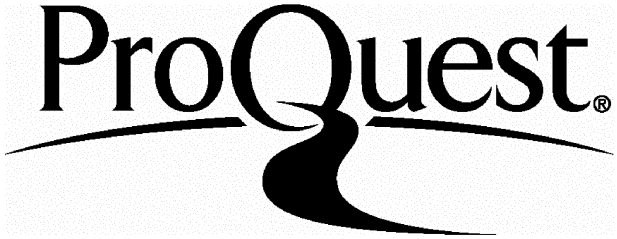

Abstract:

The generation of distinct cell types during development and their maintainance in adult multicellular organisms is achieved by the selective expression of cell or tissue specific proteins. The expression of the genes encoding these proteins are controlled primarily at the transcriptional level. This regulation is largely achieved by transcription factors which bind to specific DNA elements associated with the gene promoter. Cell-specific transcription has been attributed to families of regulatory proteins which show distinct expression patterns and interactions and which may be activators or repressors that modulate transcription activiy. The POU domain family of transcription factors have been shown to be important for the development and function of neuronal cells. Brn-3a, a member of the POU IV subfamily of POU domain transcription factors, was isolated from a brain cDNA library and later shown to be expressed in sensory neurons. Its high homology to the nematode, *C.elegans*, unc-86 gene which has been shown to be an important factor in differentiation and development of sensory neurons, suggests a conserved role for Brn-3a in sensory neuronal deveopment and function. We have isolated the POU domain of the novel but related Brn-3b protein from the sensory neuron-derived cell line, ND7. The results presented here report on the pattern of expression of these two factors, regulation of expression in ND7 cells and their role in modulation of transcriptional activity.

Brn-3a mRNA expression was found predominantly in rat brain and DRG while Brn-3b transcript were detected in these tissues but also in uterus, cervix, ovary and testis. In the ND7 cell line, there was distinct but overlapping expression of these two factors, with Brn-3b being expressed at higher levels in the proliferating ND7 cells while Brn-3a mRNA expression predominated upon differentiatiation of these cells into a sensory neuronal like cell type. The mRNA expression of both Brn-3a and Brn-3b also appeared to be regulated by combinations of growth factors and by a cyclic AMP analogue. Brn-3a may be associated with the neurite

outgrowth from these cells while Brn-3b may be characteristic of proliferating cells. Brn-3a and Brn-3b also have opposite and antagonistics effects on gene expression with Brn-3a activating transcription of a heterologous promoter via an octamer-related motif while Brn-3b repressed activity. Conditions which elevated Brn-3a mRNA also increased promoter activity in ND7 cells but not in BHK cells which lack endogenous Brn-3 expression.

Brn-3a was also an activator of the cellular α-internexin gene promoter while Brn-3b repressed its activity. Furthermore, Brn-3b appeared to interact with Brn-3a and modulated its effect on promoter activity , thus suggesting a mechanism of gene regulation by the interaction of these two factors. The amino terminus of the Brn-3a protein appeared to be necessary for efficient activation of the α-internexin promoter but not of a heterologous promoter containing the octamer-related binding site. The sequence within the α-internexin promoter that was bound by Brn-3a and Brn-3b to regulate promoter activity was distinct from the classic octamer sites recognized by many other POU proteins. This site was found to be centered at approximately -64 bases from the transcription initiation site. Furthermore, the single stranded 'coding' sequence appeared to bind the Brn-3 proteins with higher affinity than the double stranded oligonucleotide. This therefore represent a novel DNA binding site and binding pattern by these transcription factors. Thus, these transcription factors which have overlapping expression patterns and different effects on transcriptional activity in some cells may interact to modulate gene expression in specific cell types .

For my parents

Acknowledgements

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ABBREVIATIONS

APS	Ammonium persulphate
bp	base pairs
BSA	Bovine serum albumin
cyclic AMP	N6, 2'-O-Dibutyryladenosine 3':5'-cyclic monophosphate
CAT	Chloramphenicol acetyl transferase
cDNA	complementary DNA
CNS	central nervous system
CRH	Corticotrophin releasing hormone
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetra acetic acid (disodium salt)
EMSA	electrophoretic mobility shift assay
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGM	Full growth medium
HBS	Hepes buffered saline
Hepes	N-[hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IGF-1 & 2	Insulin-like growth factors 1 & 2
Internexin-L	full length α-internexin promoter
Internexin-I	intermediate length α-internexin promoter
Internexin-S	minimal α-internexin promoter
IXB3	DNA sequence with Brn-3 binding site in the α-internexin promoter
kb	kilobases
kDa	kilodalton

L-15 medium	Leibowitz's 15 medium
NBT	4-nitro-blue tetrazolium
ND	Neuroblastoma - DRG hybrid cell line
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PIC	Pre-initiation complex
POMC	Pro-opiomelanocortin
POU	Pit-Oct-Unc
SDS	Sodium dodecyl sulphate
RT-PCR	reverse transcription-polymerase chain reaction
r.p.m	revolutions per minute
TAE	Tris acetate electrophoresis buffer
TAFs	TBP associated factors
TBE	Tris-borate electrophoresis buffer
TBP	TATA-box binding proteins
TEMED	N,N,N',N'-tetramethyl-ethylene diamine
TF	Transcription factors
UV	Ultraviolet radiation
X-phosphate	5' Bromo-4-Chloro-3'indolyl phosphate

LIST OF PUBLICATIONS

1. Lillycrop, K.A., Budhram, V., Lakin, N.D., Terrenghi, G., Wood, J.N., Polak, J.M. and Latchman, D.S. (1992). A novel POU family transcription factor is closely related to Brn-3 but has a distinct expression pattern in neuronal cells. *Nucleic Acids Res.* **20** No.19 5093-5096

2. Budhram-Mahadeo, V., Lillycrop, K.A. and Latchman, D.S. (1994) Cell cycle arrest and morphological differentiation can occur in the absence of apoptosis in a neuronal cell line *Neuroscience Letts.* **165** 18-22

3. Budhram-Mahadeo, V., Theil, T., Morris, P.J., Lillycrop, K.A., Moroy, T. and Latchman, D.S. (1995) The DNA target site for the Brn-3 POU family transcription factors can confer responsiveness to cyclic AMP and removal of serum in neuronal cells *Nucleic Acids Res.* **22** No15 3092-3098

2. Budhram-Mahadeo, V., Lillycrop, K.A. and Latchman, D.S. (1995) The levels of the antagonistic POU family transcription factors Brn-3a and Brn-3b in neuronal cells are regulated in opposite directions by serum growth factors *Neuroscience Letts.* **185** 48-51

5. Budhram-Mahadeo, V., Morris. P.J., Lakin, N.D., Theil, T., Ching, G.Y., Lillycrop, K.A., Moroy, T., Leim,R.K.H. and Latchman, D.S. (1995) Activation of the α-internexin promoter by the Brn-3a transcription factor is dependent on the N-terminal region of the protein *J. Biol. Chem.* **270** No.6 2853-2858

Chapter 1

General Introduction

1.0.0 Transcriptional Regulation

Eukaryotic organisms are made up of large numbers of morphologically and functionally different cells. The appearance of these distinct cell types during development and their maintainance in adult tissues result from selective gene expression which occurs in a precise temporal and spatial pattern by a highly regulated process. Much of this regulation takes place at the transcriptional level, by modulating the production of messenger RNAs (mRNA) which encode specific proteins. The sequential activation of cascades of regulatory genes, many of which encode transcription factors control the expression of specific factors in cells [Johnson and McKnight,1989, Latchman,1990 (i) and (ii); Struhl, 1991; Zawel and Reinberg, 1995]. A brief outline of the mechanisms thought to be involved in this process will be given.

Many genes which are transcriptionally inactive are condensed and organized into chromatin structure which prevents access of the transcriptional machinery to the gene promoter. Thus, activation of gene expression requires modification of the chromatin to render the DNA open and more accessible for transcription. This is achieved by unwinding of chromatin structure and the displacement of the repressive histones from the DNA (Schlief, 1988; Wolffe, 1994), an effect which correlates with the formation of nuclease hypersensitive regions in the DNA in transcriptionally active DNA. This process requires DNA binding proteins which recognize sequence-specific elements and facilitate activation of the transcriptional process (reviewed in Latchman, 1990; Felsenfeld, 1992; Croston and Kadonaga, 1993).

1.0.1 DNA sequence elements regulating transcription

Transcriptional activity of most eukaryotic genes is regulated through a complex interplay of transcription factors (trans-activating regulators) which bind specific sites (cis-regulatory elements) within the promoter (Ptashne, 1988, Mitchell and Tijian, 1989; Latchman; 1990). The DNA sequence elements and cellular factors which are involved in transcription of genes encoding cellular proteins by the RNA polymerase II will be briefly described. For more details please refer to the references cited.

Two types of cis-active elements, the promoter and enhancer sequences, which influence the genes transcribed in specific cell, have been identified. Proximal promoter sequences which bind many constituitively expressed general transcription factors [such as the A/T rich sequence element (TATA box) which is recognized and bound by transcription factor IID, TFIID] are located immediately upstream of the transcriptional initiation site. The assembly of a basal complex of general transcription factors is sufficient for low levels of transcriptional activity but to achieve high levels of gene transcription, other cell-specific transcription factors which bind to enhancer sequences or other regulatory sequences in the promoter (which maybe located distally from the transcriptional initiation site) must be recruited to the basal transcription complex. Thus, cell-specific factors play a critical role in controlling the efficiency and rate of transcription from a given promoter.

While proximal promoter sequences are generally close to the transcriptional initiation sites, the enhancer elements may be large distances away and may be upstream or downstream of the promoter. In addition to enhancer elements which bind activators of gene expression, some negative enhancer elements (silencers), such as the sequence which binds the neuron-restrictive silencer factor, (Schoenherr and Anderson, 1995) have been identified which result in inhibition of transcriptional activity. Furthermore, some sequences which act as positive enhancers in some cells may be negative enhancers in other cell types depending

on the position of its binding site in the promoter & other transcription factors which may be co-expressed in the cells.

1.0.2 Transcription by RNA polymerase II

For efficient transcription by RNA polymerase enzyme, the promoter of the gene to be transcribed must be accessible to the transcriptional complex. The polymerase cannot bind directly to the DNA, so it is recruited as part of a complex which is formed by the step-wise assembly of general transcription factors (TFIIA, IIB, IID, IIE, IIF and IIH) on the promoter, the pre-initiation complex (PIC) (Drapkin *et al.*, 1993). A summary of the mechanisms thought to be involved in this process will be outlined. An initial complex is formed by the binding of TFIID (which consists of the TATA box binding protein (TBP) and the TBP associated factors (TAF's), to the TATA element in TATA-box-containing promoters. This appears to facilitate the assembly of other transcription factors to the complex as well as preventing the stabilization of nucleosomes in the promoter region hence enhancing the PIC formation and activity [White and Jackson, 1992 (i) and (ii); Drapkin *et al.*, 1993, Buratowski, 1994]. Thus, recruitment of this TFIID protein may be one of the rate limiting steps for transcription *in-vivo* and the TBP component of this complex has been shown to be one of the core proteins which is bound by transcriptional activators to modulate gene expression (Chatterji and Struhl, 1995; Klages and Strubin, 1995). The TFIIB protein binds to the TFIID protein which is complexed with DNA and also recruits the RNA polymerase II to this complex. The recruitment of TFIIB to the PIC has been shown to be another key rate limiting step in the transcription of many genes and this process was greatly enhanced by recruitment of enhancer-bound transcriptional activator (Lin and Green, 1991; Choy and Green, 1993). The carboxy terminal domain (CTD) of RNA polymerase II has been shown to be critical for 'enhancer-driven' transcriptional activity although the precise function of this domain is not clear. It is possible that the CTD modulates the association of the polymerase with the PIC (Drapkin *et*

al., 1993; Buratowski, 1994; Gerber *et al.*, 1995). Upon binding of the RNA polymerase other factors such as TFIIE, a DNA-dependent ATPase probably required for generating energy for transcription and TFIIF which has enzymatic activity needed to unwind DNA are also recruited to form the pre-initiation complex. Another factor, TFIIH, appears to have enzymatic activity required to initiate the transcription process (Drapkin *et al.*, 1993, Gilbert, 1994).

The mechanisms by which transcription factors or activators contribute to gene transcription are still not fully understood and are under intense investigations. However, results of some studies have suggested that activators may be involved in the removal of nucleosomes which are bound to the transcriptionally inactive DNA thus allowing access to the promoter (Workman *et al.*, 1988; Archer *et al.*, 1992). Alternatively, a second not mutually exclusive mechanism would be that these factors increase the assembly of the PIC by direct physical interactions with components of the basal transcriptional machinery. This has been demonstrated for TFIIB and TBP but any of the components of the PIC may be targetted for these interactions (Sawadogo and Roeder, 1985; Lin and Green, 1991; Drapkin *et al.*, 1993, Tjian and Maniatis, 1994).

1.0.3 Mechanisms for regulating gene expression by cell-specific transcription factors

Thus, the regulatory proteins which bind elements such as enhancers appear to be able to regulate the temporal and tissue-specific expression of differentially regulated genes. Many of these transcription factors are modular proteins which contain domains involved in the recognition and binding of specific DNA sequences as well as activation / repressor domains which allow interaction with other proteins such as the components of the PIC or other cell-specific factors. These cooperative interactions result in the modulation of transcriptional activity (Latchman, 1990; Frankel and Kim, 1991, Lin and Green, 1991;

Drapkin *et al.*, 1993). The ability of many transcription factors to interact with other proteins provides a mechanism by which the expression of a diverse range of genes can be regulated by a relatively small number of transcription factors (Struhl, 1991, Hershlag and Johnson, 1993). This cooperativity is supported by the observation that many cellular promoters contain binding sites for multiple transcription factors, thus utilizing combinations of different factors expressed in the cells at a particular time or in response to specific signals. Therefore, to effect gene transcription, a transcription factor has to be co-expressed with the target gene as well as with other factors which may be required for the combinatorial regulation of gene activity (Struhl, 1991; Xe and Rosenfeld, 1991; Hill and Treisman, 1995).

In addition to the requirement for interactions with other proteins, the activity of transcription factors may be controlled at a number of distinct stages such as the level of its synthesis, accessibility to its DNA binding site on the promoter and regulation of its ability to bind DNA by post translational modifications (reviewed in Latchman, 1990; He and Rosenfeld, 1991, Struhl,1991; Dynan and Gilman, 1993; Hill and Treisman,1995).

Families of regulatory transcription factors which show highly restricted distribution and interactions have been identified on the basis of the presence of evolutionarily conserved DNA-binding motifs which are found in all the proteins in that family. Examples include the zinc finger motif, the basic leucine zipper motif, the helix-loop-helix motif and the related helix-turn-helix motif found in the homeodomain and POU domain proteins [Latchman, 1990 (i) and (ii); He and Rosenfeld, 1991 Struhl, 1991 and Pabo and Sauer, 1992]. The homeodomain family of transcription factors have been shown to be critical for specifying the anterior-posterior body axes during development, throughout the animal kingdom .Members of this family show distinct temporal and spatial patterns of expression. The Brn-3 proteins belong to a subclasses of homeodomain proteins and hence

share some characteristics with proteins of this family. A brief discussion of the general properties of homeodomain proteins will be given to emphasize the evolutionarily conserved nature of these developmental regulators and details of the POU domain proteins will be given in more details later.

1.1.0 Homeodomain Transcription factors

Homeodomain proteins contain the highly conserved homeobox, a 183 base pair region which encodes a 61 amino acid DNA binding domain found in several classes of development-regulating genes initially identified in the *Drosophila melanogaster*. The products of these genes have been shown to be critical for specifing the identity and spatial arrangement of the body segments when establishing structural patterns during development (Gehring, 1987, Puelles and Rubenstein, 1993, Gehring *et al.*, 1994). Homeodomain proteins recognise and bind to specific A/T rich DNA elements to exert transcriptional control (see section 1.2.4) (Odenwald *et al.*,1989; Kissinger *et al.*,1990; Gehring *et al.*, 1994 ii). Studies have shown that the homeodomain folds into a helix-turn-helix structure and that conserved amino acids in this region interact with specific bases within the major groove of the DNA while the amino acids in the amino terminus of the homeodomain make contacts with bases in the minor groove (reviewed in Gehring *et al.*,1994). The recognition of specific promoters by these proteins thus depend upon the DNA binding site specific for each protein as well as the amino acids which make contact with the bases (Treisman *et al.*, 1989).

Drosophila homeobox-containing genes have been shown to regulate the expression of one another as well as other classes of developmental regulators (Puelles and Rubenstein, 1993; Gehring *et al.*, 1994). The high sequence conservation has led to the isolation of homeodomain genes from organisms as evolutionary diverse as flies, sea-urchin and mammals, with the protein products also playing a role in the normal development of these

organisms (Keynes and Krumlang, 1994). Classes of homeodomain proteins have been established on the basis of several criteria such as sequence identity, sequence similarity in the flanking regions, organizations into gene clusters and association with sequence motifs (reviewed in Kessel and Gruss, 1990; Gehring *et al.*, 1994). However related but distinct subclasses of these transcription factors have also been identified. These are characterized by composite domains (such as the *engrailed (en)*, the *paired (prd)*, the *LIM*, the *zinc finger (ZF)* and the *POU* domains) which consist of the homeodomain associated with a second distinct domain which confers different requirement for DNA binding and distinct functional characteristics. The identification of large numbers of proteins in these subgroups have demonstrated that these distinct groups of transcription factors display different tissue distribution and function when compared with the other homeodomain proteins (for reviews, see Gehring *et al.*, 1994.

The POU domain transcription factors, one of the subclass of homeodomain proteins, have been shown to be critical for development of brain and nervous tissues and display distinct spatial and temporal expression patterns both during development and in mature tissues (Rosenfeld, 1991; Wegner *et al.*, 1993). Furthermore, the expression of many POU domain proteins have been identified at earlier stages of development and with different distribution compared with the homeodomain proteins indicating defined functions for these proteins (Rosenfeld, 1991; Treacy and Rosenfeld, 1992).

1.2.0 POU domain transcription family

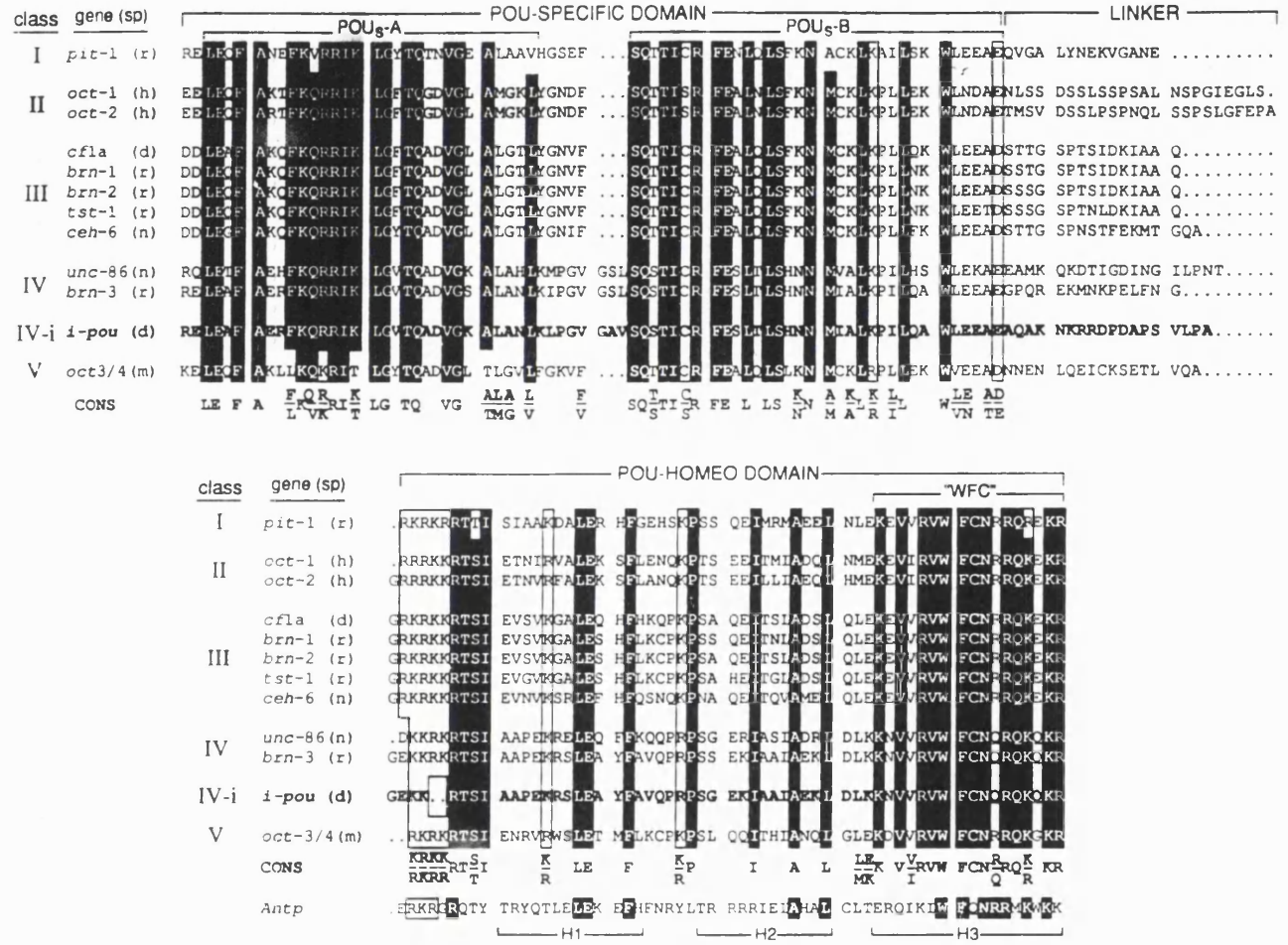
The independent isolation and characterization of three mammalian regulators, Pit-1, Oct-1 and Oct-2 and the nematode, Unc-86, led to the identification of the POU domain family named on the basis of a common highly conserved region, the POU (Pit-Oct-Unc) domain, which contained a region with distinct homology to the classic homeodomain, the POU-

homeo domain, along with another highly conserved region, the POU-specific domain present in all the members of this family (Herr *et al.*, 1988). Since the identification of the original members, a large number of other proteins belonging to this family have been isolated and characterized (He *et al.*, 1989; also reviewed in Rosenfeld, 1991; Ruvkun and Finney, 1991; Scholer, 1991; Latchman *et al.*, 1992; Treacy and Rosenfeld, 1992; Verrijzer and Van der Vliet, 1993 and Wegner *et al.*, 1993).

1.2.1 Domains of POU proteins

The POU domain transcription factors are modular proteins consisting of distinct functional domains which interact to modulate the overall structure and the functions of these proteins [Rosenfeld, 1991; Aurora and Herr, 1992, Verrijzer *et al.*, 1992 (ii)]. The common POU domain has been shown to be primarily involved in DNA sequence recognition and high affinity binding to highly conserved DNA elements in the promoter of target genes as well as mediating protein-protein interactions (Wegner *et al.*, 1993,Verrijzer and van der Vilet, 1993). In addition, many of these factors contain activation or repressor domains which contribute to the positive or negative transcriptional effects which characterize these proteins or contain domains, such as the leucine zipper motifs, which specifically mediate protein-protein interactions. The structure, functions and interactions of these domains will be discussed in more detail to highlight the properties that they confer on the proteins and their functional relevance. The overview given here summarizes the pertinent results in an effort to demonstrate the unique properties of these transcriptional regulators, the mechanisms of the regulation of their expression and some functions of members of this family of transcription factors during development and adult tissues as well as their relevance to any diseased states. For more detailed information please refer to the relevant references sited.

Figure 1.2.1: Members of the different classes of POU domain proteins as classified by homology in the linker region and the amino terminus of the POU-HD domain. Sequence homology among members of the different classes of POU-domain gene family are indicated by black highlighting. The homeodomains of Antennapedia (Antp) is shown at the bottom , with the positions of the three helical domains indicated as H1, H2 and H3. Species from which the factors were cloned are rat (r), the nematode, *C.elegans*, (n), mouse, (m), human, (h) and *Drosophila melanogaster*, (d). The consensus sequence (Cons) in the POU-specific and POUHD are also indicated. (Taken from Rosenfeld, 1991).


class
gene (sp)
POU-SPECIFIC DOMAIN
POUS-A
POUS-B
LINKER
I
pit-1 (r)
II
oct-1 (h)
oct-2 (h)
III
cfla (d)
brn-1 (r)
brn-2 (r)
tst-1 (r)
ceh-6 (n)
IV
unc-86 (n)
brn-3 (r)
IV-i
i-pou (d)
V
oct3/4 (m)
CONS
POU-HOMEO DOMAIN
"WFC"
Antp
H1
H2
H3

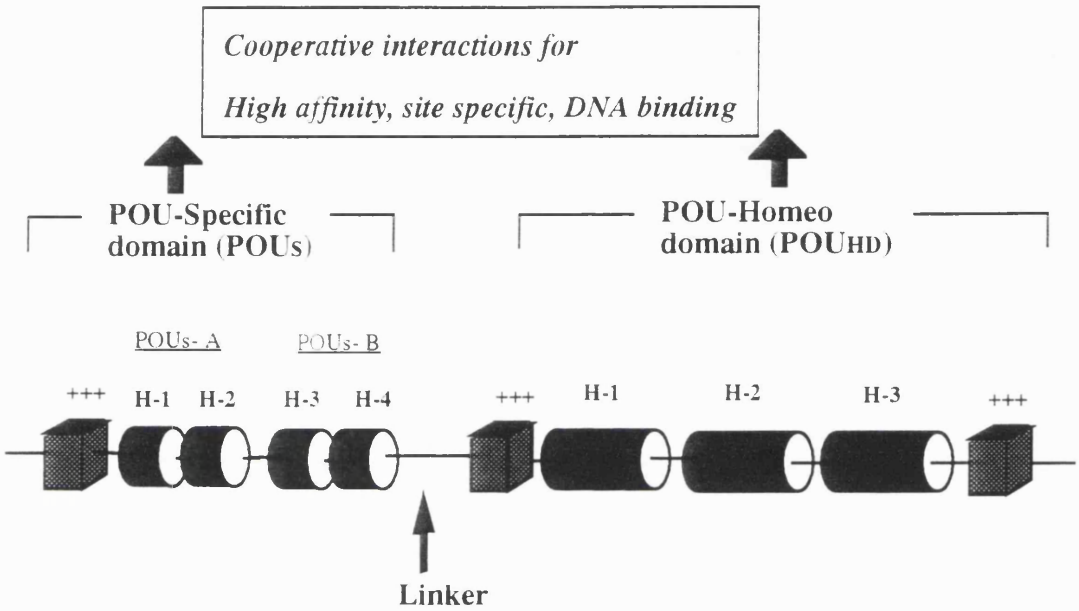
1.2.2 The POU domain

The POU domain is the only highly conserved feature which is common to all the members of this family of proteins since there is significant divergence in the sequence and structure outside this region. In its entirity, the POU domain consists of a contiguous stretch of 150-160 amino acids with two highly conserved regions; a 74-82 amino acid POU-specific domain (POU_S) at the amino terminus and the 60 amino acids carboxy terminus POU-homeo domain (POU_{HD}) (Herr et al., 1988). These domains are connected by a poorly conserved linker which may consist of 14-25 amino acid (figure 1.2.2). Based upon the sequence homology in the linker region and the main basic cluster at the amino terminus of the POU homeodomain, members of the POU domain family had been subdivided into classes, I-V (figure 1.2.1) (Rosenfeld, 1991, Treacy and Rosenfeld, 1992). Since then the cloning of genes which encode divergent POU-domain proteins such as the murine Brn-5 (Emb) (Andersen *et al.*, 1993; Okamoto *et al.*, 1993) and mPOU (Wey *et al.*, 1994), which cannot be classified into the established groups indicated that there might be additional classes of these genes.

The POU domain is primarily involved in sequence specific, high affinity DNA binding (Strum and Herr, 1988; Garcia-Blanco *et al.*, 1989; Aurora and Herr, 1992). While the binding sites of classic homeodomain proteins are generally short A/T rich sequences (Kissinger *et al.*, 1990), POU domain proteins appear to recognise longer DNA motifs such as the well characterized octamer sequence recognised by both Oct-1 and Oct-2 (figure 1.2.2) (Muller *et al.*, 1988, Strum *et al.*, 1988, Herr, 1992). A number of related octamer motifs have been identified as recognition sites for other POU factors. Functional analysis of the POU domain reveals segments which interact inter-dependently to confer flexible sequence-specific DNA binding with both the POU-specific and POU-homeo domains shown to make specific contacts with the DNA [Aurora and Herr, 1992; Verrijzer *et al.*, 1992(i)].

(A)

74 - 82 amino acids	*60 amino acids*
High affinity DNA binding	*Low affinity DNA binding*
Specific site requirement	*Relaxed specificity*
Protein-protein interaction	*Protein -protein interaction*



(B)

DNA Binding consensus sequence:

Pit-1: 5'- ATGNATA(A/T)$_{4-5}$ -3'

Oct-1/ Oct-2: 5'- ATGCAAA(AT)$_{4-5}$ -3'

Brn-2: 5'- GCATnTAAT-3'

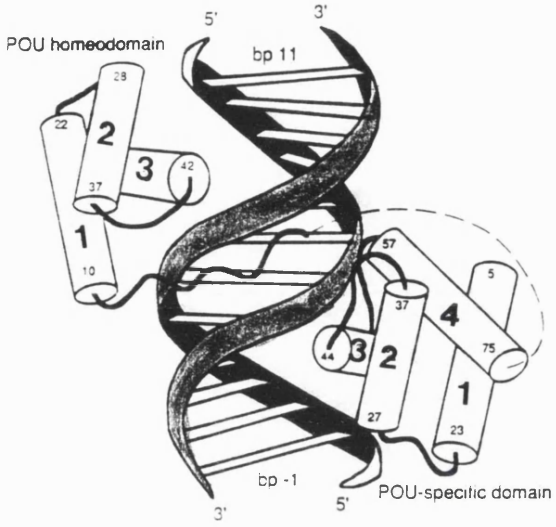
Figure 1.2.2:(A) Schematic representation of the POU domain, indicating the number of predicted helical domains (H) and the basic regions (+++) in the POU specific and POU homeo domains. The putative functions of the domains are also listed. **(B)** Examples of the A/T rich consensus sequences bound by the POU proteins, Pit-1, Oct-1/ Oct-2 and Brn-2 are also shown (see text for discussion). (Adapted from Rosenfeld,1991).

1.2.3 POU-specific domain

The highly conserved POU-specific domain can be considered as consisting of two distinct regions of particularly high homology, the POU_S-A and POU_S-B subdomains (figure 1.2.2) (Rosenfeld, 1991, Verrijzer and van der Vilet, 1993). The solution structure of the POU-specific domain of Oct-1 which was obtained by nuclear resonance spectroscopy (NMR) shows that this domain consists of a cluster of four α-helices (figure 1.2.3) with the second and third helices of the POU-specific domain forming a helix-turn-helix motif (Dekker *et al.*,1993).

The POU-specific domain binds DNA with low affinity but the sequence recognition is specific [Strum and Herr, 1988; Ingraham *et al.*, 1990; Verrijzer *et al.*, 1992 (ii) Aurora and Herr, 1992]. Results of a number of experiments including the use of deletion constructs, electrophoretic mobility shift assay (EMSA) with normal and mutated binding sites and ultraviolet cross-linking indicated that the POU-specific domain of Oct-1 recognises and binds sequences on the 'left side' of the octamer (Verrijzer *et al.*, 1990, Verrijzer *et al.*, 1992; Aurora and Herr, 1992; Verrijzer and Van der Vilet, 1993). Furthermore, analysis of the crystal structure of the POU domain of Oct-1 bound to an octamer sequence showed that the POU-specific domain made primary contacts with the ATGC sequence of the octamer motif, ATGCAAAT (Klemm *et al.*, 1994). It is thought that the third helix of the POU-specific domain makes contact with the major groove of the DNA and makes all the base contacts with the ATGC subsite (figure 1.2.3). This domain has a higher stringency for DNA recognition sites compared with the POU-homeo domain.

(A)



(B)

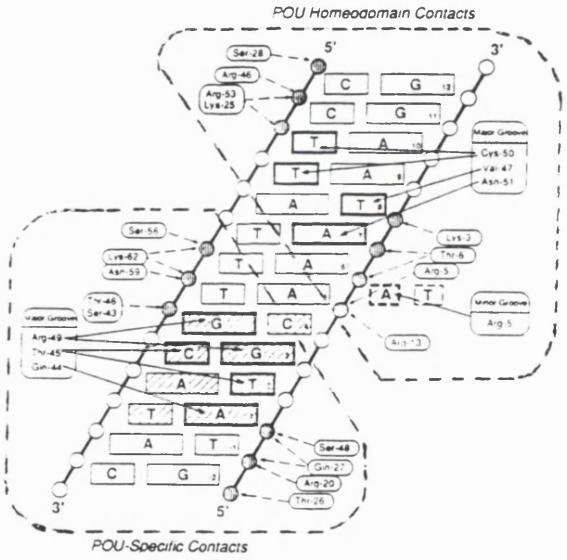


Figure 1.2.3: (A) Schematic representation of the contacts made by the POU-specific and POU-homeo domains upon binding to the DNA recognition site. Cylinders indicate the positions of the α-helices. The flexible linker which connects the two domains are indicated by the solid and dotted lines. (B) Sketch summarizing all the DNA contacts made by the POU domain of Oct-1 on its DNA recognition sequence. The DNA is represented as a cylindrical projection and the ATGC and AAAT subsites recognised by the POU-specific and POU-homeo domain, respectively, are highlighted. (Taken from Klemm et al.,1994)

The contacts made by the POU-specific domain (but not the POU-homeo domain) have also been shown to induce bending of DNA in the Oct-1 POU domain protein (Verrijzer *et al.*, 1991; Dekker *et al.*, 1993). This effect may be critical for the function of these proteins since DNA bending may results in conformational changes which can facilitate transcription complex assembly or protein-protein interactions. It was also proposed that mechanism may mediate DNA replication by causing unwinding of the DNA by torsion at the bend thus allowing the DNA replication machinery to bind to the single stranded DNA and initiate replication (Verrijzer *et al.*, 1990; Verrijzer *et al.*, 1991; Verrijzer and van der Vilet, 1993).

1.2.4 POU-homeo domain

Whereas the POU-specific domain is unique to the POU domain family, the POU-homeo domain is unambiguously related to the homeodomain with the folded structure and docking to DNA being very similar to those observed in other homeodomain-DNA complexes (Garcia-Blanco *et al.*, 1989). Studies of the crystal structure of the POU homeodomain suggest that like the classic homeodomain, the POU-homeo domain contains three α-helices with a flexible amino terminus arm (figure 1.2.2) (Kissinger *et al.*, 1990; Klemm *et al.*, 1994). As in the POU-specific domain, the second and third helices form a helix-turn-helix motif with helix 3 (referred to as the recognition helix or WFC region) making specific contacts with bases in the major groove of the DNA (figure 1.2.3). Helix 3 is particularly well conserved containing the RVWFCN residues in all known POU domain proteins.

At both the amino and carboxy terminus of the POU-homeo domain are clusters of basic amino acid residues which are critical for their function in DNA binding (Garcia-Blanco *et al.*, 1989; Pabo and Sauner, 1992, Treacy and Rosenfeld, 1992). Like classic

homeodomain proteins, the POU-homeo domain appear to recognise A/T rich sequences as exemplified by the binding of the Oct-1 POU-homeo domain to a core sequence, TAAT, in its octamer recognition motif [Verrijzer *et al.*,1992 (ii)]. However, in contrast to the classic homeodomain proteins the POU-homeo domain is only capable of low affinity DNA binding on its own and requires the presence of the POU-specific domain for the high affinity site specific DNA binding characteristic of these proteins (Ingraham *et al.*, 1990; Verrijzer *et al.*, 1990). Deletion analysis of Pit-1 and Oct-1 have shown that the POU-homeo domain can support low affinity binding on its own but with altered specificity compared with the intact POU domain (Aurora and Herr, 1992). The POU-homeo domain has been shown to be docked in the major groove of the DNA by the third 'recognition' helix which makes contact with the TAAT subsite of the recognition sequence (figure 1.2.3). This highly conserved region is critical for DNA binding as demonstrated by a mutation in the Pit-1 POU-homeo domain which result in the conversion of a tryptophan residue at position 7 to a cysteine with a resultant loss of binding to the recognition motif and the consequent non-functional Pit-1 observed in the Snell dwarf mice (Li *et al.*, 1990). Furthermore, exchange of a single amino acid in the POU-homeo domain can allow Oct-2 to mimic the interactions observed between Oct-1 and VP16 (Lai *et al.*, 1992). The highly basic and well conserved amino terminal arm of the POU-homeo domain fits into the minor groove of the DNA and contributes significantly to sequence recognition by specifying interactions with the DNA (Klemm *et al.*, 1994) .

The importance of this region for DNA binding is highlighted by the interactions of two highly related POU factors, the inhibitory POU protein I-POU, in which two amino acid residue (R, K) are deleted in the amino terminal region of the POU-homeo domain and the twin of I-POU (tI-POU) which has an identical sequence to I-POU but with the two amino acid residues present (Treacy *et al.*, 1991). Whereas I-POU is unable to bind the octamer DNA sequence element, it can interact with another POU protein, Cf1a, to form a dimer.

This interaction prevents Cf1a binding to its cognate sequence in the promoter of its target DOPA decarboxylase gene and activating it (Johnson and Hirsch, 1990). However, tI-POU which has the extra amino acids in this region, is capable of binding to the octamer motif but cannot interact with Cf1a or I-POU (Treacy *et al.*, 1992).

1.2.5 Interaction between the POU-specific and POU-homeo domains

These two domains act combinatorially to facilitate high-affinity, site-specific DNA binding. NMR data suggested that the POU-specific and the POU-homeo domains form autonomously folded structural units and make contacts on distinct parts of the DNA recognition sequences (Dekker *et al.*, 1993; Klemm *et al.*, 1994). Cooperative binding of the adjacent POU-specific and POU-homeo domains result in the recognition of the assymetrical DNA-binding motifs typical of POU domain proteins with each domain contributing to the sequence specificity of the other (Aurora and Herr, 1992; Verrijzer *et al.*, 1992; Klemm *et al.*, 1994).

The different sequence requirements for binding of the two sub-domains has been illustrated by the findings that the POU-specific and POU-homeo domain make contacts with different parts of the binding site [Verrijzer *et al.*, 1992 (ii)]. However, on binding the octamer sequences the two domains are oriented on opposite sides of the double helix with no protein-protein contacts between them (figure 1.2.3) (Dekker *et al.*,1993; Klemm *et al.*,1994). Contact point analysis as well as electrophoretic mobility shift assays (EMSA) with mutated sites indicated that the preference of the POU-specific domain overrides that of the POU-homeo domain for optimal Oct-1 binding [Verrijzer *et al.*, 1992(i) and (ii)]. In addition to contributing to the specificity of the sequence which is recognised by this domain, the POU-specific domain may also stabilize the contacts made by the POU-homeo domain.

Therefore while the POU-specific and the POU-homeo domains are capable of binding to DNA with overlapping but distinct recognition sequences, both are required for cooperative activation via the cognate element. The contacts and interactions of the various subdomains within the POU-specific and the POU-homeo domain has great implications on the function of these protein since mutation of any of the points in these domains may result in altered DNA binding which may profoundly affect the activity of these transcription factors.

1.2.6: The Linker region

Study of the crystal structure of POU domain showed the poorly conserved linker sequence as a disordered, flexible and unstructured region (Klemm *et al.*, 1994). Analysis of the amino acid sequence reveal no clear secondary structure with both the length and sequence of this region being highly variable. In addition, when the POU domain is bound to the DNA, the linker is readily accessible to proteases indicating that this region is not bound to the DNA (Aurora and Herr, 1992; Botfield *et al.*, 1992). The precise function of this region is still to be elucidated since transcription factors such as Oct-1 and Oct-2 which have the same DNA binding specificity and almost identical POU-specific and POU-homeo domains have significant differences in the linker region (Herr *et al.*, 1988). The evidence so far suggests that the linker acts as a flexible tether that provides a covalent connection between the POU-specific and the POU homeo-domains thus ensuring that binding of one domain enhances and stabilizes the binding of the other.

Interestingly, the linker regions among the members of the POU III sub-family are relatively well conserved and may thus suggest an important functional role within this class of genes (Li *et al.*, 1993). It is possible that weak structural preferences in this linker region could help orient the domains as they bind to the subsites in the target genes.

1.2.7 Binding site preferences of POU domain proteins

In addition to the specific DNA sequence recognized by POU domain proteins, the orientation and spacing between the subsites contacted by the POU-specific and the POU-homeo domains may also vary for the different POU proteins thus providing a mechanism whereby a greater diversity of target genes can be modulated by members of this family.

1.2.7(i) Orientation of POU-s and POU$_{HD}$ on core motifs

Whereas many class I (e.g. Pit-1) and class II (e.g. Oct-1, Oct-2) POU proteins recognise and bind highly related octamer motifs with the conserved ATG and (T/A)AAT (Verrijzer *et al.*,1992(i), the preferred recognition site of the class-III factor, Brn-2, which was identified in the corticotropin releasing hormone (CRH) promoter, was shown to consist of two distinct half-sites GCATnTAAT separated by a nonconserved spacer region (n) of 0, 2 or 3 nucleotides (Li *et al.*,1993). UV cross-linking experiments indicated that the POU-specific domain made contact with the CAT core sequence and the POU-homeo domain with the TAAT motif.

Interestingly, the subsite sequence, CAT, recognised by the POU-specific domain of Brn-2 is in the reverse orientation to the ATG in the octamer motif of Pit-1 and Oct-1 and Oct-2. It is therefore likely that upon binding to its site, the POU-specific domain of the Brn-2 will be in the opposite orientation to that observed when Oct-1 or Pit-1 bound to the octamer sequence. The organisation of the bipartite DNA site for Brn-2 binding in the CRH promoter appears analogous to the bipartite sites used by other receptors such as the retinoic X receptor (RXR) and retinoic acid receptor (RAR) in which core binding motifs are in direct repeat orientation (Umesono *et al.*, 1991) as opposed to the reversed orientation observed for the Pit-1 or Oct-1 binding to the octamer site (Assa-Munt *et al.*, 1993).

However, Brn-2 has also been shown to bind the Pit-1 recognition sequence on the prolactin promoter and its orientation is then similar to Pit-1 (Li *et al.*, 1993). It therefore appears that Brn-2 is capable of "switching" orientation depending on the recognition motif to which it binds thus providing the basis for regulation of a diversity of genes.

The preference of some POU domain proteins for binding to DNA sequence elements in the opposite orientation is also demonstrated by the I-POU protein. This protein which lacks two amino acids in the amino terminal arm of the POU-homeo domain and is incapable of binding the octamer DNA motif which is recognised and bound by the highly similar, tI-POU containing the extra amino acids (Treacy *et al.*, 1991; Treacy *et al.*, 1992). However, Gerrero *et al* (1993) reported binding of the I-POU protein to the Brn-2 site in the CRH promoter. The ability of this protein to bind some DNA sequence but not others suggests that the emphasis of the contacts made by the amino acids which are absent in I-POU may be related to the orientation of the POU sub-domains on the DNA element when other contacts may override the requirement for the missing amino acids. This mechanism thus provide the basis for different functions of an individual protein depending on the promoter, the orientation of the recognition motif as well as other interacting factors. The inhibitory interaction of I-POU with Cf1a, prevents the activator gaining access to its cognate element but the ability of I-POU to bind a distinct DNA site could allow a different set of target genes to be modulated by this factor by direct DNA binding although the nature of this interaction is still to be elucidated.

1.2.7(ii) Spacing between the core motifs

Further flexibility in the binding of Brn-2 to its cognate sequence is permitted by its ability to recognise DNA elements with 0, 2 or 3 nucleotides between the sites bound by the POU-specific or POU-homeo domains (Li *et al.*, 1993). Addition of these nucleotides can alter the spacing in the common 'B' form DNA conformation by changing the angle and distance

of the two core binding motifs. Therefore when Brn-2 is bound to sites with different nucleotide spacing, it may either change its own conformation to facilitate binding to the subsites or induce changes such as DNA bending to achieve the contacts required for binding. This strategy can therefore influence site specificity as well as increase the effective range of target genes modulated by a transcription factor.

Although the Brn-2 protein appears to be tolerant of the changes in the spacing between the core recognition sites, Brn-3a, a class IV POU protein, can only effectively bind its DNA recognition elements if there is three nucleotide spacing between the core motifs bound by the POU-specific and POU-homeo domains. This effect was found to depend on the contacts made by specific amino acid residues in the POU-homeo domain and in the basic region at the amino terminus of the POU-homeo domain (Li *et al.*, 1993). Replacing three variant amino acids in the basic region of Brn-2 with those from Brn-3a produced a marked switch in the spacing preferences to that of Brn-3a. Therefore the flexible amino terminal arm of the homeodomain of different POU proteins also specifies the interactions made with the DNA and contributes significantly to DNA site recognition.

1.2.8 Expression of POU domain proteins

Most genes encoding POU domain proteins have demonstrated defined patterns of expression in various regions of the nervous system during embryonic development with more restricted distribution in adults suggesting a potential role in the regulation of neuronal development and function. For instance, Pit-1 is predominantly expressed in the anterior pituitary and activates the transcription of the prolactin and growth hormone genes (Nelson *et al.*, 1988). Oct-2 was originally thought to be expressed primarily in lymphoid B cells where it was shown to be involved in the activation of the immunoglobulin genes (Muller *et al.*, 1988; Ko *et al.*, 1988). However, the recent identification of multiple spliced products

of this gene (Hatzopoulos *et al.*, 1990; Schreiber *et al.*, 1990; Wirth *et al.*, 1991) which demonstrate restricted distribution in tissues such as brain and sensory neuronal cells [He *et al.*, 1989; Hatzopoulos *et al.*, 1990; Dent *et al.*, 1991(i); Stoykova *et al.*, 1992] suggest an involvement of these isoforms in the transcriptional control of more diverse target genes which are yet to be established.

The nematode gene product, Unc-86, a genetically defined determinant of cell fate in sensory neurons is also developmentally regulated (Finney *et al.*, 1988; Finney and Ruvkun, 1988; Xue *et al.*, 1993). Some POU factors such as Oct-3 /4 and Oct-6/Tst-1 are expressed very early in development. Whereas Oct-3/4 has been shown to be characteristic of a pluripotent, undifferentiated cell phenotype (Okamoto *et al.*, 1990; Scholer *et al.*, 1990; Okazawa *et al.*, 1991; Rosner *et al.*, 1991;Scholer, 1991), Oct-6/Tst-1 has been detected in pre-implantation embryos as well as in developing brain and in specific neurons (Suzuki *et al.*, 1990; Monuki *et al.*, 1990). These factors are also expressed in adult germ line cells with Oct-3/4 detected in maturing oocytes and in the testis (Rosner *et al.*, 1990) and Oct-6/Tst-1 in the testis (He *et al.*, 1989). Other factors such as Brn-1, Brn-2 and Brn-4 (Hara *et al.*, 1992; Mathis *et al.*, 1992). Members of the Brn-3 family are also expressed during development with more restricted distribution in adult tissues (discussed in section 1.3). In contrast, Oct-1 is ubiquitously expressed (Strum *et al.*, 1988) and is thought to be involved in the regulation of a number of housekeeping genes such as histone H2B expression during the cell cycle [Segil *et al.*,1991(i) and (ii)]. A summary of these POU domain transcription factors and their expression patterns during development and in adults is given in table 1.2.1.

Table 1.2.1: POU domain proteins found in the five main classes. The alternative names by which these products may be referred are given. The chromosomal localization of the factors in mammalian genome and their expression patterns during development and in adult tissues are also indicated. Non-mammalian POU factors which are homologous to the proteins are also shown. (Taken from Wegner et al 1993)

(ES, embryonic stem cell; EC, embryonic carcinoma cell)

Class	Mammalian POU	Genomic organization	Expression		non-mammaliam homologue
			Embryo	Adult	
I	Pit-1 (GHF-1)	mouse chr16	neural tube pituitary	Pituitary	pit-1 (salmon)
II	Oct-1 OTF-1 OBP100 NFA-1 NFIII	mouse chr1 human chr 1	ubiquitous	ubiquitous	dOct-1/ (pdm-1/2), dPOU-28) (drosophila)POU-19. Oct-1(chicken) (xenopus)
	Oct-2 (OTF-2) (NF-A2)	mouse chr 7 human chr 19	neural tube. brain	lymphoid cells nervous system intestine testis kidney	
	Skn-1a/i (Oct11)	mouse chr 9	developing epidermis	epidermis	
III	Brn-1	mouse chr 1	developing nervous system	central nervous system kidney	zfPOU1 (zebra fish) XLPOU1/2 (xenopus)
	Brn-2 (N-Oct3/5)	mouse chr 4	developing nervous system	central nervous system glioblastoma neuroblastoma	Cf1a (drosophila) Ceh-6 (C.elegans)
	Brn-4 (Rsh2) (N-Oct-4)	mouse chr X	neural tube	central nervous system (forebrain)	
	Tst-1 (SCIP) (Oct-6)	mouse chr 4	blastocyst ES/ EC cells brain	nervous system myelinating glia testis	
IV	Brn-3a (Brn-3.0) (RDC1)	mouse chr 14 human chr 13	developing nervous system (brainstem, spinal cord, sensory ganglia and retina)	nervous system retina sensory ganglia neuroepithelioma Ewing's sarcoma	unc-86 (C.elegans) I-POU/ tI-POU (drosophila)
	Brn-3b (Brn-3.2)	mouse X-chr	developing brain sensory neurons retina	nervous system sensory neurons retina ovary, testis uterus, cervix	
	Brn-3c (Brn-3.1)	mouse chr 18	sensory neurons DRG, trigeminal ganglia	sensory neurons DRG, trigeminal ganglia	
V	Oct-3/4 (Oct-5)	mouse chr 17 human chr 6	ES / EC cells embryonic ectoderm primordial germ cells testis and ovaries	oocytes	Oct-60 / 25/ 91 (Xenopus)
VI	mPOU		developing brain	skeletal muscles brain, heart, lung	POU-C (zebrafish)
	Brn-V	mouse chr 15	developing brain spinal cord	brain, heart, lung, kidney testis, adrenal anterior pituitary	

The complexity of this protein family is further increased by post-transcriptional processing such as alternative splicing, a process which yields a number of isoforms as a result of differential processing of the primary messenger RNA transcript . For example, a number of Oct-2 isoforms which are generated by alternative splicing of the primary Oct-2 messenger RNA molecule have been identified (Hatzopoulos *et al.*, 1990; Wirth *et al.*, 1991). The various spliced products demonstrate distinct expression patterns in both neuronal and non-neuronal cells and also appear to have different effects on transcriptional activity of promoters (Wirth *et al.*, 1991: Dent *et al.*, 1991, Lillycrop and Latchman, 1992). Thus, this process may generate different functional products of a specific protein in the different cells. Furthermore, the use of alternative translation start sites also provide different functional proteins from a single mRNA transcript as illustrated by the two isoforms of Pit-1[Voss *et al.*, 1991(ii)] as well as Brn-3a and Brn-3b (Theil *et al.*, 1993) (see 1.3.2).

1.2.9 Functions of POU domain proteins

The highly conserved nature of the POU domain as well as the distinct spatial and temporal pattern of expression of these proteins during development and in adult tissue suggest a critical role in regulation of specific programs of gene expression which define the appearance of cellular phenotypes during organogenesis. This has been reinforced by study of developmental mutants and the finding that a number of POU domain genes have been mapped near loci of genetic developmental disorders (reviewed in Rosenfeld, 1991; Wegner *et al.*, 1993; Verrijzer and Van der Vilet, 1993). For instance, mutations have been identified in the *unc-86* gene in the nematode, *C.elegans*, which resulted in the failure of some neuronal precursor cells to differentiate with consequent absence of specific cells such as touch receptors (Chalfie and Au,1989; Finney and Ruvkun, 1990). Similarly, the *pit-1* gene which was mapped to chromosome16 in mice, was shown to be the candidate for mutations found in the dwarf (*dw*) locus in genetically transmitted Snell and Jackson

dwarf mice (Li *et al.*, 1990). In the *Jackson dwarf* (dw^J) mice the mutation resulted in loss of gene expression with consequent deficiency in growth hormone and prolactin synthesis and hypoplasia of cells that express Pit-1 in normal pituitary while in the *Snell dwarf (dw)* mice a point mutation in the POU domain resulted in dysfunctional Pit 1 expression. In humans a non-sense mutation of the *pit-1* gene present in both alleles of the affected individual was reported to produce a truncated protein lacking the POU domain resulting in cretinism with combined pituitary hormone defiency (Tatsumi *et al.*,1992). Recent studies have also demonstrated that the X-linked mixed deafness (DFN3) is associated with mutations in the gene encoding Brn-4 protein. Deletions in the POU domain which result in frameshift and premature stops in the translation of Brn-4 proteins or with non-conserved amino acid mutations in the POU domain of this protein have been identified in a significant number of patients with this condition (de Kok *et al.*,1995).

Although all POU domain factors act as transcriptional regulators some are also involved in DNA replication and cellular proliferation. For instance, Pit-1 was shown to be required for proliferation somatotrophic cell line during development as well as for establishment and maintainance of the differentiated phenotype (Li *et al.*, 1990; Castrillo *et al.*, 1991). Furthermore, in-vitro studies indicated that purified POU proteins such as Pit-1, Oct-1, Oct-2. Oct-4 and Oct-6 can stimulate adenovirus DNA replication [Verrijzer *et al.*, 1992 (iii)] while the POU domain of either Oct-1 or Oct-2 was sufficient to mediate this process (Verrijzer *et al.*, 1990) . This process may require the POU-specific domain since the POU-homeo domain on its own inhibited replication [Verrijzer *et al.*, 1992 (iii)]. Binding of the Oct-1 POU-specific domain to the DNA was also shown to induce bending and may thus provide a basis for unwinding of the DNA required for initiation of replication (Verrijzer *et al.*, 1991). Although the exact mechanism by which these proteins support DNA replication remains to be elucidated, it is thought to be a distinct process from the transcriptional regulation and may either involve conformational changes induced by the binding of the

POU domain or protein-protein interactions with other replication factors leading to formation or stabilization of a DNA replication complex.

1.2.10 Protein-Protein interaction by POU-domain proteins

In addition to its role as a DNA binding domain, the POU-domain also provides an interface for functionally important interactions with other proteins (reviewed in Rosenfeld, 1990; Verrijzer and van der Vilet, 1993; Wegner *et al.*, 1993). Many POU domain proteins have relatively weak inherent transactivation potential which is overcome by a number of strategies such as association with co-activators, multiple binding sites for the same factors on a cellular promoter or by cooperative protein-protein interactions. These interactions may require either the POU domain [Aurora and Herr, 1992; Verrijzer et al., 1992 (i)] or may be mediated via other domains such as the activation/repressor domains (Tanaka *et al.*, 1992: Verrijzer and van der Vilet, 1993)

The Oct-1 / VP16 activation of HSV immediate-early gene expression illustrates the association of Oct-1, a weak transactivator which binds DNA with a strong activator, VP16 which cannot bind DNA (Goding and O'Hare, 1989; Verrijzer and van der Vilet, 1993). The specific amino acids in the Oct-1 POU domains which are important for interactions between Oct-1 and the HSV trans-activator. VP16, have been mapped to within the first two helices of the POU-homeo domain (Stern *et al.*,1989; Lai *et al.*,1992).

Cooperative interactions of these proteins on the gene promoter also determine their activity. This can be achieved by homo-dimerization or hetero-dimerization with other POU domain proteins or with other factors whose binding sites overlap or are in close proximity to that of the POU domain protein (Shaufele *et al.*, 1990; Rosenfeld, 1991; Verrijzer and van der Vilet, 1993; Wegner *et al.*, 1993). The POU domain appears to be critical for these protein-protein interactions which [Verrijzer *et al.*, 1992(i)]. POU domain proteins have

been shown to be largely monomeric in solution but they can bind their DNA sequence elements as monomers, or cooperatively as dimers. This is demonstrated by Pit-1 which can bind to its recognition site as a monomer or as homo-dimers in DNA-dependent interactions as observed by the ability to cross-link Pit-1 dimers when bound to a DNA site but not in solution (Ingraham *et al.*, 1990). However Pit-1 can also heterodimerize with Oct-1 to synergistically activate the prolactin gene promoter in-vitro. These interactions are mediated via the POU domain and can occur in the absence of DNA (Voss *et al.*, 1991). Similarly the POU domain proteins coded for by unc-86 and mec-3 genes bind to the mec-3 promoter cooperatively to specify neuronal differentiation in the nematode, C. elegans and mutation which abolishes the site for unc-86 binding on this promoter prevents touch receptors (Xue *et al.*, 1993). Furthermore. results of studied by Lillycrop and collegues suggest that dimerization of the alternatively spliced Oct-2 isoforms which are differentially expressed in sensory neurons and which may have distinct transactivation potential may profoundly affect gene expression (Lillycrop and Latchman,1992; Lillycrop *et al.*, 1993).

Many target genes have multiple sites for a specific factor to enhance the effect by combinatorial effects. For example, the prolactin gene promoter contains four Pit-1 sites which can accomodate Pit-1 dimers to confer synergistic Pit-1 dependent, cell-specific expression (Kapiloff *et al.*, 1991; Voss *et al.*, 1991).

1.2.11 Cooperative binding involving sites outside the octamer motif

Interactions of the POU-domain proteins with DNA are not restricted to bases within the octamer sequence elements but can be influenced by sequences flanking this octamer motif such as the heptamer sequence identified in the immunoglobulin H, (IgH) gene promoter (Kemler *et al.*,1989; Poellinger and Roeder,1989). Whereas Oct-2 binds the the octamer motif in this promoter with high affinity as a monomer, cooperative binding of another Oct-

2 protein to the adjacent heptamer site is necessary for the high level of activity of the immunoglobulin promoters (LeBowitz *et al.*, 1989; Ingraham *et al.*, 1990). This strong activition is lost if the octamer or heptamer sites are mutated. The presence of such an additional site which contributes to the specificity and activity of these proteins therefore allows a certain degree of degeneracy within the DNA-binding motif in the different gene promoters modulated by these factors.

1.2.12 Interactions with cell specific factors

The POU domain also permits the association of other factors which are expressed in a highly restricted manner and which contribute to the regulation of gene expression by the POU protein. For instance, the lymphoid specific octamer coactivator from B cells (OCA-B) which is a tissue and promoter specific factor interacts with Oct-1 or Oct-2 to activate transcription from the immunoglobulin gene. This factor has been detected only in B-cells and could not activate the histone H2B promoter regulated by Oct-1 (Luo *et al.*, 1992).

More recently another tissue specific protein, the octamer binding factor-1 (OBF-1) which associates specifically with the POU domain of Oct-1 and Oct-2 but not Oct-3 or Oct-4, has been isolated from B cells (Strubin *et al.*, 1995). This protein cannot bind DNA but interacts with the POU domain of both Oct-1 and Oct-2 in a site dependent manner for highly specific modulation of gene activity. These results suggest that many cell specific factors may interact with the POU domain proteins at particular sites to facilitate transcriptional activity.

1.2.13 Interactions with the basal transcription complex

While the exact mechanisms by which the POU domain transcriptional activators mediate their effect still remain to be elucidated, results of experiments with Oct-2 have shown that there may be direct interactions with general transcription factors. In-vitro studies have

demonstrated that Oct-2 is recruited to the TATA box binding protein (TBP) in the basal transcriptional machinery during early assembly of the pre-initiation complex. Studies using deletion constructs suggested that these interactions involved the POU domain of Oct-2 and the evolutionarily conserved C-terminal domain of the component of the general transcription factor, TFIID (Zwilling *et al.*, 1994). In addition, Oct-2 is continuously required at the promoter for multiple rounds of transcription (Arnosti *et al.*, 1993).

1.2.14 Activation and Repressor domains

Activation or repressor domains which are distinct from the POU domain and which contribute to the regulation of tissue-specific gene expression have also been identified. Although there is little sequence homology within these domains, many have been found to be rich in specific amino acid residues, such as the serine / threonine rich domains of Pit-1 (Ingraham, *et al.*, 1990; Theill *et al.*, 1992), the glutamine rich activation domains of Oct-1 and Oct-2 (Gerster *et al.*, 1990; Arnosti *et al.*, 1993; Tanaka and Herr, 1990), the alanine / glycine rich regions of Oct-6/ Tst-1(Meijer *et al.*, 1992) or the proline rich domains in Oct-3 (Imagawa *et al.*, 1991).

These domains may be involved in mediating direct contact with factors in the PIC or in binding and recruitment of other secondary factors which are critical for functional preinitiation complex assembly. Studies with Oct-2 has shown that in addition to the POU domain binding to the TBP in the PIC, the amino terminal glutamine rich domain may also interact with basal transcription factors and thus affect transcriptional activation (Gerster *et al.*, 1990). This interaction may contribute to the different effects of the various isoforms of Oct-2 on transcriptional activity mediated via the octamer motif. This is illustrated by the Oct-2.1 isoform which has been shown to stimulate all octamer containing promoters, and the Oct-2.5 protein which represses some promoters but stimulates others, depending on the octamer sequence and its context within the promoter (Dent *et al.*, 1991; Lillycrop and

Latchman, 1992). Whereas Oct-2.1 has two activation domains sited in the amino and carboxyl terminals, Oct-2.5 has lost the activation site in the carboxyl terminus due to post transcriptional processing (Schreiber *et al.*, 1988; Hatzpoulos *et al.*,1990; Wirth *et al.*, 1991). The ability of Oct-2.5 to activate only some promoters therefore suggests that the promoters have different requirement for the interactions mediated by different transactivation domain (Lillycrop and Latchman, 1992). The interactions by these activation domains also helps to specify the promoters which are regulated by the POU proteins such as Oct-1 and Oct-2 which recognise a similar DNA octamer motif (Tanaka *et al.*, 1992).

A region in the amino terminus of the Oct-2 proteins which may act as a repressor of transcriptional activity has also been identified (Lillycrop *et al.*, 1993). In addition, the presence of other domains such as the leucine zipper domains which may mediate protein-protein interactions have also been identified in the Oct-2.1 protein (Lillycrop and Latchman, 1992) suggesting that a complex interaction of a number of factors achieve the final transcriptional effect by these factors. The ability of the POU domain proteins to interact with other proteins therefore provides a mechanism for the regulation of a large pool of target genes which can be differentially expressed depending on the presence of various combinations of these regulators.

1.2.11 Phosphorylation and POU protein function

In addition to the properties conferred upon the POU domain proteins by their basic physical structure, post-translational modification such as phosphorylation of the proteins can profoundly affect their properties and responses. Phosphorylation of these proteins may either change the DNA binding characteristics or the ability of these proteins to interact with other proteins (reviewed in Jackson, 1992; Hunter and Karin, 1992, Karin, 1992). For instance, phosphorylation of specific threonine residues in the Pit-1 POU-homeo

domain changes the DNA binding activity as well as in its ability to dimerize depending on the recognition site. This in turn modulates the function of this protein on different target promoters such that there is decreased binding affinity for the GH-1 element, no change for its positive 5'*pit-1* auto-regulatory site and increased binding to proximal prolactin site, 1D (Kapiloff *et al.*, 1991).

Similarly, the Oct-1 protein, which is involved in the cell cycle regulation of histone H2B gene transcription, is extensively phosphorylated by protein kinase A (possibly on the Ser^{385} residue in the POU-homeo domain) as the cells enter mitosis. This cell cycle specific phosphorylation is reversed as the cells exit mitosis and enter the growth phase [Segil *et al.*, 1991(i) and (ii)]. Mitotic phosphorylation of Oct-1 corresponds with a general inhibition of transcription that occurs during mitosis.

Phosphorylation of specific residues also appears to alter the transactivation potential on different promoters. For instance, the ability of Oct-2 to activate the transcription of the β-globin gene promoter depend on the phosphorylation of the both transactivation domains in the protein. Oct-1 which lacks the carboxyl terminal domain failed to activate this promoter (Tanaka and Herr, 1990). It seems likely that conformational changes resulting from site-specific phosphorylation in these proteins may contribute to transcriptional activity.

1.3.0 POU IV subclass of POU domain proteins

The identification of a large number of POU domain proteins by He *et al.*, (1989) was achieved using a PCR strategy whereby degenerate oligonucleotides, corresponding to the highly conserved regions of the POU domain, were used to amplify cDNA synthesized from poly (A) RNA extracted from human and rat brain and rat testis. The POU domain of Brn-3a, (originally called Brn-3) was one of the clones isolated from brain cDNA. This has since been detected in sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglia and the sensory nuclei in the brain stem.

The POU domain of Brn-3a is closely related to that of the nematode *unc-86* gene product indicating a strong evolutionary conservation of the POU proteins. Unc-86 was defined in a series of *C.elegans* behavioural mutants as a determinant of the developmental fate of some neuronal cell. Mutations have been identified in the *unc-86* gene which prevent the normal development of mechano-receptor sensory neurons (Finney *et al.*, 1988; Chalfie and Au, 1989) and the maturation of hermaphrodite-specific motor neurons (Desai *et al.*, 1988). The high sequence homology between Unc-86 and the Brn-3a suggest that the Brn-3 protein may be the mammalian homologue of Unc-86 and thus implies that it may have a role in sensory neuronal development and function.

Subsequent studies in our laboratory have led to the identification of a novel POU factor in the sensory neuronal cell line, ND7, which is closely related but distinct from Brn-3a in the POU domain and was therefore referred to as Brn-3b (Lillycrop *et al.*, 1992). Since then another factor, Brn-3c, which displayed high homology with Brn-3a and Brn-3b in the POU domain has also been isolated from a DRG cDNA library and characterized by Ninkina *et al.*, (1993) (figure 1.3.1).

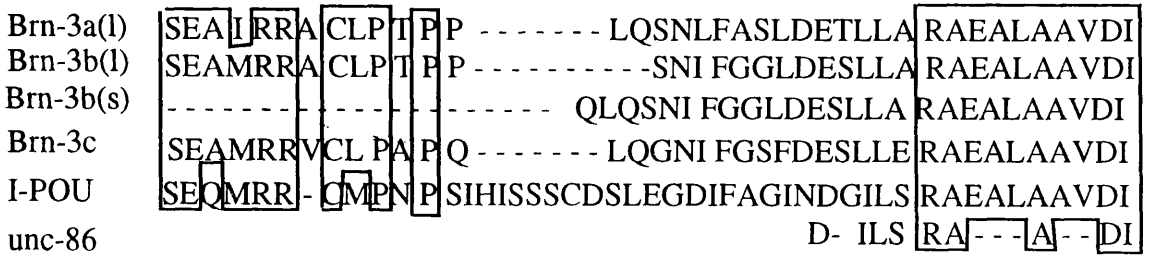
These Brn-3 proteins share particularly high homology in the POU domain with a number of other POU domain proteins such as nematode Unc-86 (86% homology), and the Drosophila proteins, inhibitory POU (I-POU) and twin I-POU (tI-POU) (83% homology) and thus constitute the POU IV sub class proteins.

1.3.1 The POU-IV box

Proteins in the POU-IV group also share another region of significant homology, the POU IV box, a stretch of about 100 amino acids found at the amino terminus that appear to be well conserved in all members of the POU IV subfamily (Theil *et al.*, 1993, Gerrero *et al.*, 1993). There is also some homology to a highly conserved domain found in the amino terminus of all c-myc family members (figure 1.3.1) which confers microtubule associated protein 2 (MAP-2) kinase dependent transactivation to the c-myc proteins (van-Beneden *et al.*,1986).

The functional significance of this domain in the POU IV proteins is being intensively investigated since the strong conservation and its putative role in the c-myc family of proteins suggest a critical function. It has been speculated that this POU IV box may be important for regulating the tissue-specific gene expression by their interaction with other co-transactivators. In the Brn-3 proteins this region was found to be critical for transactivation and transformation by Brn-3a (Theil and Moroy, 1994). This will be discussed later.

(A)



(B)

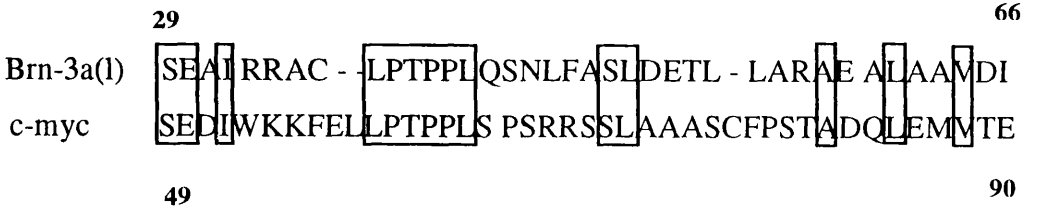


Figure 1.3.1:(A) Comparison of the amino acid sequences in the region of the POU-IV box at the amino terminal of the different Brn-3 proteins with the other members of the POU-IV family, the Drosophila I-POU and the nematode, C.elegans unc-86. The regions of homology are indicated by the boxed areas.
(B) The sequence homology within the POU-IV box of Brn-3a(l) and a conserve domains in c-Myc (from amino acid 49 - 90). (Modified from Gerrero *et al.*, 1993; Theil *et al.*, 1994 and Turner *et al.*, 1994).

1.3.2 Brn-3 POU-domain protein

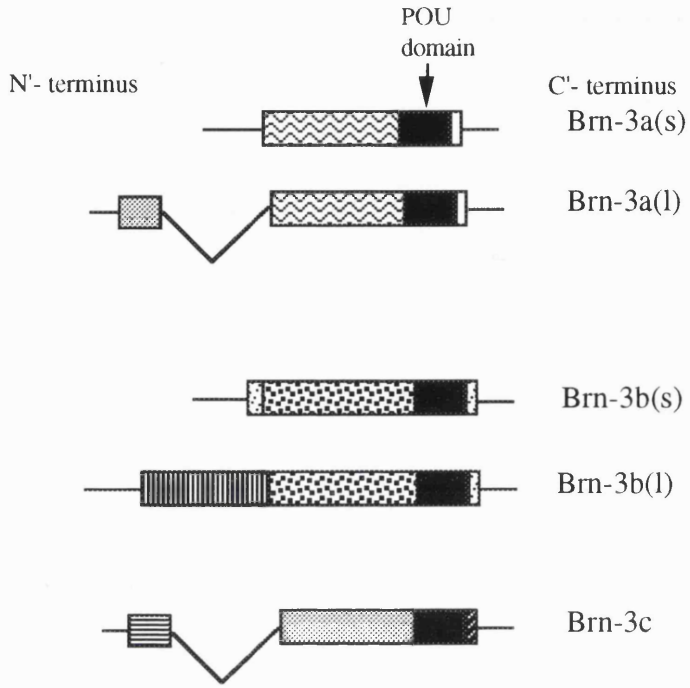
The subsequent identification and sequencing of the full length clones for both Brn-3a and Brn-3b revealed that there are two distinct isoforms of both Brn-3a and Brn-3b which differ in the amino terminal region (figure 1.3.2) (Theil *et al.*, 1993; Theil and Moroy, 1994; Gerrero *et al.*, 1993; Turner *et al.*,1994). In addition, Collum *et al.*, (1990) fortuitously isolated RDC-1, the human homolog of rat Brn-3a, following low stringency hybridization of human placental DNA with the probes from exons 2 and 3 of the human N-myc gene. To date only a single product for the Brn-3c gene has been identified.

1.3.2(i) Isoforms of Brn-3a

Transcription of the murine Brn-3a locus yields two transcripts which can be translated into long and short forms of the proteins, Brn-3a(l) and Brn-3a(s) respectively [figure 1.3.2(ii)]. The start sites of the two Brn-3a transcripts are located about 500 base pairs apart on the genomic sequence suggesting that two different promoters may be used for transcription of this locus. It is therefore possible that the expression and activity of these isoforms of Brn-3a may be differentially regulated.

A comparison of the Brn-3a(l) cDNA with the genomic clone reveals an intron and an additional exon at the 5' end which is absent from the cDNA of the Brn-3a(s). This additional exon gives rise to mRNA with a coding capacity of 421 amino acid residues and molecular weight of about 43 kDa. The shorter cDNA clone which lacks 84 amino acids at the amino terminus compared with Brn-3a(l) is entirely co-linear with the genomic sequence and constitutes an open reading frame of 1011 nucleotides capable of encoding a protein of 337 amino acids with a predicted molecular weight of approximately 33.5 kDa.

(A)



(B)

	protein (amino acids)	molecular weight (kDa)
Brn-3a(l)	421	33.5
Brn-3a(s)		43.0
Brn-3b(l)	332	35.0
Brn-3b(s)	411	42.0
Brn-3c	338	43.0

Figure 1.3.2(i): (A) Schematic representation of the process by which the different isoforms of Brn-3a and Brn-3b are generated by alternative splicing and usage of alternative translational sites respectively. A single known Brn-3c is also represented. (adapted from Theil *et al.*, 1993). **(B)** A summary of the predicted length of each protein and the approximate molecular weights

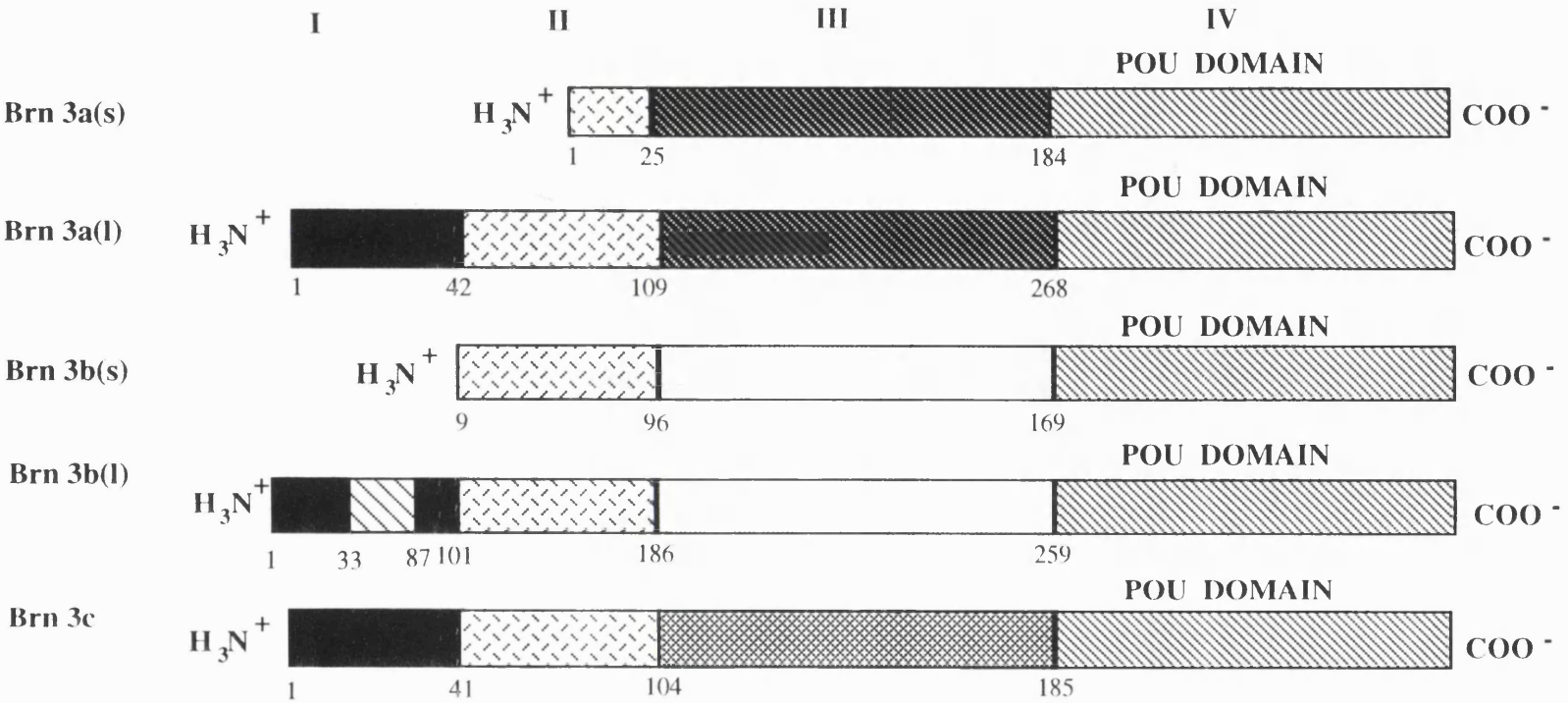


Figure 1.3.2 (ii): Schematic representation of the different domains present in the isoforms of Brn-3a Brn-3b and Brn-3c. Regions of high homology within the four domains (I- IV) are depicted by the same pattern. The numbers represent the positions of the amino acids delineating the borders of the subdomains. (Taken from Theil *et al.*,1994).

1.3.2(ii) Isoforms of Brn-3b

Two isoforms of Brn-3b generated from a single gene transcript as a result of usage of alternative translation initiation sites have also been reported. Like Brn-3a, the two isoforms differ in the amino terminus. The longer isoform was predicted to consist of 411 amino acids with an apparent molecular mass of 42 kDa (Turner *et al.*, 1994), while the shorter isoform, Brn-3b(s) is a protein of 322 amino acids with predicted molecular weight of 35 kDa (Theil *et al.*, 1993). Due to different location of exon / intron borders in the Brn-3b gene, the Brn-3b(s) protein has acquired nine amino-terminal amino acids which it does not share with the Brn-3b(l) protein (figure 3.1.2).

1.3.2(iii) Isoforms of Brn-3c

To date, one transcript arising form the Brn-3c locus has been identified. Brn-3c shows a very similar structure with almost identical exon / intron position as Brn-3a(l). Brn-3c transcripts codes for a protein which should be approximately 338 amino acids (43 kDa).

1.3.3 POU-IV BOX in the Brn-3 proteins

There is limited sequence homology outside the POU domain which is present at the extreme carboxyl terminus of all three proteins and the POU IV box at the amino terminus which is particularly well conserved between the Brn-3a(l), Brn-3b(l) and Brn-3c proteins (figure 1.3.1). Whereas the short isoform of Brn-3a lacks the entire POU IV box Brn-3b(s) has a truncated sequence. The domain containing the POU-IV box has been shown to be crucial for the long Brn-3a isoform, Brn-3a(l), to transform primary fibroblasts in cooperation with activated Ha-Ras. The results of studies using chimeras containing different domains of Brn-3a and Brn-3b indicate that the amino terminal is necessary but not sufficient for transformation (Theil and Moroy, 1994). This POU IV domain may therefore represent an interface for interaction with other co-activators. In agreement with this Brn-3a(s) or Brn-3b(s) could bind DNA but lacks any transformation potential.

Furthermore, in co-transfection studies both Brn-3a(s) and Brn-3b(s) inhibit the transformation potential of Brn-3a(l). This may be potentiated by competion for the binding site by Brn-3a(s) or by interactions of Brn-3b(s) with Brn-3a(l) which preclude Brn-3a(l) activity. In-vitro binding assays have shown that Brn-3b(s) which binds DNA more weakly, interacts with Brn-3a(l) to decrease its binding to a target DNA (Theil and Moroy, 1994). The ability of these proteins to interact may therefore provide a mechanism for modulating their expression in cells and tissues in which they appear to have overlapping expression patterns. This will be discussed in more detail in chapter 5.

1.3.4 Putative functional differences in the POU domain

Specific activity of the Brn-3 proteins in different cells or during various stages of development might depend on their interactions with other proteins via the POU domain itself. In this region of very high sequence homology, the significance of the change in a single amino acid in helix 1 of the POU-homeo domain, where Brn-3a contains a valine while Brn-3b and Brn-3c has an isoleucine residue at this position has also to be assessed. An analogous position has been shown to be critical for interactions between Oct-1 and the herpes simplex virus transactivator, VP16. Moreover, the presence or absence of two amino acid residues in this region of the I-POU and the highly related tI-POU protein result in fundamentally different DNA binding characteristics and protein-protein interactions. These amino acid changes may therefore provide an alternative mechanism for maintaining specificity of gene expression by regulating the ability of the highly related proteins to interact with DNA or other proteins.

1.3.5 Chromosomal localization of the Brn-3 genes

Analysis of genomic Southern blots have suggested that these proteins are encoded by different genes. Chromosomal mapping by a number of groups have confirmed this by localizing Brn-3a to chromosome 14 (14E1-3) (Xia *et al.*, 1993; Theil *et al.*, 1994), Brn-3b to the X-chromosome (XF1-5) (Theil *et al.*, 1994) and Brn-3c to chromosome 18 (18B3-E1) in mouse (Ninkina *et al.*, 1993, Theil *et al.*, 1994, Xia *et al.*, 1993). To date the only human homologue which has been mapped is RDC1 (Brn-3a) and it is located on chromosome13 (13q14-22) (Collum *et al.*,1992). This is in agreement with the conserved linkage relationship between human chromosome 13 and mouse chromosome 14 (Lyon and Kirby 1993). Using comparative mapping data it was suggested that human homolog of Brn-3b maybe localized to the short arm of the human X-chromosome and Brn-3c to the long arm of chromosome 5 (Theil *et al.*, 1993).

1.3.6 Expression of the Brn-3 proteins

Some of the results in chapter 3 reports on the expression of Brn-3a and Brn-3b mRNA in various tissues and cell lines. However during this time the published works of other groups on the distribution of Brn-3a and Brn-3b have supplemented and supported our data. A brief discussion of these results will be given here.

He *et al.*, (1989) isolated the original Brn-3 POU domain which constitutes the protein we now refer to as Brn-3a and reported on the expression of the mRNA in developing brain and other tissues using the POU domain sequence as a probe. Collum *et al.*, isolated and characterized the human homologue of the rat Brn-3a, RDC -1, while Gerrero *et al.*, (1993) studied the expression of Brn-3a(l) (referred to as Brn-3.0) during development, as well as in some adult tissues and cell lines by using RNAse protection assay and in-situ hybridization. Our group initially isolated the POU domain of Brn-3b (Lillycrop *et al.*,

1992) and Theil *et al.*, (1993) later cloned the full length cDNA of both Brn-3a(l), Brn-3a(s), Brn-3b(s) and Brn-3c. Turner *et al.*, (1994) reported the isolation, characterization and expression of the long Brn-3b isoform, Brn-3b(l), (referred to as Brn-3.2) while Xiang *et al.*, (1993) identified and studied the expression of Brn-3b protein in retinal ganglion cells. Bhargava *et al.*, (1993) identified two clones in activated and PMA (phorbol 12-myristate 13-acetate) treated Jurkat T cell lines which are identical to Brn-3a (Oct-T1) and Brn-3b (Oct-T2). Nikina *et al.*, (1993) characterized the Brn-3c POU domain initially and studies were also carried out by Gerrero *et al.*, (1993) (referred to as Brn-3.1).

1.3.6(i) Brn-3a expression

The expression of Brn-3a has been studied during murine development and in adult tissues by different groups using ribonuclease protection assay or northern hybridization studies (Ninkina *et al.*, 1992; Gererro *et al.*, 1993; Theil *et al.*, 1993 and Turner et al., 1994). Brn-3a mRNA is first detected at e9.5 (embryonic day 9.5) in total embryonal RNA. By e12.5 it is readily detectable in RNA obtained from both embryonal head and body but by e 17.5 it is only detected in brain RNA. Study of the distribution in more discrete tissues show high levels of mRNA in the eye, the trigeminal ganglia and the brain in embryonal tissues which decreases in adult eye and brain but remains high in the trigeminal ganglia (Turner *et al.*, 1994).

In-situ hybridization studies have allowed a more precise localization of Brn-3a mRNA expression and the results have revealed that structures of the developing nervous system show high expression at different stages of development. For instance, at e10, Brn-3a mRNA is detected in the neural plate at very low levels but by e12.5 much higher expression is detected in the brain stem, the spinal cord and the sensory ganglia. At e15.5 there is strong hybridization to the inferior olivary complex of the hindbrain, the outer layer of postmitotic cells in the tectum of the midbrain, the interpeduncular nucleus of the

midbrain and in the trigeminal ganglia. Lower levels are also observed in the retina, the superior and inferior colliculus, the medial habenula and the brainstem. By e17.5, Brn-3a is uniquely detected in the medial habenula of the diencephalon, thus defining a more anterior limit of Brn-3a expression compared with Brn-3b in the brain (Gerrero *et al.*, 1993; Nikina *et al.*, 1993; Turner *et al.*, 1994).

In the spinal cord, Brn-3a mRNA is localized to a subset of post-mitotic neurons and many cells of lamina 4 / 5. In the dorsal root ganglion Brn-3a transcripts were detected at e13-e14, when some neurons were still dividing, and in postmitotic neurons of the neonatal animal.

Brn-3a mRNA is also developmentally regulated in the retina. There is no detectable Brn-3a transcripts in undifferentiated neuroblasts but high levels appear at the inner edges of the retinal neuroblastic layer at e15 when the ganglion cells begin to differentiate and axonal outgrowth is initiated (Gerrero *et al.*, 1993). High levels of Brn-3a mRNA are maintained at e17 and e19 but this decreases after birth.

In adult tissues the expression of Brn-3a mRNA appear to be more restricted with high expression in brain structures such as the medial habenula, the nucleus ambiguus of the hindbrain and the inferior olivary complex and more moderate expression in regions of the midbrain such as the red nucleus, the mesencephalic nucleus and the superior colliculus. Brn-3a transcripts are also detected in the retina, the trigeminal ganglia, the dorsal root ganglia and at much lower levels in the pituitary but is undetectable in tissues such as adult liver, spleen, kidney, heart, adrenal glands or lung (Ninkina *et al.*, 1993; Gerrero *et al.*, 1993; Theil *et al.*, 1993) suggesting that Brn-3a may be more characteristic of neuronal cells.

The human Brn-3a homologue, RDC-1, is also expressed in a stage and tissue specific manner during development in the neurogenic tissues such as brain, spinal cord and eye. In contrast, there is a marked absence in non-neurogenic tissues tested such as, adrenal, heart, liver, kidney and thymus during development or in adult tissues (Collum *et al.*,1992).

1.3.5(ii) Brn-3a expression in cell lines

Collum *et al.* (1992) have also reported the expression of the human RDC-1 mRNA in cell lines with neuroendocrine characteristics and found that it is readily detected in the neuroepithelioma cell line (CHP100) with lower levels found in the small cell carcinoma cell line (H510) and the monocyte cell line (U937). However RDC-1 RNA is not detectable in other cell lines derived from tumours of kidney, colon, stomach or myelomas. Both the CHP100 and H510 cell lines are derived from cells arising from the neural crest thus paralleling the restricted distribution of this factor in cells of neural crest or neuronal origin in normal tissues. Lower levels of the mRNA are detected in neuroblastomas cells compared with neuroepitheliomas possibly suggesting a link between the expression of this factor and the neurotransmitter produced by the cells since neuroepitheliomas were shown to be cholinergic while neuroblastomas were generally more adrenergic (Collum *et al.*, 1992).

Low levels of Brn-3a mRNA have been detected in the pituitary gland and in the corticotroph cell line ((AtT20) but not in somatotroph (GC), lactotroph (235, MMQ) or thyrotroph (TtT-97) cell lines (Gerrero *et al.*,1993). Detectable levels are also found in the B-cell line, BCL1, splenic B-cells and the T-cell line, EL4 but not in the macrophage like cell line, P388D or in adult thymocytes. These results indicates that Brn-3a expression is highly regulated during development and appears to be restricted to tissues with neural characteristics. It is possible that this protein may be associated with active growth and differentiation.

1.3.6(ii) Brn-3b expression

Much of the results in chapter 3 will report on the expression of Brn-3b in this study and so the summary of results given here will reflect the data of other research groups whose works have been published.

Brn-3a and Brn-3b mRNA demonstrate overlapping expression during development (Turner *et al.*, 1994). Ribonuclease protection assay using RNA from mouse tissues, Brn-3b mRNA is first detectable in the embryonic head at e10.5 and in the body at e11.5 compared with Brn-3a mRNA which is detectable at e9.5. At e17, high expression of both Brn-3a and Brn-3b mRNA is found in the developing brain but with a two fold lower level of Brn-3b transcripts compared with Brn-3a. There is a gradual decrease of mRNA encoding both proteins after birth.

In-situ hybridization in mouse embryos detects Brn-3b mRNA in the medulla of the hindbrain and in the midbrain by e13.5. By e15.5, both Brn-3a and Brn-3b mRNAs are detected in the inferior olivary nucleus of the hindbrain and in the midbrain sections with most intensive hybridization in the outer layer of the postmitotic cells but not the neuroepithelial layer of the tectum. By e17.5, Brn-3b mRNA, but not Brn-3a, is found in the interpeduncular nucleus of the midbrain, a site for exclusive Brn-3b expression in adult brain (see figure 1.3.3 for summary). Xiang *et al.*, (1993) used Brn-3b antibodies to study the protein localization in brain sections taken from a day 1 post natal rat. Results of this study shows strong signals in the cerebellum, inferior colliculus and periaquaductal gray of the midbrain, as well as in the brain stem and the pons and medulla of the hindbrain.

In the retina Brn-3b mRNA expression has been shown to be two to four fold lower than Brn-3a with the highest expression of both factors present at birth followed by a gradual decline to p30. Brn-3b expression is confined to the precursors of the ganglion cell population but is absent from the neuroblast layer of cells (Turner *et al.*, 1994). By e17.5, Brn-3b is confined to the postmitotic layer. At the protein level, Brn-3b is also shown to be localized to a subset of the ganglion cells (Xiang *et al.*, 1993).

Brn-3b mRNA expression is similar to Brn-3a in the sensory neurons of the trigeminal and dorsal root ganglia by e13.5 although Brn-3a mRNA expression preceeds Brn-3b by one to two days in the mice embryo. In the trigeminal ganglia, Brn-3b mRNA remains elevated at e17.5 then gradually decrease after birth. By e15.5, Brn-3b transcripts are also detected in the ganglion of the VIII cranial nerve and in the glossopharangeal nerve. In the spinal cord, Brn-3b mRNA appear to be widely distributed at e12.5 but by e15.5 it is confined to the intermediate gray area (Gerrero *et al.*, 1993; Turner *et al.*, 1994)

In adult tissues Brn-3b mRNA has been detected in brain structures such as the superior colliculus, the lateral interpeduncular nucleus of the midbrain and in the pontine gray. It is also expressed in the sensory neurons of the dorsal root ganglia and the trigeminal ganglia (Turner *et al.*, 1994). However, Brn-3b mRNA is not detectable in a number of adult tissues including the pituitary gland, thymus, neocortex, basal ganglia, thalamus, cerebellum, spleen, liver, kidney, heart or lung. Therefore Brn-3b also appears to be developmentally regulated with its mRNA expression pattern showing considerable overlap with that of Brn-3a both during development and in adult tissues. A summary of the expression of Brn-3b is shown in table 1.3.1. Results of the expression studies undertaken for this project will be discussed in chapter 3.

	Brn-3a		Brn-3b		
	e 15.5	Adult	e 15.5	Adult	Localization
Brain structures					
Thalamus	++	+++	-	-	medial habenula lateral habenula
Midbrain	++	+/-	+++	+++	superior colliculus inferior colliculus parabigeminal n. periaquaductal gray
-interpeduncular n.	-	-	++	+++	
-mesencephalic n.	++	+++	-	-	
Hindbrain	+++	++ /+	+++	-	brain stem (motor) nucleus ambiguus medulla pons inferior olivary n.
Cerebellum (and related)					
-red nucleus	++	++	-	-	
Other neurons					
Retina	++	++	++	+	post mitotic ganglion cells
Sensory neurons					
-Trigeminal ganglia	+++	+++	++	+	
-Dorsal root ganglia	+++	+++	++	+	
Spinal cord	++	++	++	+	Brn-3a- dorsal gray Brn-3b- intermediate gray

Figure 1.3.3: Summary of the expression patterns of Brn-3a and Brn-3b in selected neuronal cells. (-) indicates no detectable expression; +/-, equivocal signal; +, weak signal; ++ moderate signal; +++, strong signal. (n.) indicates nucleus.

(Adapted from Mandel and McKinnon. 1993 with additional data from Lillycrop et al, 1992, Ninkina et al, 1993, Xiang et al, 1993; Gerrero et al.,1993 and Turner et al.,1994).

1.3.6(iii) Brn-3c expression

Analysis of Brn3c mRNA expression in murine tissues show that the transcripts are restricted to a sub-set of sensory neurons in the dorsal root ganglia and the spinal cord (Nininka *et al.*, 1993, Gerrero *et al.*, 1993). However the transcript levels are lower compared with the other two Brn-3 proteins. Brn-3c expressing cells are first detected in the dorsal root ganglion at e12 and Brn-3c mRNA continue to be expressed throughout development (Nikinka *et al.*, 1993). Expression is restricted to a subset of neurons which may correspond to mechanosensory neurons but this has to be confirmed since the different subclasses of sensory neurons have not been not unambiguously distinguished. In the spinal cord Brn-3c mRNA is first detected at e16 in the spinal cord when a small number of post-mitotic cells in lamina 4/5 hybridizes to the probe. This pattern persists during later development also.

1.3.7 Regulation of Brn-3a and Brn-3b mRNA expression

The regulation of the expression of Brn-3a and Brn-3b mRNA has been studied in F9 teratocarcinoma cell line and in N18 neuroblastoma cell (Turner *et al.*, 1994). Addition of retinoic acid or cyclic AMP to F9 or N18 cells results in their differentiation to a neuronal like phenotype. Following addition of retinoic acid (10^{-6}M) and 0.5 mM cyclic AMP to the F9 cultures, a significant reduction of the Brn-3b mRNA levels was detected as early as two hours suggesting a direct transcriptional effect. By ninety six hours, Brn-3b had decreased to undetectable levels. This effect is reversible since addition of retinoic acid free medium resulted in the return to the previous levels. In the N18 neuroblastoma cells which express both Brn-3a and Brn-3b mRNA, retinoic acid differentiation also result in the decrease of the Brn-3b transcripts but not to the low levels observed in the F9 cells. The retinoic acid had no significant effect on the expression of Brn-3a mRNA suggesting that the control of Brn-3a and Brn-3b mRNA expression by this hormone may not be dependent on cellular factors but may reflect differences in promoter or enhancer elements

recognised by these two factors. This may provide a possible mechanism for the differences between Brn-3a and Brn-3b mRNA expression observed during development. Gerrero *et al.*, (1993) has reported the expression of Brn-3a but not Brn-3b in the T-cell line, EL-4, which show a transient decrease after 1 hour of stimulation of these cells with PMA. In contrast, Bhargava *et al.*, (1993) reported strong stimulation of Brn-3a (Oct-T1) expression in the human T-cell line, Jurkat, following PMA treatment. A significant increase is observed after eight hours, peaking after 36 hours and remaining high up to 62 hours. This may indicate a bi-phasic response to PMA in these cells whereby there is a transient decrease followed by the sustained high levels observed. Brn-3b (Oct-T2) is expressed at very low levels in untreated Jurkat cells and remained at a low but with detectable level up to 62 hours following treatment (Bhargava *et al*, 1993).

Brn-3a has been shown to activate the corticotropin-releasing hormone (CRH) gene promoter (Gerrero *et al.*, 1993) as well as a reporter construct containing various octamer sequences (Theil *et al.*, 1993; Morris *et al.*, 1994 and results chapter 4 and 5). However Bhargava *et al.*, (1993) have shown that this protein is capable of repressing the activity of the IL-2 promoter when co-transfected into activated Jurkat cells. This may therefore reflect different activity depending on the cell type and other factors which may be present in the cells (discussed in chapter 5).

1.4.0 Characteristics of sensory neuronal cells and cell lines

The nervous system consist of many types of neurons that differ structurally and biochemically in features such as shape, pattern of connectivity, neurotransmitter, receptors and channels. In addition, neurons display remarkable plasticity both during development and during the subsequent remodelling of synaptic connection. During development, the neural crest cells from which sensory neurons are derived are also the precursors for a large number of other cellular phenotypes including the neurons and glia cells of the sympathetic and parasympathetic nervous system, the adrenaline producing cells (medulla) of the adrenal glands, the pigment containing cells of the epidermis and skeletal and connective tissue components of the head (in Gilbert, 1994).

While the molecular mechanisms involved in this process are not fully understood, a number of factors which influence cell commitment and differentiation have been identified and widely studied. These include (i) the pattern of the crest cells migration, (ii) the local neuronal environment determined by trophic factors which are encountered by the growth cone of the developing neuron, (iii) the expression of structural and secretory proteins which mediate growth cone development and (iv) migration of the neurite and establishment of functional synaptic complexes (Black, 1984).

Although the mechanisms for generating the complexity and diversity of nervous system are not fully understood it is possible that an interplay of environmental factors and intrinsic changes in the expression pattern of factors within the cells may determine cell fate (Anderson, 1994). This is achieved by a precise and highly regulated process which involves the expression and combinatorial interactions of a large number of transcription factors with diverse sequence specific interactions which are modified in response to a variety of environmental cues and intracellular signals at any point in this process (Johnson

and McKnight, 1989; Kessel and Gruss,1990; He and Rosenfeld, 1991; Struhl, 1991; Scholer, 1991; Treacy and Rosenfeld, 1992).

The detection of Brn-3a in the neural plate at during early development, together with the restricted distribution at later stages suggests a role in cell commitment. Furthermore, the presence of Brn-3a, Brn-3b and Brn-3c in the sensory neurons of the dorsal root ganglia and trigeminal ganglia and their homology to *unc-86*, a determinant of neuronal specificity in the nematode, imply that they may have an important role in the development and function of these cells.

Regulatory factors such as the Brn-3 proteins may control the expression of specific proteins which are involved in cell commitment during development. One strategy of identifying the target genes modulated by these proteins may be to study proteins which show a similar pattern of expression to the Brn-3 proteins and which contain the DNA recognition sequences for these proteins in their promoters. A number of such factors have been investigated as possible targets and the results are discussed in chapter 5. A brief outline of the expression and the putative functions of groups of these proteins which may have a role in determining the development and differentiation of sensory neurons will be given to clarify aspects of this process and to highlight the areas of interest in this study.

1.4.1 Trophic Factors and Neuronal Development

During development of the nervous system, cells acting as targets for developing neurons produce limiting amounts of specific molecules, termed neurotropins, which are required for the survival of the neurons and for which the nerve terminals appear to compete (Barde, 1989; Oppenheim, 1991; Anderson, 1993). In the target field, competition for limited quantities of neurotropins result in the survival of some neurons while others are eliminated by apoptosis, a process which occurs during the period immediately following the arrival of the axons (Martin *et al.*, 1988; Johnson and Deckworth, 1993; Oppeinheim, 1994).

Neurotropins so far charactrized include: Nerve Growth Factor (NGF), Brain Derived Neurotrophic factor (BDNF), neurotropin 3 (NT-3) and ciliary neurotropic factor (CNTF) (Barde, 1989; DiCicco-Bloom *et al.*, 1993; Ernfors *et al.*, 1994 Barbacid, 1995). The neurotropins and other factors such as Fibroblast Growth Factor (FGF) and platelet derived growth factor (PDGF) appear to have a variety of actions on their physiological targets which include the sympathetic and sensory neurons including promoting neuronal survival (Lindsay, 1988; Davies *et al.*, 1991; Ernfors *et al.*, 1994; Barbacid, 1995). These factors have been shown to profoundly affect the expression of a number of transcription factors. This is illustrated by the NGF treatment of PC12 cells which causes a rapid induction of immediate-early genes encoding transcription factors such as c-Fos and NGFIA (Milbrant, 1986; Sheng and Greenberg, 1990; Armstrong and Montminy, 1993; Kendall *et al.*,1995). Similarly in sensory neurons, NGF has been shown to regulate the expression of the neuronal Oct-2 proteins (Wood *et al.*, 1992; Kendall *et al.*, 1995).

While neurotrophic factors such as NGF are required by a subpopulation of neural crest derived neurons for survival during development, in the post-natal period NGF appears to be more important for the normal biochemical function of sensory neurons (Johnson *et al.*, 1986; Lindsay, 1988, Ruitt *et al.*, 1992; Kang and Schuman, 1995). During development, an important process which the trophic factors play a role in is mediating the promotion and maintainance of neurite outgrowth and axonal arborization. A brief discussion of the mechanism by which NGF achieves this effect will be given since it underlies a number of other changes accompanying process formation in the maturing neuron.

1.4.2 Neurite outgrowth and structural proteins

While the neurites of developing neurons must be capable of exploratory and locomotive functions, they must also maintain a stable and fairly rigid shape. Structural proteins such as the tubulins and actin filaments are modified and rearranged to facilitate the changes in

the labile growth cones and to achieve the more stable structures seen later (Diaz-Nido *et al.*, 1991, Langford, 1995, Mandelkow and Mandelkow, 1995). Factors such as NGF appear to regulate both growth cone movement and neurite stability (reviewed in Thoenen and Barde, 1980; Greene *et al.*, 1984; Barbacid, 1995). A number of groups have shown that the neurites of sympathetic neurons cannot grow or be maintained *in-vitro* in the absence of NGF and withdrawal of NGF from *in-vitro* cultures resulted in the degeneration of the neurites already present in the field (reviewed in Anderson, 1993).

The mechanism by which neurite outgrowth is accomplished may be viewed as comprising of two separate and distinguishable components (Greene *et al.*, 1984). The first process involves a rapid, transcription independent regulation of growth cone shape, motility and locomotion. The second mechanism entails a slow, transcription dependent stabilization of the neurites resulting from the re-organization or modification of the microtubule proteins and the microtubule associated proteins (MAPs). The assembly of microtubules which are stabilized by the MAPs are required for the inner cytoskeletal scaffolding of neurites and provides the basis for axonal transport (Drubin *et al.*, 1985; Hernandez *et al.*, 1989; Mandelkow and Mandelkow, 1995). Phosphorylation of different microtubule components such as tubulin, MAP1B factor and tau-related proteins in neuroblastoma cells has been demonstrated to parallel the assembly of dynamic microtubles in the growth cones and their progressive stabilization within the neurite shaft (Diaz-Nido *et al.*, 1991; Mandelkow and Mandelkow, 1995).

In addition, structural proteins such as the intermediate filaments which are present in most neurons of the central and peripheral nervous system are thought to play a role in maintainance of the structural integrity of the cells during development and differentiation (reviewed in Flienger and Liem, 1991; Raats and Bloemendal, 1992; Fuchs and Weber, 1994; Klymkowsky, 1995). These proteins are differentially regulated throughout

development and in the mature neurons and may be involved in functions such as axonal transport in neuronal cells. Some widely studied intermediate filament proteins include, the neurofilament (NF) proteins, NF-L, NF-M and NF-H (low, medium and high molecular weight subunits, respectively), peripherin, α-internexin and vimentin. NF-L, NF-M and NF-H are expressed predominantly in the axons of mature neurons of the central and peripheral nervous systems. Results of recent studies have demonstrated that disruption of neurofilaments such as NF-H expression may be associated with neuro-degeneration (Collard *et al.*,1995). Peripherin is found mainly in the peripheral nervous system and in CNS neurons with peripheral projections. The α-internexin protein is expressed in most neurons of the CNS and developing PNS, while vimentin is highly expressed in early development but demonstrates a restricted pattern subsequently. The α-internexin gene promoter has been shown to be regulated by Brn-3a and Brn-3b and a more detailed discussion will be given in chapter 5.

Other proteins such as the growth associated factor, GAP-43, have been shown to induce filipodia formation even when transfected into non-neuronal cells (Zuber *et al.*, 1989). This factor may exist either as a soluble protein or membrane bound components of the growth cones which form the tips of elongating axons in developing neurons (Basi *et al.*, 1987; Zuber *et al.*, 1989; Strittmatter *et al.*, 1990; Baetge and Hammang, 1991). GAP-43 shows restricted expression pattern, being highly expressed in neuronal cells during normal development but decreasing sharply as the neurons mature. The expression of this protein is also upregulated during successful nerve regeneration following injury of peripheral nerves (Strittmatter *et al.*, 1992). This increased expression occurs primarily at the mRNA level indicating that it is a direct target for the regulatory pathway conveying information about axon integrity to the neuron cell body. GAP-43 expression remains high only in defined parts of the adult brain such as the hippocampus, an observation which supports the theory that this protein may be involved in the remodelling and placticity that

accompanies learning and memory.

1.4.3 Cell Adhesion Molecules

During development, neurites extend across different surfaces often over considerable distances to reach the innervation site or target field. This is achieved by the extension of filopodia by the growth cones. These transient, dynamic structures make contact with varying degrees of adhesion to the surfaces through which they traverse to reach their targets. They can progress or retract depending on environmental stimuli which may contain diffusable chemoattractants, such as netrins (Serafini *et al.*, 1994), or molecules which have been shown to mediate growth cone collapse, such as collapsin (Luo *et al.*, 1993) or nitric oxide (Hess *et al.*, 1993).

The firm adherence of the filopodia to the surface determines the direction of innervation and this is partly governed by cell adhesion molecules (CAMs) (reviewed in Doherty and Walsh, 1994). These molecules are present on the neuronal growth cone and bind to CAMs on other axons or in non-neuronal cells. Many CAMs such as NCAM (neuronal CAM), N-cadherin and L1 glycoprotein have been shown to promote axonal growth and migration over cells such as the Schwann cells, astrocytes and muscle cells . Stable adhesion involves the accumulation of the CAMs at the sites of cell-cell contact and their direct or indirect binding to the actin based cytoskeleton (Martini *et al.*, 1994). The binding of CAMs promotes neurite outgrowth by activating second messenger pathways rather than modulating adhesion per se (Doherty and Walsh, 1994). Calcium appears to be a principle second messenger involved in the promotion or inhibition of axonal growth and migration by the filopodia in the migratory processes and this may be mediated by the production of arachidonic acid (Williams *et al.*, 1994).

There is also evidence for the role of these molecules in stabilizing synaptic connections and hence inhibiting plasticity, depending on the interactions with other factors or alternative splicing which gives rise to slightly different N-CAM with different properties (Doherty and Walsh, 1994). The expression of N-CAM was shown to be regulated by the homeobox *Hox* genes in-vitro (Jones *et al.*, 1992) suggesting that these transcription factors may play a role in the expression and function of these proteins during development and differentiation. Recent studies also indicated that the CAMs can mediate signals via the FGF receptor which promotes neurite outgrowth without necessarily modifying adhesion, an observation which may indicate that these molecules play a more diverse role in cellular processes (O'Brien, 1995, Doherty and Walsh, 1995).

1.4.4 Regulated vesicle fusion and exocytosis

During development, the trafficking from the cell body to the axonal surface of materials which are required for growth cone expansion of the neurites is achieved by regulated vesicular transport. The constituitive fusion and exocytosis of transport vesicles result from the association of N-ethylmaleimide-sensitive factor (NSF) and its associated proteins α-, β- and γ-SNAPs (Whiteheart and Kubalek, 1993) with the SNAP receptors, SNAREs (Warren, 1993; Sollner *et al.*, 1993, Bark and Wilson, 1994). The SNAREs are membrane receptors complexes of proteins such as SNAP-25, syntaxin (Sytx), Synaptotagmin (Sytg) and synaptobrevin (Syb).

The differential expression patterns and the various isoforms of the component proteins which form the receptor complex provide a model for directing the specificity of these vesicle fusion complexes. For instance, two isoforms of the SNAP-25 protein are expressed. A low level of the SNAP-25a isoform accumulates mainly in the cell bodies and fibre tracts of the noenatal brain and is associated with neurite outgrowth. Blocking the expression of SNAP-25 with antisense oligonucleotide in a number of neurons does not

perturb the initial neurite outgrowth but prevents the extension of the neurites (Osen-Sand *et al.*,1993). During synaptogenesis and brain maturation there is a dramatic induction of SNAP-25 mRNA with preferential expression of the SNAP-25b isoform which is relocated to the nerve terminals. This isoform may participate in docking and fusion of secretory granules and is considered to be important for neurotransmission since it is highly expressed in synaptosomes of adult brain and cleavage by the botulinum neurotoxin (BoNT/A and BoNT/C) blocks calcium dependent -glutamate release (Catsicas *et al.*, 1991; Bark and Wilson, 1994).

The SNAP-25 promoter appears to be regulated by Brn-3a and Brn-3b, with Brn-3a behaving as an activator and Brn-3b repressing the promoter activity (Lakin *et al.*, 1995). Furthermore, the reduction of the functional Brn-3a expression in stably transfected neuronal cell line, ND7, with an inducible construct containing the antisense to the Brn-3a POU domain resulted in a reduction of the level of SNAP-25 protein compared with the control cells. Differentiation of these cells to a neuronal phenotype resulted in shorter neurite outgrowth compared with cells transfected with the control sense construct. This parallels the observation of inhibited axonal growth in primary cultures of newborn rat cortical cells treated with antisense oligonucleotides to block SNAP-25 expression (Osen-Sand *et al.*, 1993). The decrease in synaptic marker proteins such as synaptophysin (Sudhof *et al.*, 1987) also support the role of the SNAP-25 protein in the differentiation events in these cells. It is likely therefore that the Brn-3a and Brn-3b transcription factors may be involved in the regulation of the expression of SNAP-25 thus directing an effect on cell differentiation. The functional significance of this observation will be discussed in more detail in chapter 5.

Synaptic proteins such as synapsins, which are important in cytoskeletal organisation during early neuronal differentiation, may be involved in the tethering of synaptic vesicles

to cytoskeletal actin (Ferreira *et al.*, 1994) while the function of synaptophysin and other members of the rab family of small GTP binding proteins in vesicular trafficking remains to be elucidated. Preliminary data from in-vitro studies in our laboratory suggests that the promoters of rab3A and synaptophysin may also be regulated by these Brn-3 proteins (Morris and Latchman-unpublished data: Lakin *et al.*, 1995).

The interaction of these different factors, which may act at different stages to specify neuronal development, differentiation and function, provides a number of possible levels at which the Brn-3 transcription factors may be involved in determining the fate of cells which express the proteins.

1.5.0 Sensory neuronal cell lines

To study the mechanism of differentiation of sensory neurons and the role of the Brn-3 protein family at the molecular level requires large amounts of a homogeneous population of neuronal cells. While it is relatively easy to dissect the dorsal root ganglia (DRG), the primary cultures produced contain a highly heterogeneous population of cells. To circumvent this problem, attempts have been made to obtain neuronal-derived cell lines. Neuroblastoma cell cultures such as C1300 (Augusti-Tocco and Sato, 1969) are derived from neural crest precursors of sympathetic neurons that continue to proliferate and show defective differentiation *in vivo*. By modifying the culture medium *in-vitro* these cells can be induced to differentiate and display some properties of neuronal cells which are often representative of cells in the central nervous system (CNS). Other cell lines such as the phaechromocytoma cells, PC12, provide a good model for sympathetic neurons since in the presence of nerve growth factor (NGF) these cells readily differentiate from an adrenal medullary cell type to a mature sympathetic neuron (Greene and Tischler, 1976).

However, neither of these cell lines are suitable models for studying sensory neuronal cell development, characteristics or behaviour. Hence, a number of groups have endeavoured to develop hybrids of immortalized neuroblastoma cells with primary sensory neuronal cells from DRG. Hybrids resulting from this process have been studied and some of these clones, such as the ND7 cells, (Wood *et al.*, 1990), provide useful models for studying the sensory neurons *in vitro*, since they can be maintained indefinitely in culture using the appropriate medium but can be induced to differentiate and take up the characteristics and behaviour of sensory neurons.

1.5.1 ND cell lines

The ND (Neuroblastoma + DRG) cell lines were derived by a fusion of neonatal rat primary DRG and the immortalized mouse neuroblastoma cell line, N18Tg2 (a subclone of the C1300 cell line) (Wood *et al.*, 1990). Cloning by limiting dilution allowed the isolation of several ND cell lines that exhibited some properties not displayed by the parental neuroblastoma cells, such as presence of specific surface markers and neuropeptides present in differentiated DRG neurons. These cells were also characterized in terms of their biochemical and pharmacological responses such as the ability to depolorize in response to bradykinin and capsaicin (figure 1.3.0) (Wood *et al.*, 1990, Wheatley *et al.*, 1992).

Some of these cells, ND3, ND7 and ND26 could be induced to differentiate when transferred to specifically defined medium (Wood *et al.*, 1990). The cells were able to exit the cell cycle and ceased proliferation while undergoing morphological changes such as the flattening of the cell body and the extension of neurite processes which are characteristic of sensory neurons. Other changes such as the secretion of neuropeptides indicated that some cell lines were more characteristic of the sensory neuronal parent. In addition, ND3 and ND7 cells could be latently infected by the herpes simplex virus (HSV) in a manner that was similar to the asymptomatic latent infection observed in sensory neurons (Wheatley *et*

al., 1991, Latchman, 1990). The ND7 cell line was widely studied and found to be the best model for sensory neuronal cells.

1.5.2 ND7 cells

ND7 cells maintained in medium containing 10% foetal calf serum (referred to as full growth medium, FGM) continue to proliferate but on transfer to low serum medium (0.5% FCS) supplemented with 1mM cyclic AMP, or into serum free medium, these cells cease to proliferate and by forty eight to seventy two hours assume the morphology of sensory neuronal cells with flat cell bodies and extensive processes (figure 1.5.1). In addition, the electrophysiological responses are similar to that observed in sensory neurons when they were exposed to activators of nociceptive neurons such as bradykinin and capsaicin.

Immunocytochemical studies also show that upon differentiation there is a selective pattern of redistribution of neuropeptides such as neuropeptide Y (NPY) and its C'- flanking peptide (CPON), chromogranin A and B, which are associated with secretory granules and synaptophysin which is a marker of synaptic vesicles. Chromogranin A and B as well as NPY and CPON appear to be localized to the tips of the cell processes in differentiated ND7 cells (Suboro *et al.*, 1992). Whereas NPY and CPON are also found in the differentiated parental N18Tg2 cell line, they are present in the cell body instead of being relocalized to the axons. These findings, combined with the detection of synaptophysin, a marker of differentiated synapses (Sudhof *et al.*, 1987), in ND7 cells but not in the N18Tg2 cells reflects the more complex neuronal differentiation in ND7 cells compared with the neuroblastoma cells under similar conditions. Furthermore, these cells have also been shown to transcribe the latency associated transcripts (LATs) which support latent HSV infection by contributing to the failure of transcription of the immediate-early genes necessary for a lytic infection and which are found in sensory neurons but not in the neuroblastoma cell line, C1300 [reviewed in Latchman, 1990(ii)].

(1) (2) (3)

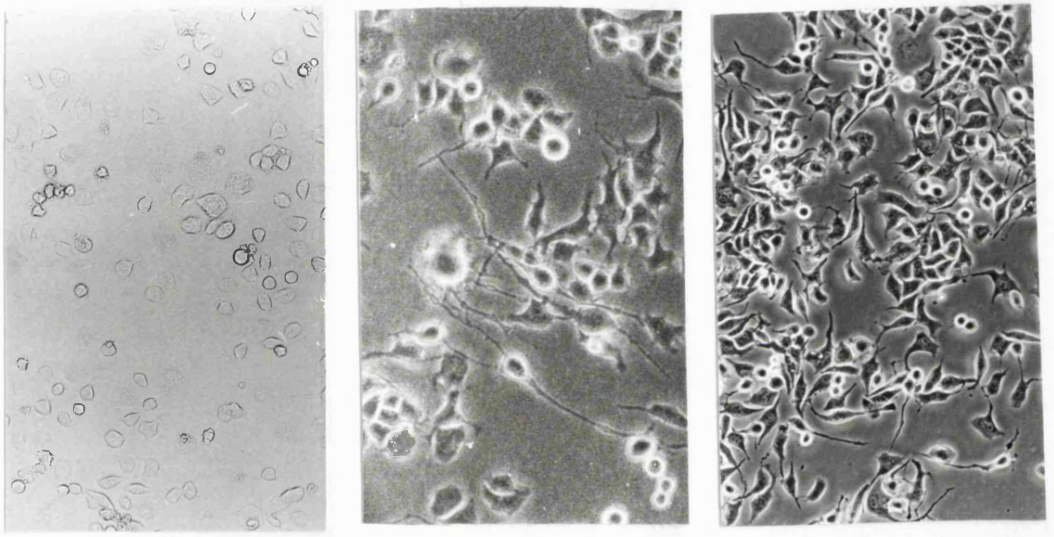


Figure 1.5.1: Morphology of ND7 cells grown in (1) full growth medium containing 10% foetal calf serum, (2) differentiation medium (serum-free) or (3) full growth medium supplemented with cyclic AMP.

Therefore ND7 cells appear to provide a suitable model which parallels the characteristics and responses of DRG sensory neurons. Furthermore, expression of a number of POU domain proteins such as Oct-1, the different isoforms of Oct-2 and Brn-3 proteins which have been identified in sensory neurons have validated the use of this cell line for studying the modulation of these factors *in-vitro* (Hatzopoulos *et al.*, 1990, Wirth *et al.*, 1990; Latchman *et al.*, 1992; Lillycrop *et al.*, 1992). ND7 cells were therefore used for studying the mechanism of sensory neuron differentiation *in-vitro* and the roles that the Brn-3 proteins may have in this process. The results are discussed in chapters 3 to 6.

1.6.0 Aims of Research

The importance of the POU domain proteins in sensory neuron development and function impelled us to try and identify novel POU domain proteins in the sensory neuron derived cell line, ND7. The identification of Brn-3a and the novel Brn-3b mRNA in these cells provided the basis for this study.

The aims of this project were therefore:

1. To characterize the expression of Brn-3a and the novel Brn-3b protein in tissues and cell lines (Chapter 3).

2. To establish the relationship between these two factors in ND7 cells and identify the factors which may regulate their expression in these cells. Study of the functional effect of over-expression of Brn-3a, Brn-3b and Brn-3c on promoter activity (Chapter 4)

3. To identify cellular promoters regulated by these transcription factors and locate the binding sites through which these proteins mediate transcriptional regulation (Chapter 5).

4. To study of the role of the different functional domains of Brn-3a and Brn-3b on transcriptional modulation of different target promoters. Study of the interaction of the Brn-3a and Brn-3b protein and the effect on transcription by these factors (Chapter 5).

5. To optimize conditions for the growth and survival of ND7 cells (Chapter 6).

Chapter 2

Materials and Methods

2.0 Materials:

2.0.1 Bacterial strains and growth conditions

The *Escherichia coli* strains used as cloning hosts were XL1, JM83 and DH4a which were growth in L-broth. For preparation of plasmid clones transformed cells were grown or on LB plates with the appropriate concentrations of antibiotics such as ampicillin at 50mg/ml for isolation of recombinants possessing selectable characteristics. Where appropriate, recombinants were identified by plating transformed *E.coli* on LB (ampicillin) agar plates that contained 5-bromo 4-chloro 3-indolyl b-D-galactoside (BCIG or X-Gal) at 60 mg/ml and isopropyl b-D-thio galactopyranoside (IPTG) at 72 mg/ml (both obtained from Boehringer Mannheim) (Sambrook *et al,* 1989).

2.0.2 Buffers and Solutions

The following general solutions were used. All solutions were made up in double distilled, deionized (dd) H_2O and either sterilized by autoclaving at 15 lb/sq. in. liquid cycle or by filter sterilization unless otherwise stated.

Luria-Bertani medium (L-Broth or LB)	x1	1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast, 1% NaCl. Adjusted to pH 7.2 with sodium hydroxide (NaOH). Ingredients from Pharmacia, Difco

Agar for plates were prepared from this by the addition of 1.5% (w/v) bacto-agar

M9 salts		0.6% (w/v) $NaHPO_4$, 0.3% (w/v) KH_2PO_4, 0.1% (w/v) NH_4Cl and 0.05% (w/v) NaCl
Denhardt's solution	x100	2% (w/v) polyvinyl pyrolidine 360,000, 2% (w/v) BSA and 2% (w/v) Ficoll 400,000. The solution was filter-sterilized and stored at -20°C.

SSC	x 20	3 M NaCl and 300 mM trisodium citrate
TE	x1	10 mM Tris-HCl (pH 7.5) and 1mM EDTA (pH 8.0) (pH 7.5)
TBE	x10	100 mM Tris.Cl , 100 mM boric acid, 2mM EDTA (pH 8.3)
TAE	x50	40 mM Tris.Cl, 1 mM EDTA pH 8.0
PBS	x10	104 mM NaCl, 1.8 mM KCl, 5.4 mM Na_2HPO_4, +1.25 mM KH_2PO_4

2.0.3 Cell lines

BHK cells (clone 13) (Macpherson and Stoker, 1962) and PC12 cells (Greene and Tischler, 1976) were kindly provided by the Imperial Cancer Research Funf (ICRF), London

ND cells - ND3, ND7, ND11 and ND21 (Wood *et al.*,1990) were kind gifts from Dr. J. Wood, Sandoz Institute, London.

2.0.4 DNA (Plasmids)

Bluescript vector SK (Stratagene)

Bluescript KS^+ plasmids containing the Brn-3a (long) and Brn-3b (short) cDNA were kindly provided by Dr. T.Theil, Institut fur Molekularbiologie and Tumorforschung, Universitat Marburg, Germany) (Theil and Moroy, 1994)

pGEM-T vector - (Promega) contains a single overhanging 3' dT deoxyribonucleotide base which is compatible with the single overhanging 3' dA base often produced by Taq DNA polymerase thus allowing rapid cloning of PCR products .

Eukaryotic expression vectors -

LTR poly (American Type Culture Collection, U.S.A.) contains the moloney murine leukaemia virus (MoMuLV) promoter which has been modified by deletion of a cryptic splice site in the SV40 3' untranslated region (Gorman, 1985). Can be transformed in DH5α *E.coli* cells.

LTR poly expression vector containing the full length cDNAs of Brn-3a (long and short isoforms), Brn-3b(s) short isoform and Brn-3c were provided by Dr. T.Theil, Germany (Theil *et al.*, 1993).

pLTR poly expression vector containing the Brn-3 chimeras were also a kind gift from T.Theil. These constructs were prepared by the ligation of different segments of Brn-3a and Brn-3b sequences generated by polymerase chain reaction using primers that allowed them to be in frame when ligated (Theil *et al.*,1993, Theil and Moroy, 1994). The position of Brn-3a or Brn-3b segments is indicated by A or B, respectively. Chimeras used were AAAB, AABB, AABA, and -BBA where (-) indicates no domain present.

pJ7 expression vector (Morgenstern and Land, 1990) contains the constituitive immediate-early cytomegalovirus (CMV) IE94 promoter flanked 3' by a polylinker, an intron and a transcriptional termination signal.

pJ7 expression vector into which the POU domains of Brn 3a and Brn-3b with an added ATG sequence were cloned by Dr. N. Lakin. The 'antisense' sequence of the Brn-3a POU domain was also cloned into pJ7 vector.

CAT constructs - pBLCAT 2 and pBLCAT3 vector (Luknow and Schutz, 1987). pBLCAT 2 contains a multiple cloning site flanking the herpes simplex virus thymidine kinase (tk) promoter from -105 to +51 driving the expression of the CAT gene while

pBLCAT3 did not contain the tk promoter thus allowing the cloning of cellular promoters to drive the expression of the CAT gene.

pBLCAT 2 vector was used for cloning the octamer related binding sites oligo A and PAP 3/4 (see oligonucleotides for sequences). The empty vector was used as a control for transcriptional activity.

p-α (internexin) CAT - (a kind gift from Dr. R.K.H. Leim, Columbia University College of Physicians and Surgeons, New York)
The different lengths of the a-internexin promoter sequences were cloned into the pBLCAT3 vector, upstream of the CAT gene and regulatory sequences so that the promoter the expression of the CAT gene.

Internexin (Long) - (Int'x-L) (-1219 bases to +73bases)

Internexin (Intermediate)- (Int'x-I) (-254 bases to +73 bases)

Internexin (Short) - (Int'x-S) (-77 bases to +73 bases)

The GAP-43 gene promoter cloned into pBLCAT3 was a kind gift from Dr.L.Schrama (Laboratorum voor Fyscologische Chemie, Utrecht, Holland)

Luciferase reporter plasmid (Nordeen, 1988)
The POMC promoter and the CE-2 sites cloned into the luciferase vector (JA 300 and JA 522, respectively) (Therrien and Drouin) were kindly provided by Dr. J. Drouin, Clinical Research Institute of Montreal, Canada.

The L1 promoter cloned into pCAT-Basic (Promega) was a kind gift from Dr M. Schachner (Swiss Federal Institute of Technology, Zurich, Switzerland)

2.0.5 Enzymes and Markers

Restriction and modifying enzymes were generally purchased from Gibco BRL, Promega or New England Biolabs while Taq DNA polymerase, PCR reagents were provided by Promega. Alkaline Phosphatase, acetyl coenzyme A (1units/ul), DNAase1 (RNAase free) (35 units/ul), Proteinase K, RNAse Inhibitor (40 units/µl), T4 DNA ligase transfer RNA (tRNA) and poly A+ RNA were obtained from Boehringer Mannheim. DNA polymerase (Klenow) (7 units/ul), M-MLV Reverse Transcriptase (200 units/µl), RNAase-1 (1000 units/µl), RNA polymerases, SP6, T3 and T7 (10-20 U/µl) and the restriction enzymes were obtained from Gibco BRL or Promega. Lysozyme and RNAase A were obtained from Sigma.

1 kb DNA ladder, l-HindIII DNA markers were supplied by Gibco-BRL
Protein molecular weight (Rainbow) markers were obtained from Amersham International, U.K.

2.0.6 Equipment

Densitometer and phosphor imager -BioRad GS-250 Molecular Imager (Bio Rad)
Luminometer - Turner TD-20e (Pharmacia)
PCR machine - Hybaid Omigene Thermal reactor
Spectrophotometer - Unicam SP1800 UV spectrophotometer or
GeneQuant RNA/DNA calculator (Pharmacia)
UV stratalinker 2400 (BioRad)
Heating block - Techne Dri Block; Scientific Laboratory Supplies (SLS), U.K.

2.0.7 Growth Factors:

The acidic and basic FGFs, IGF I and II and PDGF growth factors were obtained form Boehringer Mannheim, Germany. The NGF was a kind gift from G.Kendall, Sandoz Institute, U.K. Stock solution of all growth factors were aliquoted and stored at -20^{o}C since freeze-thaw cycles destroyed the activity.

Fibroblast growth factors (acidic and basic FGF) were human recombinant proteins acctive in murine, bovine and human cells. The acidic FGF was reconstituted in sterile dd H_2O to a concentration of 100 µg/ml stock solution. This was further diluted with PBS and BSA (1mg/ml) and used at 5-25 ng/ ml in cell cultures. Basic FGF was reconstituted in sterile PBS to 100 µg/ml and stored or diluted for further use as for acidic FGF 5-10ng of basic FGF was used in the treatment of ND7 cells.

Insulin-like growth factor (IGF I and IGF II) were also human recombinant proteins active in most mammalian cells. IGF I and II were reconstituted in 0.1 mol/l of sterile acetic acid to a concentration of 100 µg/ml, aliquoted and stored for later use. Further dilutions were done with medium containing 10% serum for IGF I and in PBS for IGF II. Both IGF I and II were used at 25 ng/ml in treatment of ND7 cells.

Platelet Derived Growth factor A/B (PDGF A/B) human recombinant was found to be active in murine or human cells. The protein was in a sterile solution of 20 mM Tris.HCl pH 7.4 and 700 mM NaCl and was diluted in the culture medium containing serum and was use at 5-10 ng/ml in cell cultures.

2.0.8 Oligonucleotides

Deoxyribonucleotides, ribonucleotides and random hexamer oligonucleotides were obtained from Pharmacia. All specific oligonucleotide sequences are given as 5' to 3' as they were synthesized. For the oligonucleotides used as double stranded DNA, the reverse complementary sequence of the primer given was also synthesized then annealed as described in 2.1.8.

Primers	Sequence 5' 3'	Source
Brn-3 POUs (common to Brn-3a and 3b)	GTGGCTCGGCGCTGGC	(Dr K.A Lillycrop)
Brn-3a POU specific	CGGGGTTGTACGGCAAAA	
Brn-3b POU specific	CTTGGCTGGATGGCGAAAG	
L6ribosomal-1 (rib-1)	ATCGCTCCTCAAACTTGAAC	
L6ribosomal-2 (rib-3)	AACTACAACCACCTCATGCC	
Brn-3a (3a3')-1	TTGGATTATTAGTATGAGATAC	(Ms J.Begbie)
Brn-3a (3a3')-2	CAAATAGGTCTGCACTTATCCG	"
Brn-3b (3bN)-1	GGTCTGCATCCACGTCGCTC	"
Brn-3b (3bN)-2	GGAGGGCGAGCTGCTTGA GC	"

Brn-3a competition primer

CGGGGTTGTACGGCAAAAGGCCTCCTCCAGCCAGGC

Brn-3B competition primer

GTTGGCTGGATGGCGAAGAGCTCTCCAGCCACGC

octamer like sequence (oligo A)	GATCATGCTAATGAGAT
HPV-16 Octamer related motif (PAP 3/4)	GATCAAGCCAAGTATGCAATTAC
Internexin-Brn-3 site (IXB3)	TCGATCTGAAGATGAAGCTCCACGGATC

Heat Shock Element (HSE)

GAGCCCGGCTGGAATATTCCCGACCTG-
GCAGCCGA

CRH (Brn-3 site) GCATAAATAAT

Synthesis of some oligonucleotides were carried out on an Applied Biosystems 318A DNA synthesizer (Applied Biosystems Inc.). Columns were manually flushed through with ammonium hydroxide for an hour then incubated in a tightly sealed tube at 55°C overnight to remove the protecting groups. Prior to use the oligonucleotides were precipitated at -70°C with ethanol and 0.2 M sodium chloride, vacuum dried then resuspended to an appropriate concentration, usually 0.5 nM/ml for PCR primers.

2.0.9 Other materials and reagents

Chemicals were supplied by British Drug House (BDH), Sigma chemical company or Fisons Laboratories.

The components of culture medium, sera and plasticware -Gibco BRL (unless otherwise stated)

0.22 μm and 0.45 μm filters - Millipore, U.K.

Sequenase kits - United States Biochemicals Corporation (obtained through Cambridge Biosciences U.K.)

Sequagel concentrate, sequagel diluent and Protogel polyacrylamide - National Diagnostics, Bucks, U.K.

Nitrocellulose filters - Hybond N - Amersham International, U.K.

Thin layer chromatography (TLC) plates - Camlab, U.K.

3MM chromatography paper - Whatman

Wizard™ PCR Preps DNA purification System - Promega

B.C.A. Protein Assay Kit - Pierce Co. U.S.A.

RNAzol™ B RNA isolation kit - Biotecs Laboratories U.S.A.(distributed by Biogenesis, U.K.)

Polaroid 667 film -Polaroid U.K.

X-ray film - X-omat AR fast film - Kodak, U.K. or Hyperfilm-MP - Amersham International (U.K.)

Film developer and fixer were obtained from Photosol

2.0.10 Radiochemicals

Radiochemicals were purchased from New England Nuclear Inc. (NEN) or Amersham International. These were [α-^{32}P] dCTP (3000 Ci / mmol), [α-^{32}P] CTP (3000 Ci / mol), [γ-^{32}P] ATP (3000 Ci / mol), [α-^{35}S] dATP (800-1500 Ci / mmol) and ^{35}S methionine (^{35}S Met) and tritiated Thymidine (Thymidine [methyl-3H]). ^{14}C-chloramphenicol (D-Thero-{dichloroacetyl-1,2-^{14}C), 1μCi/ul was obtained from Dupont.

2.0.11 Tissues

Rat tissues were obtained from adult Sprague-Dawley rats for RNA extraction and for in-situ hybridization by Ms. E. Ensor.

2.1. METHODS

2.1.1 General Procedures

2.1.1 (i) Preparation and storage of Chemicals

All solid chemicals were dissolved in ddH_2O, adjusted to the correct pH with HCl, glacial acetic acid or NaOH and were either filter sterilized or autoclaved before use unless otherwise indicated. SDS and NaOH were not autoclavedwhereas guanidinium isothiocyanate was filter sterilized before using. Solutions such as chloroform, ethidium bromide were kept in light-tight containers while MOPS, RNAzol solution and phenol were stored in the dark at 4^oC. $CaCl_2$, deionized formamide, growth factors, cyclic AMP, ampicillin, DTT, APS, diluted nucleotides and all the enzymes and reaction buffers were stored at -20^oC. RNA markers and the transcription-translation kit were kept at -70^oC.

2.1.1 (ii) Extraction and precipitation of nucleic acid preparations

Removal of proteins from nucleic acid preparations was achieved by extraction of the preparations with equal parts of phenol and chloroform: isoamyl alcohol (IAA) (24:1). This was vortex mixed then centrifuged for 5 minutes. The top aqueous phase was retained and re-extracted until it appeared clear. Chloroform:IAA extraction (x 1-2) was then carried out to remove residual phenol from the preparation.

The aqueous phase was then precipitated by addition of 2.5 volumes of cold ethanol and 0.1 M salt (sodium chloride for DNA preparations or sodium acetate for RNA) and storing at -20^oC or -70^oC respectively for 1 hour but can be left overnight. The precipitated nucleic acid was pelleted by spinning at 12000g for 10-15 minutes. RNA samples were always spun at 4^oC and kept on ice at all other times during preparation. The pellets were washed in 500 µl of 70% ethanol to remove residual salts, freeze dried for 1-2 minutes then resuspended in ddH_2O (RNA samples were dissolved in autoclaved DEPC treated ddH_2O.

2.1.1 (iii) Estimation of nucleic acid concentration

DNA and RNA concentrations were determined by measuring the optical density (OD) at 260 nm using a double beam spectrophotometer (see equipment). An OD_{260} of 1 indicated 50 µg/ml of double stranded DNA, 40 µg/ml of single stranded RNA or 20 µg/ml of oligonucleotides. Measurement of the OD at 280 nm gave an estimation of the amount of proteins or contaminants present in the sample since the ratio of OD_{260} / OD_{280} should be 1.8 and 2.0 for pure DNA and RNA respectively (Sambrook *et al.*,1989).

Both DNA and RNA samples could also be visualized on a UV transilluminator following resolution on an agarose/ ethidium bromide gel.

2.1.1 (iv) Preparation and storage of Organic solutions

(Phenol, Chloroform, deionized formamide)

Chloroform used for extraction of nucleic acids always contained 4% v/v isoamyl alcohol (IAA). Phenol for use in DNA extraction was buffered by shaking with equal volumes of 0.5M Tris pH 8 (x3) and with 0.1 M Tris pH 8, 0.2% v/v b-mercaptoethanol. The top aqueous phase was aspirated and this process repeated until the pH of the phenol was greater than 7.8. After removal of the final aqueous phase, 0.1volumes of 0.1 M Tris.Cl pH 8.0 containing 0.2% β-mercaptoethanol was added to the equilibrated phenol. This was stored for up to 4 weeks in a dark, cold place. Formamide was deionized by stirring with 10% w/v duolite MB6113 mixed resin for 30 minutes and then filtered through 3MM Whatman paper. The deionized formamide was then aliquoted and stored at -20^{o}C.

2.1.2 BAL-31 nuclease digest

The deletion constructs used to study the position of the DNA binding site for Brn-3 proteins in the α-internexin promoters were obtained following BAL-31 nuclease digestion which generated clones with bases removed at the 5' end of the minimal promoter construct (Internexin S). BAL-31 enzyme (New England Biolabs, USA.) degrades double stranded DNA sequentially from both termini. It has a rapid exonuclease activity that removes mononucleotides from the 3' termini of duplex DNA followed by a slow endonucleolytic activity on the complementary strand so that double stranded DNA with blunt or protruding 3' hydroxyl termini are degraded to shorter double stranded molecules.

To generate the shortened sequences, the construct containing the short internexin sequence (-77 to +73) was linearized at the 5' end using Pst 1 restriction enzyme and this was checked by gel electrophoresis. To stop the reaction 1x Phenol / chloroform and 2 x chloroform / IAA extractions were performed and the DNA precipitated with 2 volumes of ethanol and 0.2 M sodium chloride at -70°C. Following centrifugation the DNA pellet was dried, resuspended in water and then used for the BAL 31 nuclease digest according to the manufacturers recommendation.

The Bal-31 digestion reaction was carried out in 50 ul total volume containing 10 ug DNA, 25 ul of 2 x reaction buffer (600 mM NaCl, 12 mM CaCl, 12 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA), and 1 unit of enzyme. One unit of enzyme is defined as the amount of enzyme required to remove 200 bases from each end of linearized ds DNA øX174 in 10 minutes at 30°C in a 50 ul so this reaction was carried out for 1, 2 and 3 minutes using 1 unit of enzyme. This should theoretically remove 20 - 60 base pairs from dsDNA. To stop the reaction 20mM of the specific chelator of essential $Ca2^+$, EGTA, was added. Any staggered ends which were generated were end-filled using Klenow DNA polymerase. The insert was cut out of the vector with Xho 1 enzyme present at the cloning site flanking the 3' end. The fragments which

were of slightly differing sizes were resolved on a high percentage, low melting point agarose gel, cut out of the gel and re-cloned into pBLCAT3 vector (see 2.1.4 ii) linearized with the Xho 1 and Sal 1 enzymes (which provide blunt ends following digestion). Clones were selected on Amp^r LB agar plates and sequenced to confirm the number of bases deleted.

2.1.3(i) Conditions for cell culture

BHK cells

The hamster fibroblast BHK cells were routinely grown in RPMI medium supplemented with 10% foetal calf serum (FCS).

ND7 cells

ND7 cells were routinely grown in L15 medium (Gibco) supplemented with 10% FCS, 0.3% D-glucose, 0.37% sodium bicarbonate, 0.2 mM L-glutamine and 1%penicillin/streptomycin (10,000 units/ml). This will be later referred to as the full growth medium, FGM. To differentiate the ND7 cells to a neuronal phenotype, the cells were transferred to serum-free medium consisting of DMEM/HAMS (1:1) (Gibco) supplemented with 5 ug/ml transferrin, 250 ng/ml bovine insulin and 30mM sodium selinite.

Cyclic AMP treatment of ND7 cells were carried out either in the FGM containing 10% FCS and 1mM dibutryl cyclic AMP or the growth medium with 0.5% FCS (instead of 10%). Some studies were also carried out using serum-free medium containing 1mM cyclic AMP.

PC12 cells

The pheochromocytoma PC12 cell line was routinely grown in DMEM medium (Gibco) containing 10% FCS, 5% horse serum and 1% L-glutamine. To differentiate into sympathetic neuronal-like cells, these cells were transferred to medium containing 1mM cyclic AMP and 200 ng/ml nerve growth factor (NGF).

2.1.3(ii) Cell culture for RNA extraction

Cells were grown in the FGM or in defined medium as described. For the routine cultures required for RNA extraction, cells were plated out in 15 cm plates at a density of 5 x 10^5 - 1 x 10^6 cells depending upon the type and duration of treatments. For instance cells which were to be differentiated would undergo limited replication and were thus plated at a higher density. Specific treatments such as addition of growth factors or cyclic AMP to the medium were carried out for the duration indicated.

2.1.4 Cloning of DNA fragment into plasmid vectors

2.1.4 (i) Preparation of DNA fragments for cloning

DNA fragments to be cloned were excised from the low melting agarose gel under ultraviolet transillumination. Care was taken to minimise the time of exposure of the DNA to ultraviolet radiation to minimize UV mediated damage. The DNA was recovered from the agarose using Geneclean glass milk (Bio 101 Inc., California, U.S.A) or using the Wizard™ PCR prep DNA purification system (Promega) in accordance with the manufacturers' instruction. Alternatively, the gel slice containing the DNA fragment was weighed and 0.1 ml of ddH_2O was added for each 0.3 g of gel for use in ligation reactions (see later).

2.1.4 (ii) Preparation of the vector DNA

Plasmids were digested with the appropriate restriction enzymes to generate sites which were compatible with the ends of the fragment of DNA to be cloned.The effectiveness of the reaction was monitored by analysis of an aliquot by agarose gel electrophoresis. The remaining DNA was extracted with phenol / chloroform and chloroform then precipitated with ethanol. The pellet was resuspended in at the appropriate concentration.

2.1.4 (iii) Dephosphorylation of linearized vectors

Where necessary the DNA was dephosphorylated to increase the efficiency of the ligation using CIP (calf intestinal phosphatase). A typical reaction contained 20 mM Tris.Cl, pH 8.0, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, ~20 pM of DNA termini and 0.1 unit of CIP.The reaction was carried out at 37°C for 30 minutes followed by heating to 75°C for ten minutes to stop the reaction. The DNA was ethanol precipitated as described before.

2.1.4 (iv) Ligation of DNA fragments and Plasmid Vectors

The method of ligation depended on the way in which the insert DNA was recovered:

1. DNA fragments which were gel purified were combined with the vector DNA in a 5:1 molar ratio so that the total mass of DNA did not exceed 200 ng in the final reaction volume of 10 µl. Ligase buffer (5 mM Tris.Cl pH 7.8, 1 mM $MgCl_2$ and 2 mM DTT), ATP to a final concentration of 1 mM and 10 units of T4 DNA ligase were added and the reaction incubated overnight at 15°C.

2. 'In gel' ligations were performed with DNA fragments that had been excised from a low melting point agarose gel. The gel containing the DNA was melted in the volume of dd H_2O [see 2.1.4(i)] at 60°C for approximately 10 minutes. A volume of this molten gel was added to the vector such that a 5:1 molar ratio was maintained but the total mass of DNA did not exceed 100 ng. The volume of the reaction was adjusted with pre-warmed H_2O so that the concentration of the agarose did not exceed 0.2% (w/v). T4 ligation buffer was added to 1x concentration and ATP to 1 mM followed by addition of 100 units of T4 ligase. The reaction was incubated for a minimum of 5 hours at room temperature and may be left overnight. This reaction mixture was then heated to 65°C immediately prior to addition to competent cells for transformation.

2.1.5 Transformation of *E.Coli* cells using the Calcium chloride method

2.1.5 (i) Making competent cells

Most bacterial cultures were grown in LB medium at 37°C in a shaking incubator. *E.coli* cells were prepared for transformation by growing up a single colony in overnight cultures then growing a 1:100 dilution of this overnight culture in 100 mls of fresh LB medium in a sterile flask shaking at 37°C until the OD_{600} was between 0.5 and 0.7 absorbance units (from 3 to 5 hours). Cells were pelleted at 3,000 x g in an IEC Centra- 4R benchtop or equivalent centrifuge at 4°C for 10 minutes then resuspended in 100 mls of ice-cold 100 mM calcium chloride and recentrifuged as above but for 5 minutes. The cells were resuspended in a smaller volume of about 1 ml of ice cold 100 mM calcium chloride. At this stage the cells could be stored at -70°C after diluting with 0.15 volumes of sterile glycerol, mixing well, and aliquoting, and could be used for up to one month later. Alternatively the cells could also be used straight away for transformation with homogeneous plasmid DNA.

2.1.5 (ii) Transformation of Bacterial cells with plasmid DNA

Transformation of the competent cells was achieved by using calcium chloride and heat shock to render the membrane transiently permeable. Routinely, 100 µl of competent cells were incubated with 10-20 ng of plasmid DNA or with the ligation mix and left on ice for 30 mins. The cells were then subjectedto heat shock at 42°C for 2 minutes, both the time and temperature being critical for efficient transformation. Cells were then replaced on ice for 30 minutes immediately after removal from the water bath. 200 ul of LB media (with no ampicillin) was then added and the cells incubated at 37°C for 30 minutes. These cells were then plated on 1% LB agar plates containing 50 µg/ml of ampicillin and incubated overnight at 37°C. If selectable plasmids such as Bluescript-SK were used for cloning then 0.02% w/v X-gal (Gibco BRL) was spread onto the plates prior to plating of the cells.

2.1.6 DNA extraction (Plasmid)

2.1.6 (i) Large Scale PEG precipitation of plasmid DNA

Bacteria were grown to confluence overnight in as orbital shaker in 400 ml LB medium containing ampicillin (50 μg/ml). The bacteria was sedimented by centrifugation at 4000 rpm for 10 minutes in a Beckman J-6B centrifuge and the pellet was then resuspended in 4 ml of ice cold solution containing 50mM Tris.Cl (pH 8.0) and 25% (w/v) sucrose. Freshly prepared lysozyme was added to a final concentration of 1 mg/ml and the cells allowed to lyse whilst incubating on ice for 15 minutes. EDTA was added to the solution to a final concentration of 10 mM and left on ice for another 15 minutes. 0.5 volumes of Triton lysis buffer (150 mM Tris.Cl pH 8.0, 375 mM EDTA, Triton X-100 (v/v) was added to this and mixed gently then incubated on ice for a further 30 minutes followed by centrifugation at 18,000 rpm for 60 minutes in a Beckman JA20 rotor. The supernatant which was removed to a fresh tube and NaCl was added to a final concentration of 0.5 M to followed by 1x phenol:chloroform and 1x chloroform extractions. The aqueous phase was removed to a clean tube and the DNA was precipitated overnight with 10% (w/v) PEG 6000. The DNA was pelleted at 12,000 rpm for 10 minutes at 4^{o}C in a Sorval RC-5B (DuPont) rotor then resuspended in 500 μl 0.1 M Tris.Cl (pH 8.0). RNA was removed from the preparation by incubation with 0.2 mg/ml RNAse A (which was previously heated at 100^{o}C for 10 minutes to inactivate DNAse) for 30 minutes at 37^{o}C. Phenol/chloroform and chloroform:IAA extractions were performed to inactivate the enzyme and the DNA was re-precipitated with an equal volume of PEG buffer (10 mM Tris.Cl pH 8.0, 1mM EDTA, 1M NaCl and 20% (w/v) PEG 6000) for 1 hour on ice then pelleted by centrifugation in a microfuge for 10 minutes. The pellet was re-dissolved in 400 μl of 10 mM Tris.Cl (pH 8.0), containing 0.5 M NaCl and the DNA was then sequentially extracted with phenol, phenol/chloroform and chloroform:IAA prior to ethanol precipitation at -20^{o}C (1 hour to overnight). The pellet obtained following

centrifugation was washed with 70% ethanol, vacuum dried briefly then resuspended in ddH_2O at the appropriate concentration.

2.1.6 (ii) Small Scale Alkaline lysis plasmid DNA preparation

For small scale plasmid preparation, 10 ml of overnight bacterial cultures which were grown to saturation were pelleted. The cells were then resuspended in GTE buffer (25 mM Tris.Cl, pH 8.0, 50mM glucose and 10mM EDTA) and then lysed with freshly prepared 0.2 M NaOH/ 1% (w/v)SDS. Potassium acetate (pH 4.8) was then added so that the final concentration of the solution was 3M with respect to potassium was and 5M with respect to acetate. After shaking well, this mixture was stored on ice for 10 minutes then centrifuged at high speed in a microfuge for 10 minutes. The DNA in the supernatant was precipitated in 0.6 volumes of iso-propanol at room temperature for 10 minutes then pelleted by centrifugation for a 10 minutes at 12,000 rpm. The DNA pellet was washed in 70% ethanol vacuum dried briefly and resuspended in 200 µl of TE. To remove contaminating RNA, this preparation was subjected to DNAse free RNAse A digest for 30 minutes at 37^oC. Phenol/chloroform and chloroform extractions were performed to inactivate the enzyme and the DNA was ethanol precipitated pior to resuspension in H_2O at the appropriate concentration.

2.1.7 Analysis of DNA

2.1.7 (i) Restriction endonuclease digestion of DNA

Restriction endonuclease digestions were carried out according to the manufacturers instructions in the appropriate buffer supplied by the manufacturers. The incubation conditions were 37oC for 1-2 hours unless specified otherwise in the protocol.

2.1.7 (ii) Non- denaturing gel electrophoresis of DNA

DNA fragments were analysed by separation on horizontal agarose gels. The concentration of the agarose in the gels used depended on the size of the fragment to be separated and varied from 0.8% for the very large fragments to 2.5% for very small

fragments. Gels were usually prepared in 1x TBE using electrophoresis grade agarose and prior to pouring the gel 1ng/ml of ethidium bromide was added generally. Where the DNA was to be used for cloning, the gels were prepared using low melting point agarose and the electrophoresis was carried out in TAE. Prior to loading the samples on the gel, 0.1 volume of 6x loading buffer (0.25% bromophenol blue, 15% Ficoll 400) was added to the samples. DNA markers such the 1 kb DNA marker was always loaded onto the gel to help identify the DNA fragment sizes. The gels were run at 5-20 V/cm for 1-4 hours. The DNA was then visualized on an ultraviolet transilluminator (wavelength 254 nm) and photographed using Polaroid 667 film through an orange filter.

2.1.7 (iii) DNA Transfer (Southern Blotting)

The transfer of DNA from agarose gels to nitrocellulose membranes was carried out as described by carried by Sambrook *et al.*, 1989 but with slight modification. Agarose gels on which the PCR products were separated were photographed and marked for orientation. They were then denatured in 0.4% Sodium Hydroxide (NaOH) for up to one hour then washed in distilled water before placing in neutralisation solution (Tris.Cl pH 7.5/NaCl) for up to 1 hour. A large shallow tray was lined with 6 pieces of 3MM Whatman paper cut to be slightly larger than the gel. The 3MM Whatman paper was soaked in 20 x SSC for a few minutes and any air bubbles removed by gently rolling a pipette over the surface. Any excess SSC was poured out and the gel was placed on Whatman paper, lower side facing upwards. A piece of Hybond N nitrocellulose membrane cut to the size of the gel was overlaid on the gel and any air bubbles removed. The edges were covered with saran wrap to prevent diffusion around the gel. Three pieces of 3MM Whatman paper was then placed on top of the filter followed by even layers of absorbant paper towels. A flat tray was placed on top of this layer of paper and held in place by evenly distributed weights. This was left overnight to allow maximal transfer of the DNA to the filter. After removal of the filter from the

gel the DNA was fixed to the membrane by UV cross linking in a UV stratalinker (Biorad) and kept dry until ready for hybridization.

2.1.7 (iv) Hybridization of Southern Blots

Pre-hybridization and hybridization were carried out in Hybaid bottles in a Hybaid oven set at the specified temperature. If multiple filters were being hybridized, these were layered in between nylon meshes. Nitrocellulose membranes were pre-hybridized in pre-hybridization buffers (0.01 M EDTA, 6x SSC, 5x Denhardt's, 0.1% SDS, 10% (w/v) dextran sulphate and 100 μg/ml herring sperm DNA (dissolved in ddH_2O and denatures by heating at 100°C for 5 minutes) for a minimum of 2 hours generally at 65°C. Filters were transferred to hybridization solution (similar to the pre-hybridization solution but with no EDTA) containing sufficient heat denatured probe at a final activity of approximately 10^6 cpm/ml. Hybridization was also carried out at 65°C for a minimum of 4 hours and generally done overnight. The filters were washed with a solution containing 1x SSC / 0.1% SDS initially. The stringency and subsequent numbers of washes were determined by the activity on the filter as determined by the counts on a Geiger-Muller counter. Increasing the stringency was achieved by decreasing the concentration of the SSC, such that a typical high stringency wash was 0.1x SSC / 0.1% SDS at 65°C for 30 minutes. The filters were then wrapped in Saran wrap while still damp and exposed to film at -70°C for ^{32}P labelled probes.

If the filter was to stripped and reprobed, care was taken to prevent it from drying and hence prevent irreversible binding of the probe. To strip the probe, the filter was washed in ddH_2O containing 0.1x SSC / 0.1% SDS at 95°C for a minimum of 2 hours or until there was no counts on the filter.

2.1.7 (v) Colony Hybridization

This technique was used to screen large number of colonies for the presence of recombinant plasmids containing the gene of interest. Briefly, 5 µl of bacterial cultures were spotted onto a piece of Hybond-N filter (which may be marked with grids and numbers to assist with orientation after hybridization). The filter was washed in 0.5 M NaOH twice for 3 minutes each, twice in 1.0 M Tris.Cl pH 7.2 and twice in 0.5 M Tris.Cl pH 7.5 / 1.5 M NaCl for the same duration of 3 minutes. Following a final wash in 2x SSC the filter was briefly air dried and the DNA was UV cross linked to the filter. This was then hybridized with labelled probe in a similar manner to Southern hybridization.

2.1.7 (vi) Probe preparation

Labelled double stranded DNA probes were most commonly used for Southern hybridization. The enzyme used for labelling the larger DNA fragments obtained by restriction digestion depended on the types of ends generated such that fragments with 3' overhanging ends (generated by enzymes such as Kpn1 or Sst1) and blunt ended fragments (e.g. by Sma1 and Rsa1) were labelled using T4 DNA polymerase which possesses 3' to 5' exonuclease activity also. Fragments possessing 5' overhanging ends (such as those generated by BamH1 and EcoR1) were labelled using the Klenow fragment of the DNA polymerase 1.

The labelling of fragments possessing this 5' overhang with Klenow fragment were more commonly used and the protocol for labelling will be described briefly:

The oligo labelling buffer used for this reaction was prepared by mixing 100 µl of buffer A (containing 125 mM $MgCl_2$ and 1.25 M Tris.Cl pH 8.0, 5 µl each of dATP, dTTP and dGTP and 18 µl of β-mercaptoethanol) with 250 µl of 2 M Hepes (pH 6.6) and 150 µl of 10mg/ml random oligonucleotides (Pharmacia). 8 µl of oligo labelling buffer and 2 µl of BSA (2 mg/ml) was added to 100 ng of DNA which was denatured by heating in a boiling H_2O bath for about 5 minutes then placed immediately on ice. To this mix, 10 Ci/µMol of [^{32}P] α-dCTP and 1 µl (2.5 units) of Klenow fragment of

DNA polymerase 1 were added mixed well and incubated at 37°C for 1 hour or overnight at room temperature. The unincorporated label was removed by centrifugation through a 1 ml Sephadex-G50 column The DNA was heated at 95°C for 5 minutes prior to addition to the hybridization buffer.

1 ul of the final labelled probe could be used to measure the level of incorporation by adding to 5 ml of scintillation fluid and measuring the counts in a scintillation counter. Prior to hybridization the probe is heated for about 5 mins at 80 - 100°C on a heating block to render the labelled fragments single stranded.

2.1.7 (vii) Gel Filtration chromatography to separate probes

This technique was used to separate high molecular weight DNA from smaller molecules such as unincorporated labelled nucleotides. Sephadex G-25 or G-50 resins were commonly used for smaller and larger fragments respectively. The Sephadex beads were rehydrated in sterile ddH_2O and washed several times to remove soluble dextran. The resin was then equilibrated in 1x TE (pH 7.6), autoclaved at 10 lb/sq.in. for 15 minutes and then stored at room temperature.

To prepare the columns, a 1 ml syringe was plugged with siliconized sterile glass wool and the resin added until the column was filled. This was then supported in an appropriate tube and centrifuged at 2,500 rpm for 5 minutes to allow compaction of the resin and to remove any air bubbles. The excess TE was removed and the sample to be seperated was added to the top of the column and centrifuged as before.

2.1.8 DNA Sequencing

All sequencing was by the dideoxy chain termination method, performed using modified T7 DNA polymerase (Sequenase Version 2.0) and the 'Sequenase' kit (USB Corp.) according to the manufacturers instructions. PCR products intended for sequencing and which contained linkers in the primers were either digested and then ligated into the cloning site of Bluescript II or cloned straight into the modified EcoR1 site of pGEM-T. Plasmid purified by PEG precipitation method were used for double stranded sequencing. When sequencing fragments in Bluescript the -40 primer provided in the Sequenase kit could be used. Some additional oligonucleotides corresponding to internal sequences were synthesized to facilitate sequencing of fragments in other vectors such as primer synthesized to sequences in the CAT gene (see 2.0.8).For each reaction, 2-3 μg of double stranded DNA was made up to 20 μl with ddH2O and denatured by addition of 20 μl of a solution containing 0.2 M NaOH, 0.2 mM EDTA. This was mixed well, spun briefly to collect any droplets, then left at room temperature for 10-30 minutes. The reaction was neutralized by adding 0.1 volumes of 3M sodium acetate (pH 4.5) and the DNA precipitated with 2.5 volumes of ethanol at -70^{o}C for 10-30 minutes. The DNA obtained after centrifugation was washed in 70% ethanol, dried briefly then resuspended in 7 μl of ddH_2O and used for the sequencing reaction as instructed in the manufacturer's protocol.

Sequencing reactions were electrophoresed on denaturing 6% polyacrylamide gels.These were prepared in TBE buffer using pre-made sequagel acrylamide solutions (33 ml sequagel diluent, 12 ml sequagel concentrate and 5 ml TBE polymerized with 220 μl of 10% Ammonium persulphate (APS) and 12 μl of N,N,N',N'-tetramethyl-ethylene diamine (TEMED). The samples were electrophoresed at ~1250 volts, 40 mA for 2-7 hours. After running, one of the plates was prised off and the DNA fixed by soaking in 10% acetic acid / 10% methanol (v/v) for approximately 20 minutes. A piece of 3MM chromatography paper was placed on the gel which was then carefully lifted

off the plate and dried on a heated vacuum drier. Dried gels were expose to X-ray film (Kodak, XAR-5) at room temperature, overnight.

The DNA sequence analysis was performed using the EMBL data bank or using the Wisconsin software package at Daresbury.

2.1.9 Electrophoretic Mobility Shift Assay (Bandshift)

2.1.9 (i) In-Vitro translated Brn-3a and Brn-3b

The in-vitro translated proteins were obtained using the TnT™ Coupled Wheat Germ Extract kits (Promega) and the reactions were carried out according to the manufacturer's protocol using the full length cDNA of Brn-3a and Brn-3b cloned into the Bluescript KS^+. All reagents which were stored at -70°C were hand thawed just prior to use and then kept on ice (RNA polymerase was removed from -70°C immediately before using since the enzymes are very heat labile).

Briefly, for a 50 µl reaction, the following reagents were prepared for each reaction: 25 µl of the wheat germ extract, 2µl of the reaction buffer, 1 µl of the appropriate RNA polymerase, 1 µl of the amino acid mixture minus Methionine (1mM), 4µl of translation grade ^{35}S-Methionine (1000 Ci/mmol at 10mCi/ml), 1µl of RNasin inhibitor (40 u/µl), 1 µg of the DNA template and ddH_2O (nuclease-free) to 50 µl. The reaction which was carried out at 30°C for 2 hours and the products were analysed by SDS-polyacrylamide gel electrophoresis. The products were stored at -20°C for later use.

The orientation of the Brn-3a cDNA required the T3 RNA polymerase to be used for the transcription of the sense RNA while Brn-3b transcription required the T7 RNA polymerase. For all reactions carried out, a positive control was done using the control DNA provided in the kit to check that the reaction mixture and conditions were satisfactory.

2.1.9 (ii) Analysis of the In-Vitro translated product

5 µl of the reaction products including the control product were removed and analysed by denaturing SDS gel electrophoresis. In preparation for loading the samples onto the gel, 20 µl of SDS sample buffer (2 ml glycerol, 2 ml 10% SDS, 0.25 mg bromophenol blue, 2.5 ml stacking buffer (4x) and 0.5 ml β-mercaptoethanol) was added to the sample and this was heated at 100°C for 2 minutes to denature the proteins. An aliquot of this sample was then loaded onto 12.5% SDS polyacrylamide separating gel. Protein markers such as the Rainbow markers (Amersham International) were also subjected to electrophoresis. Typically, electrophoresis was carried out at a constant current of 15 mA in the stacking gel and 30 mA in the separating gel. The gels were dried and exposed to X-ray film at room temperature overnight.

The 100 x 70 mm slab minigels were composed of the resolving gel - 7.5 ml 30% polyacrylamide, 4.5 ml lower gel buffer (1.5M Tris, pH 6.8 / 0.4% SDS) 6 ml ddH_2O, 74 µl APS (10%) and 8 µl of TEMED and the stacking gel - 1 ml 30% acrylamide, 3 ml lower gel buffer (0.5 M Tris pH 6.8 / 0.4% SDS) 7 ml ddH_2O, 100 µl APS (10%) and 10 µl TEMED.

2.1.9 (iii) Total Cellular Extract

Cell extracts were prepared using a modified method of Dignam *et al.*, (1983). All procedures were carried out at 4°C. Cells (1 x 10^6) were harvested after washing in ice-cold PBS and spun at low speed to pellet the cells which were then resuspended in 25 - 50 µl of buffer containing 50 mM Tris HCl (pH 7.9), 50mM KCl, 0.1 mM EDTA (pH 8.5) 2 mM DTT and 2 µg / ml each of aprotinin, leupeptin and 25% glycerol. The cells were homogenized using a small, glass homogenizer then spun at high speed for 10 minutes at 4°C. The supernatant was aliquoted and stored at -70°C for later use. 4 - 8 µl was used in the bandshift reactions.

2.1.9 (iv) Preparation of labelled oligonucleotide probes

The oligonucleotides were synthesized as two complementary single-stranded sequences. The single stranded DNA were precipitated with 2.5 volumes of ethanol and 0.2 M NaCl for 10 minutes at -70^{o}C. The pellet recovered was washed in 70% ethanol, dried briefly then resuspended to a concentration of 500 ng/µl. The two oligonucleotides were annealed by mixing equimolar amounts in 1x buffer containing 2.5 mM Tris.Cl, pH 7.8, 10 mM KCl, 1 mM $MgCl_2$, heating to 80^{o}C for 5 minutes and allowing the oligonucleotides to cool slowly to room temperature.

End-labelling of the oligonucleotides with T4 kinase was the most commonly used method of labelling probes for the EMSA reaction. Typically, 100 ng of the annealed oligonucleotide was incubated with 20µCi [^{32}P] γ-ATP in the presence of 50 mM Tris.Cl (pH 7.6), 10 mM $MgCl_2$, 5mM DTT, 0.1 mM EDTA and 5 - 10 units of T4 DNA kinase. The reaction was incubated at 37^{o}C for 30 minutes. 50 µl of TE buffer was added to the reaction and the labelled DNA was then separated from the unincorporated label by spin-eluting through a Sephadex G-25 column (2.2.6 vii). The final volume should be approximately 100 µl with the probe concentration at 1 ng/µl.

2.1.9 (v) Gel Mobility Shift Assays (Bandshift)

This procedure was carried out according to the method described by Theil *et al.*, (1993) using either the in-vitro translated Brn-3a or Brn-3b proteins or the total cellular extracts obtained from ND7 cells. To prepare for this reaction, the following reagents were mixed together without the labelled probe and left at room temperature for 5-10 minutes: 2.5 ul *in-vitro* translated Brn 3a or Brn 3b or 3.5 ul cell extract, 2.0 ul 10x EMSA Buffer (10 mM Hepes (pH 7.9), 60 mM KCl, 4% w/v Ficoll, 1 mM EDTA, 1 mM DTT), 2.0 ug poly (dI-dC) (Pharmacia) (to prevent non-specific interactions), 50-100 ng cold oligonucleotide (50 -100 fold molar excess specific competitor) or

50 ng non-specific competitor (HSE oligonucleotide) and sterile dd H_2O to 20 ul. 1 ng of kinase labelled oligonucleotide probe was then added, mixed well, spun at 12000 rpm for about 5 seconds then incubated on ice for 1 hour.

The protein-DNA complexes were resolved from the free probe on a 7% non-denaturing polyacrylamide gel run in 0.5% TBE at 30-50 mAmp for 2-2.5 hours at 4^{o}C. Gels were dried and exposed to X-ray film at -70^{o}C.

2.1.10 In-Situ Hybridization

All glassware containers used for in-situ hybridization were washed thoroughly, rinsed in ddH2O and baked at 180^{o}C overnight. Most solutions used were autoclaved and kept sterile. Sterile DEPC treated ddH_2O was used for most procedures.

2.1.10 (i) Glass slide preparation

After thorough cleaning with detergent, the glass slides were rinsed in dd H_2O, soaked in 10% HCl for 20 minutes, rinsed again in water then baked at 180^{o}C overnight. After cooling the slides were dipped in a fresh solution of 2% (v/v) 3-amino-propyl-triethoxysaline (TESPA) in acetone for 10 minutes followed by 2 rinses in acetone for about 1 minute each and a final rinse in ddH_2O. After draining the excess H_2O, slides were air dried used within 4 days of TESPA treatment.

2.1.10 (ii) Fixing and Sectioning of tissues

Rat tissues were provided by Ms E. Ensor immediately following sacrifice of the animal. The tissues were mounted in OCT embedding compound (Tissue Tec - Miles Inc. Diagnostics, USA) in a container such as a plastic mould and frozen by slowly placing the mould into isopenthane (which was cooled in a flask of liquid Nitrogen), until the OCT compound was completely frozen. The tissues were immediately transferred to liquid nitrogen to prevent thawing and may be stored long term at -70^{o}C until ready for sectioning.

Sections which were 7 μm thick were obtained from most tissues using a cryostat at -20^{O}C. The testis which were cut at 20 μm to provide satisfactory sections. The sections were collected onto the TESPA coated slides and fixed in 4% paraformaldehyde (pH 7.2) at 4^{O}C for at least 30 minutes and may be left overnight. The following washes were carried out at room temperature: 3x washes in 1x PBS were carried for 5 - 10 minutes each followed by a wash in 70% ethanol for 10 minutes and 2 washes in sterile dd H_2O. Sections were permeabilized in freshly made 0.1M HCl for 5 - 10 minutes, washed in 1x PBS twice then soaked in a freshly prepared solution of 0.1M triethanolamide containing 0.5 ml of acetic anhydride in 200 ml for 20 minutes. This was followed by 2 washes of 5 minutes each in 1x PBS and dehydration through graded ethanol washes in 70%, 80% and 95% ethanol for 5 minutes each. The sections were air dried briefly and were either used immediately for hybridization or were stored in a sealed box containing silica gel to prevent condensation until ready for use.

2.1.10 (iii) Pre-Hybridization and Hybridization of the labelled probe

The in-situ hybridization was carried out using the Digoxigenin-11-uridine-5'-triphosphate (Dig-11-UTP) label (Boehringer Mannheim) with reagents and the DNA labelling and detection kits also obtained from Boehringer Mannheim. The in-situ hybridization protocol used was described by Ulrich Dorries ((Institute for Neurobiology, Zurich).

The sections on the slides were encircled with a grease pen and placed in an incubation chamber, the base of which was lined with strips of filter paper soaked in the 1:1 PBS (x1) : Formamide. 200 μl of 1:1 mixture of the and prehybridization solution (see section 2.1.9 vi) and formamide was added to each slide so that the sections were covered and there were no air bubbles. This was incubated for at least 3 hours at 37^{O}C.

The hybridization mixture for each section was prepared as follows: 50% deionized formamide, 1 x hybridization buffer, 0.33M NaCl, 0.1M DTT, 10% dextran sulphate and 4µl of the riboprobe made up to 200 µl with sterile ddH_2O. For a large number of sections a 'bulk mix' was prepared to ensure an equal amount of probe was placed on each section. This mixture was kept at the temperature at which the hybridization would be carried out. After 3 hours the prehybridization solution was poured off and the grease reapplied if necessary followed by addition of the hybridization mix to each section. Care was taken to prevent any air bubbles and a piece of nescofilm was placed over the solution to prevent concentration of the probe at the edges of the meniscus. This was carefully placed in the incubation chamber, sealed and incubated overnight at the hybridization temperature (55^oC for the Brn-3 probes).

2.1.10 (iv) Post-hybridization washes

The hybridization mix was poured off and the slides replaced in a slide rack for the washes. 2 x washes in 0.2x SSC for 30 minutes each followed by 3 x washes in 0.1 x SSC: 50% formamide for 60 minutes each were carried out at the hybridization temperature. A final wash was carried out in 0.2 x SSC for 10 minutes at room temperature.

2.1.10 (v) Incubation with the anti-digoxigenin antibodies and development of signals

The sections were equilibrated in buffer 1 for 10 minutes at room temperature then blocked in buffer 2 for 1 hour at room temperature. 150 µl of the anti-DIG antibody (diluted 1:500 in buffer 2) was added over the sections, sealed in the incubation chamber and incubated overnight at 4^oC. This antibody solution was poured off and the slides washed twice in buffer 1 for 15 minutes each at room temperature followed by 1 wash in buffer 3 for 15 minutes at room temperature. Grease was re-applied as necessary prior to the addition of 150µl of colour detection buffer (2 ml of buffer 3, 9 µl of 4-nitro-blue tetrazolium (NBT), 7 µl of 5' Bromo-4Chloro-3'indolyl phosphate

(X-phosphate) and 2 µl of 1M Levamisole) to each slide. This was incubated in the dark for up to 16 hours. In initial experiments the colour reaction was monitored at intervals by viewing colour development in the sections using a light microscope. The reaction was stopped by submerging the slides in buffer 4. The slides were then rinsed in H_2O air dried and mounted in 90%glycerol/10% PBS and could then be viewed using a light microscope and photographed.

2.1.10 (vi) Solutions for ISH with digoxigenin-11-UTP labelled probe

Pre-hybridization solution

30.5 ml of sterile dd H_2O

5 ml 50x Denhardt's

5 ml 0.5M EDTA

5 ml 1 M Tris (pH 7.6)

2.5 ml transfer RNA (10 mg/ml)

2 ml of 1M NaCl

10 x Hybridization solution

2 ml of 1M Tris.Cl (pH 7.5)

200 µl of o.5 M EDTA

2 ml of 50x Denhardt's solution

2 ml of transfer RNA (25 mg/ml)

1 ml of poly A RNA (10 mg/ml)

2.8 ml sterile dd H_2O

Pre hybridization and hybridization solutions were aliquoted and stored at -20^{o}C.

Buffer 1

100 mM Tris.Cl (pH 7.5)

150 mM NaCl)

Buffer 2

500 mls of Buffer 1

5g of Blocking Reagent (Boehringer Mannheim)

2.5 g BSA fraction V (Sigma)

dissolved at 60oC then cooled and stored at -20^{o}C

Buffer 3

0.1M Tris pH 9.5

0.1M NaCl

0.05M $MgCl_2$

Buffer 4

1x TE pH8.0

2.1.10 (vii) Riboprobe synthesis using DIG-11-UTP

The plasmids containing the DNA to be transcribed were linearized using the appropriate restriction enzymes required to generate the antisense probe and the control sense transcripts (Brn 3a antisense probe was generated from plasmid linearized with Not 1 enzyme and the sense probe with Sac 1; Brn 3b plasmid was linearized with Not 1 to generate the antisense probe, and Nco 1 for the sense probe). Following restriction digest, 1 µl of the linearized plasmid was run on a gel to check for complete linearization while the rest of the reaction was subjected to 1x phenol/chloroform and 1x chloroform:IAA extraction followed by ethanol precipitation at -20^{o}C. The DNA pellet obtained following centrifugation was washed in 70% ethanol, dried briefly then resuspended in sterile ddH_2O at 1 µg/µl for use in the in-vitro transcription reaction.

For the in-vitro transcription reaction the following reagents were added together: 8.5 µl of sterile ddH_2O, 5µl of 10x transcription buffer (400mM Tris.Cl pH7.5; 60mM MgCl2; 20 mM spermidine.HCl; 50 mM NaCl). 2.5 µl of 100mM DTT, 2 µl each of rATP, rGTP and rCTP, 1.3 µl of rUTP, 0.7 µl of DIG-11-rUTP, 0.5 µl of RNAse inhibitor (40 units/µl), 1µg DNA template and 1 µl of the appropriate RNA polymerase. This mixture was incubated at 37oC for 1 hour after which 1 µl was removed for analysis by gel electrophoresis while the rest of the reaction was subjected to DNAse 1 digest to remove the DNA template by addition of 10 µg of transfer RNA (carrier) and 1 µl of RNAse-free DNAse 1 enzyme. 1 µl of this reaction mix was also removed and resolved on an agarose gel along with the undigested sample. 2 µl of EDTA was added to stop the transcription reaction and the probe was precipitated with 2-3 volumes of ethanol and 0.1 volume of lithium chloride at -70^{o}C. The pellet obtained following centrifugation was washed in 70% ethanol, briefly vacuum dried in a freeze dried then resuspended in 50 µl of TE pH 7.5 and stored at -20^{o}C.
The concentration of the probe was checked using the protocol described by the manufacturer.

2.1.11 Polymerase Chain Reaction (PCR)

Transcripts were amplified in the Hybaid thermal cycler and the conditions for the reaction depended on the target sequences to be amplified and the purpose of the reaction.

2.1.11 (i) Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA isolated from the cells or tissues (described in 2.1.12) which were used for RT-PCR were subjected to DNAse 1 treatment to remove contaminating genomic DNA (2.1.12 vi).

2.1.11 (ii) cDNA synthesis from total cellular RNA

To ensure an equal amount of RNA was used for each cDNA synthesis reaction, a small aliquot of RNA from groups of cells which were subjected to different treatment were resolved on denaturing agarose gel electrophoresis and Northern hybridization with ^{32}P α-CTP labelled probe for the 18s ribosomal RNA. The levels were quantified by densitometric scanning and equal amounts of RNA were used to synthesised cDNA. The cDNA synthesis reactions were routinely carried out using sterile equipment and reagents. The RNA sample must be in the single stranded conformation to facilitate the synthesis, so to denature the RNA, 1ug of each RNA sample was made up to 4 ul, with DEPC treated, sterile dd H_2O, heated at 65°C for 2 mins then placed on ice immediately and kept there until ready for use. Samples were spun briefly prior to the addition of the reagents mixture described below. If a large number of RNA samples were to be used a 'bulk mix' was prepared by addition of the following reagents together for each sample of RNA used for cDNA synthesis.Typically the reaction was carried in a volume of 50 µl consisting of 10 µl DEPC treated water, 10 µl of 5 x Reverse transcription buffer (50mM Tris.Cl, pH 7.6, 60 mM KCl, 10mM Mg_2Cl, 1mM DTT), 1 µl (40 units) of RNAse Inhibitor and 10 mM of each NTP, 1 ul (200 units) of Reverse transcriptase

enzyme was added immediately before the mix was added to the RNA samples. Following gentle mixing and a brief spin, the reaction was incubated at 37°C for up to 1 hour. This was then stored at -20°C for later use or was used straight away.

2.1.11 (iii) PCR conditions

All PCR were carried out in 100 ul reaction mix containing either plasmid DNA or cDNA synthesised from cellular RNA, 10 ul of 10 x PCR buffer (100 mM Tris.Cl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatin), 200 μM of each NTP, o.1 μM of each of the upstream and downstream oligonucleotide primers and 2-5 units of Taq DNA polymerase. The volume was made to 100 ul with sterile ddH_2O. Mineral oil was layered on top of the reaction mix to prevent evaporation. The thermal profile and number of cycles carried out depended on the sequences being amplified.

2.1.11 (iv) Isolation of Novel POU-domain factors from ND7 cells by RT-PCR

PCR amplification were carried out according to the method of He *et al.*, (1989) using degenerate oligonucleotides containing all possible nucleotide sequences encoding two regions of the POU domain, each consisting of nine amino acid which are highly conserved in all POU proteins. One of these regions, Phe-Lys-Val/Gln-Arg-Arg-Ile-Lys-Leu-Gly, is located near the amino terminus of the POU-specific domain while the other, Arg-Val-Trp-Phe-Cys-Arg-Gln/Arg-Arg-Gln, is located near the carboxyl terminus of the POU homeodomain and should therefore allow amplification of the entire POU-domain (He *et al.*,1989, Lillycrop *et al.*,1992). PCR was carried out in 100 ul reaction mix described above) containing cDNA synthesised total RNA obtained from proliferating ND7. The thermal profile used was 94°C for 30 seconds (denaturing), 52°C for 30 seconds (annealing primers) and 72°C for 30 seconds (extension). This was carried out for 30 cycles. The PCR products obtained were resolved on low-melting point agarose gel and visualized and the products of the expected size cut out of the gel and cloned into the Bam H1 site of Bluescript vector

using the GATC overhang present in the primer sequences (section 2.1.4). Clones obtained were screened by colony hybridization with probes to known POU proteins and positive clones were sequenced using the Sequenase DNA sequencing kit and primers flanking the multiple cloning site of the plasmid.

2.1.11 (v) Optimizing conditions for Brn-3a and Brn-3b PCR

The relatively low abundance of the mRNA for the Brn-3a and Brn-3b transcription factors as well as the limited cDNA sequence of the POU domain which was available initially meant that the expression patterns of the mRNA of these two factors could not be effectively studied by ribonuclease protection assays on Northern blots. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was optimized to measure the levels of expression of the two transcription factors in tissues and cell cultures. PCR amplification was carried out using an upstream primer common to both proteins (Brn-3 POUs) and one of two downstream primers from the most diverged region of Brn-3a and Brn-3b (Brn-3a specific or Brn-3b specific) (2.0.8).

Conditions for specific and efficient amplification of Brn-3a and Brn-3b were optimized by using various annealing temperatures to amplify a plasmid containing Brn-3a or Brn-3b POU domain cDNA sequence. The specificity of the annealing reaction at the optimized temperature was tested by carrying out PCR of Brn-3b POU domain containing plasmid with the Brn-3a primers. Similarly PCR was carried out using the Brn-3b primers and both the Brn-3a and Brn-3b plasmid. The PCR products were resolved and visualized on 2% agarose gels since the expected sizes were both approximately 272 bases.

2.1.11 (vi) Semi-quantitative PCR (Quantifying Brn-3a and Brn-3b mRNA)

cDNA was synthesized from RNA which was extracted from treated cells and this product was then used for RT-PCR. Each set of cDNA from an experiment was tested by trial PCR using primers to the control L6 ribosomal or cyclophilin mRNA mRNA. Samples taken at different cycles indicated the efficiency of the cDNA synthesis and also allowed an estimate of the variation of cDNA between samples when resolved on agarose gel. The samples were transferred to membrane filters by Southern blotting and hybridized with with the labelled control probe. Densitometric scanning to quantify the differences between samples allowed the volume of cDNA used for amplification of the Brn-3a and Brn-3b to be adjusted to equalize the amount of cDNA used in each sample.

For each set of Brn-3 PCR carried out, the same volume of cDNA was used in parallel amplification of the control, L6 ribosomal product or cyclophilin product also. Aliquots of the PCR products were taken at different cycles to ensure that the reaction was in the exponential phase. In addition, duplicates of the reactions were performed on each sample. To confirm the identity and to facilitate quantification, the amplification products were run on 2% agarose gel, transferred to nitrocellulose filter by Southern blotting and hybridized with the specific random prime labelled probes of the Brn-3a and Brn-3b POU domain cDNA clones. The amplified products on were either quantified on the phosphor imager or by scanning the autoradiographic film using a densitometer. Any variation in the amplification of the control samples were used to correct the values obtained for the Brn-3 mRNA amplification. Each set of experiments included a control untreated culture and the expression of the Brn-3 mRNA were then presented as a percentage of the control values.

2.1.11 (vii) Brn-3 PCR (POU domain)

Brn-3a

Denature at 94°C for 30 seconds

Anneal at 56°C for 30 seconds

Extend at 72°C for 30 seconds

Brn-3b

Denature at 94°C for 30 seconds

Anneal at 58°C for 30 seconds

Extend at 72°C for 30 seconds.

Control L6 ribosomal PCR

Denature at 94°C for 30 seconds

Anneal at 56°C for 30 seconds

Extend at 72°C for 30 seconds.

Aliquots of products taken at 15, 20 and 25 cycles ensured that the reaction was being measured in the exponential phase. Later, primers for the cyclophilin gene () were also used to equalize for mRNA variation as testing the amplification gave similar results to the L6 ribosomal primers.

2.1.11 (viii) Brn-3 PCR with primers outside the POU domain

The oligonucleotide primers designated 3A3' 1 and 2 and 3BN and 3BF were designed to amplify sequences outside the POU domain of Brn-3a (3' non-coding sequences) and Brn-3b (region 5' to the POU domain), respectively. PCR was carried out as described above but the thermal profiles were:

Brn-3a (3' non-coding)

Denature at 94°C for 30 seconds

Anneal at 62°C for 30 seconds

Extend at 72°C for 30 seconds

Brn-3b (5' POU domain)

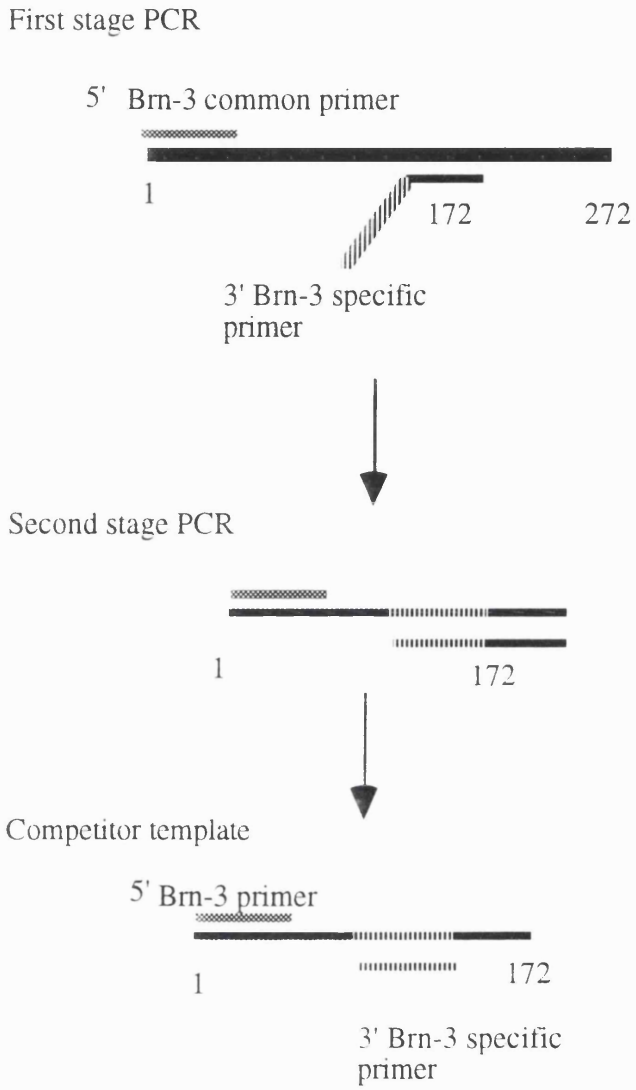
Denature at 94°C for 30 seconds

Anneal at 58°C for 30 seconds

Extend at 72°C for 30 seconds.

2.1.11 (ix) Competitive PCR

An internal control was designed to control for tube to tube variation in the amplification process by using specific competitor primers designed for Brn-3a and Brn-3b according to the method of Celi *et al* (1993). The competitor template was designed to be amplified using the same primers required for the target gene amplification but was smaller than the target product and could thus be distinguished upon resolution of the PCR products by gel electrophoresis. To generate the competitor template, the common 5' oligonucleotide primer used for Brn-3 PCR was used with new 3' primers were designed to contain the sequence of the primer used for Brn-3a or Brn-3b specific amplification linked to a sequence which was about 100 bases upstream of this sequence. These primers were used for two sets of amplification with the initial PCR carried out using plasmids containing the cDNA of either Brn-3a or Brn-3b. The conditions were as before except for the annealing temperature and time which were set at 52°C for 45 seconds to allow low stringency annealing of the primer to the target. The products of this reaction were resolved on low melting point agarose gel, and the product of the right size was cut out of the gel, purified using the PCR magic ™ protocol (Promega) and used in the second round of amplification with the same primers. In this PCR the annealing temperature was raised to 60°C to ensure specific amplification and the product was gel isolated and purified. This could then be cloned into pGEM-T cloning vector and sequenced to check the product.



The amount of competitor DNA product used in the competitive PCR was optimized by using a dilution series of the DNA in PCR in which the primers and cDNA concentration were kept constant. The concentration of the competitor which allowed approximately equal amplification of the target and competitor products was later used. Co-amplification of an equal amount of these competitor templates with each experimental samples acted as an internal control for the efficiency of the PCR since the product of the competitor template can be distinguished from that of the target when analysed on a high percentage (2%) agarose gel on the basis of its smaller size.

2.1.12 RNA Extraction:

Sterile equipment and solutions were used for all procedures in which RNA is used or extracted. DEPC dd H_2O (double distilled water which has been treated to inactivate nucleases by autoclaving it after the addition of 200 ml/l diethyl pyrocarbonate (Sigma) was used in solutions or for resuspention of RNA preparations.

2.1.12(i) Total RNA preparation (Phenol / Chloroform Method)

Cells in culture were harvested in 1x PBS and pelleted by centrifugation at 1500 rpm for 5 mins at 4°C then resuspended in 500 ul of Solution 'D' (4 M guanidinium isothiocyanate, 25 uM Sodium Acetate pH 5.2 dissolved in water with 14% v/v β-mercaptoethanol added). If tissues were used for RNA isolation, they were snap frozen in liquid nitrogen immediately after dissection. 100 mg of tissue was ground in liquid nitrogen using a pestle and mortar and the resultant powder dissolved in 1 ml of solution 'D'.

The homogenate was transferred to an eppendorf tube and 0.2 M Sodium acetate, (pH4.0) was added and mixed by inversion. An equal volume of water-saturated phenol was then added to this and mixed thoroughly. A tenth of the volume of chloroform / isoamyl alcohol (24:1) was added, mixed well and then left on ice for 15 mins. This mixture was spun for 20 mins at 12000 rpm at 4°C. The aqueous phase was removed to a fresh tube and RNA precipitated by addition of an equal volume of isopropanol This was left on ice for 20 mins then centrifuged at 12000 rpm for 10 mins at 4°C. The pellet was washed with 70% ethanol, freeze-dried for 2 mins to remove residual ethanol then resuspended in 300 ul Solution 'D'. The isopropanol precipitation step was repeated. Centrifugation was repeated at 12000 rpm for 5 mins at 4°C. The pellet was washed in 70% ethanol dried, redissolved in DEPC H_2O and either stored at -70°C until ready for use or subjected to DNAse 1 digestion.

2.1.12(ii) Mini-RNA preparation - Cesium Chloride Method

This method was mainly used for RNA extraction from small amounts of cells in cell culture. The medium was removed from cells in culture which were then washed with 1 x PBS and harvested in 1ml PBS. Cells were pelleted by spinning at low speed (6000 rpm) for 5 mins at 4°C. The pellet was resuspended in 500 ul of Solution 'D'. This extract was then used immediately in the procedure described below but could be frozen at -70°C for later use.

A cushion of 220 ul 5.7 M $CsCl_2$ (containing 5.7 M cesium chloride ($CsCl_2$), 0.1 M EDTA pH 7.5, 0.1% DEPC mixed well, allowed to stand for 30 mins, then autoclaved for 20 mins and cooled prior to use) was placed on the bottom of a Beckman SW40 polyallomer tube and the cell extract was layered on this. The tubes were balanced using Solution 'D' then spun in a Beckman TL-100 ultracentrifuge at 55,000 rpm for 3 hrs at 18°C.

The supernatant was decanted off carefully and 150 ul of DEPC treated H_2O added to the pellet and left on ice for 30 mins to rehydrate then resuspened by pipetting.
The RNA was precipitated with 2.5 volume of ethanol and 0.1M of sodium acetate at -70°C for 1 hour then spun at 12000 rpm for 15 mins at 4°C. The pellet was washed in 70% ethanol, freeze dried for 2 mins then resuspended in 30 µl DEPC water. The RNA could then be stored at -70°C until ready for use or subject to DNAse 1 treatment to remove any residual DNA.

2.1.12(iii) RNA preparation using RNAzol™ B solution

This method was carried out according to the manufacturers instructions. Briefly, issues were homogenised in the RNAzol™ B solution (2ml / 100 mg tissue) using a polytron probe (Ultra turrax T25, Jank & Kunkel IKA Labrotechnik). For cell lines the media was removed, cells were washed in PBS and harvested in the RNAzol™ B solution (0.2 ml / 10^6 cells). Samples were kept on ice following homogenization /

harvesting. 200 ul chloroform:IAA was added to the homogenate, shaken vigorously and left on ice for 5 mins. The suspension was centrifuged at 12,000 g at 4°C for 15 minutes. The upper aqueous phase was transferred to a fresh tube (taking care not to transfer any of the interface or lower organic phase as this appeared to affect later precipitation) and equal volumes of isopropanol was added, mixed well and stored at 4°C for 15 mins. The samples were then centrifuged at 12000 g for 15 mins at 4°C. The RNA pellet was washed with 75% ethanol by vortexing and subsequent centrifugation. The pellet was briefly freeze-dried and resuspended in DEPC water.

2.1.12(iv) Northern Blotting and Hybridization

Total RNA samples were resolved on 1.0% agarose / formamide gels prepared in 1x MEA buffer (20 mM MOPS, 1 mM EDTA, 0.5 mM sodium acetate in H_2O adjusted to pH 7.2 with NaOH) and made 2.2M with respect to formaldehyde before casting. RNA samples were denatured by treating with the sample buffer (1x MEA, 50% formamide, 2.5 M formaldehyde) and heating to 65oC for 15 minutes prior to loading on the gel. 0.2 volume of 6x loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue) was added to the RNA samples and this was loaded and the gel was electrophoreses until the blue dye had migrated about 100 mm from the wells. The gel was washed in 20x SSC and the RNA transferred onto Hybond-N filters by a procedure similar to that described for Southern blotting (2.1.6 iv). After transfer the RNA was cross-linked to the membrane the U.V. Stratalinker. Filters were incubated in the pre-hybridization solution (4 x SSC, 1 x Denhardt's solution, 5% Dextran sulphate, 0.001 M EDTA, 0.05 M phosphate buffer, 100 µg/ml salmon sperm DNA and 0.1% SDS) at 42°C for 4 hours. Hybridization was carried out overnight in hybridization solution containing 50% deionized formamide, 5x SSC, 1 x Denhardt's solution, 5% Dextran sulphate, 0.05 M phosphate buffer, 500 µg/ml salmon sperm DNA, 0.1% SDS, 100 µg/ml of yeast total RNA and 10 µg/ml poly A RNA with radioactively labelled riboprobe or random prime labelled probes (see 2.1.12) .

The washing procedure is similar to that described for Southern hybridization and typically include 2 x washes in 1 x SSC / 0.1% SDS at 65°C for 30 minutes each and 2 x washes in 0.1 x SSC / 0.1 % SDS at 42°C. Filters were sealed to prevent drying (especially if the filter has to be reprobed) then exposed to film for up to one week depending on the abundance of the messenger RNA.

2.1.12(v) Probes used for Northern Hybridization

Random prime labelled probe (Klenow fragment of DNA polymerase 1) was commonly used for northern hybridization. However when high activity probes were required (for detection of low abundance mRNA) [^{32}P] labelled riboprobes were used. Riboprobes were also used for experiments such as ribonuclease protection or in-situ hybridization which required the synthesis of both the antisense and sense strands.

Riboprobe preparation (cRNA synthesis)

Templates were linearized with the appropriate enzyme and following the restriction digest the DNA was extracted with phenol / chloroform: IAA once, then once with chloroform / IAA. Ethanol / sodium acetate precipitation was carried out at -20oC for 1 - 2 hrs followed by centrifugation at 12000g for 15 mins. The pellet was washed in 70% ethanol, dried and resuspended in DEPC treated ddH_2O at 1 ug/ul.

[32P] labelled full length RNA transcripts of high activity were synthesized in 25 µl reaction containing 8.5 µl DEPC water, 100 ng - 1 ug template DNA, 500 µM each rATP, rGTP, rUTP, 200 µM rCTP, 1 x Transcription buffer (supplied with the enzyme and containing 40 mM Tris.Cl pH7.5, 10 mM $MgCl_2$, 1 mM DTT, and 100 ug/ml BSA) 1.0 ul DNAse free RNAse inhibitor (10 units), 50 µCi a- ^{32}P rCTP and 1 µl bacteriophage DNA-dependent RNA polymerase (7-12 units). The reagents were mixed, centrifuged briefly then incubated for 1 - 2 hours at 37°C (T7 and T3 polymerases) and 40°C for SP6 polymerase.

To remove the DNA from the reaction 1ul of RNAse free DNAse 1 (1mg/ml) was added to the reaction, mixed well and incubated at 37°C for 15 mins. 100 ul of DEPC treated dd H_2O was added and phenol / chloroform:IAA extraction performed followed by a chloroform:IAA extraction. The aqueous phase was transferred to a fresh tube and the cRNA precipitated with 2.5 volumes of ice-cold ethanol and 0.2 M ammonium acetate added at -20°C for 30 mins. Following centrifugation at 12,000g for 10 mins at 4°C the pellet was briefly vacuum dried or air dried while stored on ice then resuspended in 100 ul of DEPC water. Repeat of the precipitation with ethanol removed much of the unincorporated rNTPs from the RNA. This was stored an ice until ready for use. 1µl of the labelled probe was removed and used to determine the specific activity by TCA precipitation as described by Sambrook *et al.*,1989. The specific activity of the probe used for hybridization was 0.5 - 1 $x10^5$ counts/ min/µl.

2.1.12(vi) DNAse1 treatment of RNA samples

All reactions used DEPC-treated dd H_2O and sterile materials. A typical 100 ul reaction was prepared by addition of the following reagents to the RNA preparation: 20 ul 5xDNAse 1 buffer (50mMTris HCl pH 7.6, 10mM $MgCl_2$, 0.1mM DTT), 1 ul DNAse free - RNAse Inhibitor (10 units/ul), 1 ul RNAse free - DNAse 1 enzyme (1 ug/ul) and the total volume was made to 100ul with DEPC water. This reaction mixture was incubated at 37°C for 30 mins.

To stop the reaction and remove the enzyme, 1 x Phenol / chloroform:isoamyl alcohol (IAA) (24:1) extraction was performed followed by 1 x chloroform: IAA extraction. The aqueous phase was then precipitated in 2.5 x volume of ethanol and 0.1 volume of 3M sodium acetate at -70°C for about 1hour. The RNA was pelleted by centrifugation at 12000g for 15 mins at 4°C. After briefly freeze drying the pellet was resuspended in 20 - 30 ul of DEPC water and stored at -70°C for use as required.

2.1.13 Transient transfections and CAT assays

Transfections were carried out according to the calcium phosphate method described by Gorman, 1985. Briefly, cells were plated out at a density of 5 x 10^5 in petri dishes (90 mm diameter) in the medium being used for treatment on the day prior to the transfection. On the following day the cell grown in L15 containing medium (ND7 cells) were washed in serum free DMEM medium then incubated with 5 ml of DMEM medium containing 10% FCS for about 3 hours to stabilize the pH of the medium. Prior to transfection the calcium phosphate DNA precipitate was prepared as follows: 10 µg of each plasmid DNA (reporter plasmid and expression vector) and sterile sonicated herring sperm DNA were added to give a total of 30 µg of total DNA per sample. This was added to 31 µl of 2 M $CaCl_2$ and made up to a final volume of 250 µl with sterile dd H_2O. This solution was then added in dropwise to an equal volume of freshly prepared 2 x HBS [1.64% (w/v) NaCl, 1.19% (w/v) HEPES, 0.04% (w/v) Na_2PO_4 adjusted to pH 7.12 with 1 M NaOH] then immediately added to the cells and the DNA precipitate was left on the cells for 3 - 6. At the end of this incubation the medium was removed and the cells were washed in serum free medium then transferred to fresh growth medium. At this time if the cells were being treated with cyclic AMP or growth factors these factors were also added to the medium. After 48 - 72 hours the cells were washed with PBS, harvested, pelletted by low speed centrifugation and resuspended in 100 µl of 0.25 M Tris. Cl (pH 7.8).

Cell extracts were obtained by three cycles of freeze-thawing in liquid nitrogen and a 37^{o}C water bath. The cell debris were removed by low speed centrifugation and the supernatant was stored at -20 until required for used. Aliquots of these extracts were used to measure the DNA uptake in the different transfected cells and the protein content in each sample. To measure the DNA uptake, the extracts were treated with RNAse A and Proteinase K before immobilizing on nitrocellulose filter, Hybond N, by slot blotting. The amount of plasmid per sample was quantified following hybridization with a probe which specifically detected the plasmid vector, such as the probe derived

for the ampicillin resistance gene in the(Sca 1 / Bgl II fragment) which is present only in the plasmids transfected. This values obtained were used in conjunction with the protein levels obtained using the BCA protein assay to normalize the values obtained in the CAT assays.

2.1.13 (i) CAT assay

Assays of the chloramphenicol acetyl transferase activity were carried out according to the method described by Gorman, 1985. Briefly, the promoter activity of the transfected reporter construct was measured by the level of activity of the chloramphenicol acetyl transferase enzyme (CAT) gene product which catalyses the transfer of the acetyl group from acetyl co-enzyme A to the ^{14}C labeled chloramphenicol. The conversion to the acetylated form can be detected and quantified by thin layer chromatography. For the typical reaction (x) µl of cell extract (volume equalized on the basis of protein and DNA uptake level) was made up to 90 µl with 0.25 M Tris-HCl (pH 7.8). To this 20 µl 4mM acetyl Co-enzyme A, 1 µl ^{14}C chloramphenicol (40 - 50 Ci/mmol) (NEN or Amersham) and 35 µl dd H_2O were added.This reaction mix was incubated at 37^{o}C for 30 - 60 minutes, followed by extraction of the chloramphenicol with 1 ml of ethyl acetate. Following adequate vortexing, the layers were resolved by high speed centrifugation for 10 minutes. The upper organic phase which contains the different forms of chloramphenicol, i.e., the unconverted as well as the converted mono acetate and di-acetate chloramphenicol was removed and dried down in a vacuum dryer. The samples were resuspended in 20 µl of ethyl acetate, and applied to silica gel thin layer chromatography plates which were then subjected to ascending chromatography in a 95:5 mixture of chloroform:methanol until the solvent had reached the top of the TLC plates. After air drying, the plates were either used for analysis of the converted and unconverted products on the phosphor image analyzer or exposed to X-ray film at room temperature which could then be scanned on a densitometer.

2.1.14 Tritiated Thymidine [^{3}H] Incorporation

Cell cultures were incubated in the presence of [^{3}H] thymidine for 4 hours. The cells were washed in serum free medium then harvested since proteins in serum were shown to result in lower measurements of thymidine incorporation compared with cells which were washed medium prior to counting (possibly caused by proteins in serum which sequester the tritium so giving rise to lower counts).[^{3}H] thymidine incorporation was determined by precipitation of cellular DNA with 10% trichloroacetic acid (TCA) as described in Stambrook *et al.*,1989.

1.1.15 Trypan Blue Exclusion

Cells were aspirated and spun for 10 minutes at 4000 rpm at 4°C. The cell pellet was resuspended in 50 ul of medium and an equal volume of 0.4% trypan blue in PBS was added. The mixture was incubated at room temperature for 5 minutes and the proportion of cells able to exclude trypan blue dye counted. This was a measure of the viable cells and was expressed as percentage of the total cells to indicate percentage survival.

Chapter 3

Results

Isolation of the novel Brn-3b and the expression of Brn-3a and Brn-3b POU domain protein

3.0.0 Background

The cooperative interactions of various transcription factors which are differentially expressed within the nervous system contribute to the development and differentiation of specific neurons. Therefore to understand the role of families of these proteins in neuronal development and function, it is necessary to identify all such factors in the tissue of interest. We were interested in studying the POU domain transcription factors in sensory neuronal cells since some proteins in this family have been demonstrated to be critical for normal development and differentiation of specific neuronal cells (see introduction).

He *et al.*, (1989) had isolated a number of novel POU domain proteins using cDNA synthesized from mRNA obtained from rat and human brain and rat testis using degenerate PCR primers which corresponded to highly conserved regions in the POU domain. Three of the seven clones isolated were found to be sequences encoding the POU domain of the known proteins Oct-1, Oct-2 and Pit-1, the other three were novel clones isolated from brain cDNA amplification and were named Brn-1, Brn-2 and Brn-3 while the seventh clone was derived from testis cDNA and was called Tst-1.

The Brn-3 protein (herein referred to as Brn-3a) was found to be expressed in sensory neurons as well as in specific parts of the brain (He *et al.*, 1989; Ninkina *et al.*, 1993; Gerrero *et al.*, 1993). Brn-3a was the most homologous mammalian factor to the nematode POU factor, unc-86, which was shown to be crucial for normal development and differentiation of specific neuronal phenotypes in *C. elegans* (Xue *et al.*, 1993; Finney and Ruvkun, 1990) suggesting a conserved role for the Brn-3 proteins in the development of sensory neurons. The results in this chapter report on the isolation of a novel Brn-3 transcription factor, (Brn-3b), from the sensory neuronal derived cell line, ND7, and the patterns of expression observed in various tissues and cell lines.

Results:

3.1.0 Isolation of Novel POU factors from ND7 cDNA

The initial work carried out which resulted in the isolation and sequencing of the novel Brn-3b factor was done by Dr. Karen Lillycrop. This will be described to give a complete picture of the project. However, all the other studies reported constituted the work carried out for this thesis.

In an effort to identify novel POU-domain proteins expressed in sensory neuronal cells a strategy similar to that described by He *et al.*, (1989) was used. cDNA synthesized from RNA obtained from the sensory neuron derived cell line, ND7 was amplified by PCR using degenerate primers which were designed to contain all possible sequences encoding two highly conserved nine amino acid regions of the POU-domain (He *et al.*, 1989; Lillycrop *et al.*, 1992). One of these regions, Phe-Lys-Val/Gln-Arg-Arg-Ile-Lys-Leu-Gly, was located at the amino terminal of the POU-specific domain while the other, Arg-Val-Trp-Phe-Cys-Arg-Gln/Arg-Arg-Gln was at the carboxyl terminus of the POU-homeodomain. The position of these primers were selected to allow the amplification of the entire POU-domain. The reaction was carried out at a low stringency which should facilitate the amplification of all POU domain factors present in the cDNA. 30-40 cycles of PCR were performed at 94°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes. The PCR product was gel-isolated and cloned into the BamH1 site of Bluescript vector using the GATC overhang present in the original oligonucleotides.

The resultant PCR product was approximately 400 bases, consistent with the distance between the primers in the POU-domain. The 45 clones obtained from sub-cloning the PCR products into the Bluescript vector were classified into five groups (Class 1-5) as a result of the hybridization patterns and sequences (summarized in table 3.1.1).

Class	Number	Comments
1	4	Closely related to human and mouse Oct-1
2	7	Identical to mouse Oct-2
3	12	Identical to rat and mouse Brn-3
4	8	Seven amino acid difference from mouse Brn-3 and Class 3 clones
5	14	Unrelated to POU-factors

Table 3.1.1: A summary of the POU factor clones obtained by polymerase chain reaction using degenerate primers to two highly conserved regions in the POU domain and cDNA from ND7 cells.

The POU domain clones isolated are represented in Groups 1-4 since clones in class 5 did not hybridize to any known POU factor probe and showed little or no sequence homology outside the primer. These products resulted from the amplification of sequences unrelated to POU factors and maybe caused by random homology within the primers used for the PCR. As such these clones were not studied any further. He *et al.*, (1989) also reported a number of such spurious clones using this strategy. Group 1 and 2 represented the rat homologue of the mouse Oct-1 and Oct-2 mRNA, respectively.

Oct-1 is ubiquitously expressed in proliferating cells and was previously shown to be expressed in ND7 cells (Strum *et al.*,1988 Latchman *et al.*,1992), while Oct-2 was shown to be expressed in brain, sensory neurons (He *et al.*,1989; Scholer et al., 1991; Hatzopoulos *et al.*,1990) and in ND7 cells (Lillycrop and Latchman, 1992).

The clones in Group 3 and 4 hybridized strongly to the Brn-3 cDNA probe but further analysis revealed differences in the DNA sequences and predicted amino acids. Whereas the group 3 clones were identical at both the DNA and protein levels to the previously characterized rat and mouse Brn-3 POU-domain (He *et al.*,1989), group 4 clones differed by 39 bases with a resultant change in seven amino acids in the protein sequence (figure 3.1.1). Most of these changes (five of the seven amino acid) were in the poorly conserved linker region. One of the two other amino acid changes, a valine to isoleucine alteration, was in the first helix of the POU-homeodomain. This domain is generally highly conserved in homeodomain proteins and has been shown to be critical for protein-protein interactions between other POU-domain proteins, such as CF1a and I-POU / tI-POU (Treacy *et al.*, 1991, 1992). This change may therefore modify the protein-protein interactions of this novel protein compared with the original Brn-3 protein. To indicate that these were two distinct but closely related factors the previously characterized Brn-3 was renamed Brn-3a and the novel protein then referred to as Brn-3b.

```
          V   T   Q   A   D   V   G   S   A   L   A   N   L
Brn-3a:  GTG ACG CAG GCC GAC GTG GGC TCG GCG CTG GCC AAC CTC
Brn-3b:  --- --C --- --A --T --- --- --- --- --- --- --- ---

  K   I   P   G   V   G   S   L   S   Q   S   T   I   C   R
: AAG ATC CCG GGC GTG GGC TCG CTC AGC CAG AGC ACC ATC TGC AGG
: --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

  F   E   S   L   T   L   S   H   N   N   M   I   A   L   K
: TTC GAG TCG CTC ACG CTC TCG CAC AAC AAC ATG ATC GCG CTC AAG
: --T --T --- --A --G --A --- --- --- --- --- --- --- --- ---

  P   I   L   Q   A   W   L   E   E   A   E   G/K A/S Q/H
: CCC ATC CTG CAG GCC TGG CTG GAG GAG GCC GAG GGC GCG CAA
: --A--- --- --- --G --- --- --- --A --T --- AAA T-C --C

  R   E   K   M/L N/T K   P   E   L   F   N   G   G/A E
: CGT GAG AAA ATG AAC AAG CCG GAG CTC TTC AAC GGC GGC GAG
: --C ------G C-C -CT --- --- --- --- ------T --- -CT ---

  K   K   R   K   R   T   S   I   A   A   P   E   K   R   S
: AAG AAG CGC AAG CGA ACT TCC ATC GCC GCG CCC GAG AAG CGC TCC
: --- --- --- --- --C --G --- --- --G --- --G --- --- --- --T

  E   A   Y   F   A   V/I Q   P   R   P   S   S   E   K   L
: CTC GAG GCC TAT TTT GCC GTA CAA CCC CGG CCC TCG TCT GAG AAG
: --G --A --- --C --C --- A-C --G --AA-- --- --C --G --- ---

  I   A   A   I   A   E   K   L   D   L   K   K   N   V   V
: ATC GCC GCC ATC GCC GAG AAA CTG GAC CTC AAA AAG AAC GTG GTG
: --- --G --- --- ------A --G --- --T ------G --A --T --- ---
```

Figure 3.1.1: Sequences of the Brn-3a and Brn-3b POU domains isolated from ND7 cells. The predicted amino acid sequence of Brn-3a is shown along with the differences in the Brn-3b sequence. The linker region is indicated in italics. The underlined sequences indicate the regions to which PCR primers were designed. The specific primers which determined amplification of Brn-3a or Brn-3b in RT-PCR were derived from the underlined sequence in the linker region while the common POU-specific primer which is underlined in the amino terminus were used for both reactions.

3.1.1 Optimizing conditions for Brn-3a and Brn-3b specific PCR

When the novel Brn-3b was isolated only the POU domain cDNA sequences was available for both the Brn-3a and Brn-3b and so for further analysis of the mRNA expression, PCR primers were designed and conditions optimized to facilitate the specific amplification of the regions encoding the POU domain of each factor (see methods). One of the primers which was common to both Brn-3a and Brn-3b was located in the POU-specific domain while the second oligonucleotide corresponded to the most divergent sequence in the linker region (figure 3.1.1). The twelve base differences between these two factors in this region resulted in efficient but specific amplification of the two factors under the optimized conditions.

This specificity was confirmed by the restriction enzyme digestion of the PCR products with the enzyme Fok 1 since digest of Brn-3a POU domain sequence gave rise to fragments of 154 and118 base pairs while digest of Brn-3b produced fragments of 218, 43 and 11 base pairs (Lillycrop *et al.*, 1992). In addition, PCR with these primers resulted in specific amplification when using the specific primers and plasmid DNA containing either the POU domain of Brn-3a or Brn-3b. There was no amplification from the Brn-3a plasmid using Brn-3b primers and vice versa (figure 3.1.2).

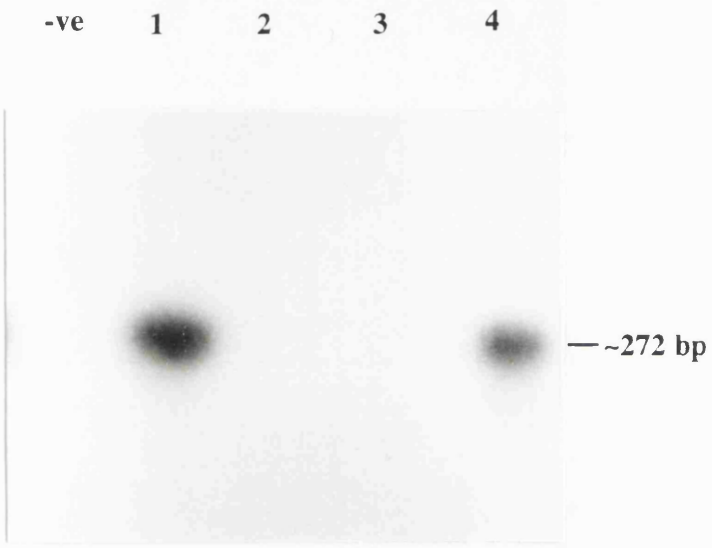


Figure 3.1.2 : PCR amplification of Brn-3a and Brn-3b POU domain showing specificity of the reaction when using the Brn-3a or Brn-3b specific primers and the conditions optimized for these primers. PCR was carried out with either the Brn-3a primers and plasmid containing the Brn-3a cDNA (1) or Brn-3b cDNA (2) or with these plasmids and the Brn-3b primers (3 and 4) . Amplification with the Brn-3a primers resulted in product only with the plasmid containing Brn-3a cDNA (1) but not Brn-3b (2). Similarly there was no amplification of the Brn-3a cDNA using the Brn-3b primers (3) but specific product was obtained with the Brn-3b cDNA under the same conditions (4). The first track was the negative control.

3.1.2 Expression of Brn-3a and Brn-3b

The expression patterns of these two factors were next examined in various cell lines and tissues. RT-PCR amplification of mRNA of Brn-3a, Brn-3b and the constituitively expressed L6 ribosomal proteins (which was used as an invariant control) was carried out using cDNA synthesized from total cellular RNA under the optimized conditions. Aliquots of the PCR samples were taken at different cycles to ensure that the reaction was in the exponential phase of the reaction (figure 3.1.3). The expected products of about 272 bases were observed for both Brn-3a and Brn-3b while the L6 ribosomal control product was about 150 bases. In most experiments the PCR products were resolved by agarose gel electrophoresis, transferred to nitrocellulose membrane by Southern blotting and hybridized with the specific random primed labelled probe. The levels were quantified as described in the methods.

3.1.3 Expression in proliferating and differentiated ND7 cells

Both Brn-3a and Brn-3b clones were isolated from the cDNA obtained from undifferentiated ND7 in the initial experiments. However, since these cells can be induced to differentiate into neuronal like cells on transfer to defined medium (see introduction) we were interested in comparing the level of expression of these two transcription factors in ND7 cells in the proliferative, undifferentiated state and upon differentiation. Therefore, cDNA synthesized from the RNA which was extracted from either proliferating or differentiated ND7 cells and were used for RT-PCR to amplify Brn-3a, Brn-3b and the control L6 ribosomal protein.

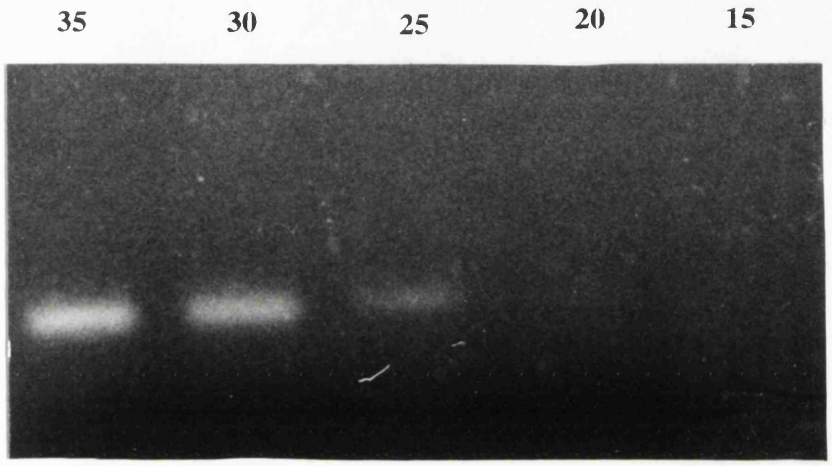


Figure 3.1.3: Amplification of the Brn-3a mRNA with increasing number of PCR cycles of 15, 20, 25, 30 and 35 cycles from right to left. The increase observed between 20 and 30 cycles indicate an exponential increase in the PCR product. Aliquots of subsequent experiments were taken at various cycle number within this range.

In these experiments Brn-3a and Brn-3b showed distinct but overlapping expression patterns in proliferating and differentiated ND7 cells (figure 3.1.4). Whereas Brn-3b mRNA was highly expressed in the proliferating immature cell type, Brn-3a transcripts were in much lower abundance. Upon differentiation, this pattern is reversed, such that Brn-3a mRNA expression was upregulated with Brn-3b showing a concomitant decrease. This effect was observed at 24, 48 and 72 hours after transfer into differentiation media (figure 3.1.5). For Brn-3a the changes were observed as early as 6 hours following treatment and the levels then remained relatively constant. However Brn-3b showed a more gradual decrease over time, reaching a plateau after 48 hours.

The changes in the expression of these two transcription factors were reversible in ND7 cells as observed when the cells were induced to differentiate then transferred back to full serum medium. As expected, differentiation of the cells by transfer to serum-free media resulted in the upregulation of Brn-3a transcripts and a decrease in Brn-3b. However, transfer of these cells into medium containing serum resulted in a reversal of the Brn-3 levels in a manner that was dependent upon the time that the cells were left in serum free medium and the time which had elapsed after replacement of the full growth medium at which the cells were harvested (figure 3.1.6). Thus the cells maintained in serum free medium for 24 hours reverted back to the original levels of Brn-3a and Brn-3b by 24 hours following replenishment of serum. However serum deprivation for 48 hours or more required up to 72 hours to achieve this reversal. Replacement of the full growth media also appeared to impel the cells back into the cell cycle with resultant morphological changes and increased cell numbers. It is possible that in the ND7 cells, Brn-3a may be characteristic of the mature, non-dividing, neuronal phenotype, and may either be involved in the process of differentiation or in bringing about the changes in the morphology of the cells. In contrast, Brn-3b may be more representative of the less mature, proliferating cells of neuronal origin. This will be discussed in more detail in the next chapter.

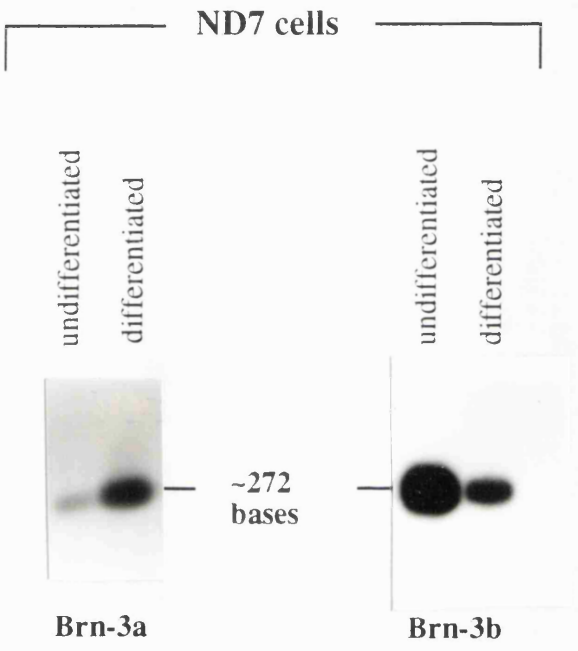


Figure 3.1.4: Expression of Brn-3a and Brn-3b in ND7 cells grown in full growth media containing 10% FCS (undifferentiated) and in serum free media (differentiated) for 72 hours . Levels of RNA were measured by RT -PCR and the bands represent the PCR product following 25 cycles of amplification of cDNA which were equalized on the basis of the amplification of control L6 ribosomal mRNA. The marker indicates the size of the PCR product.

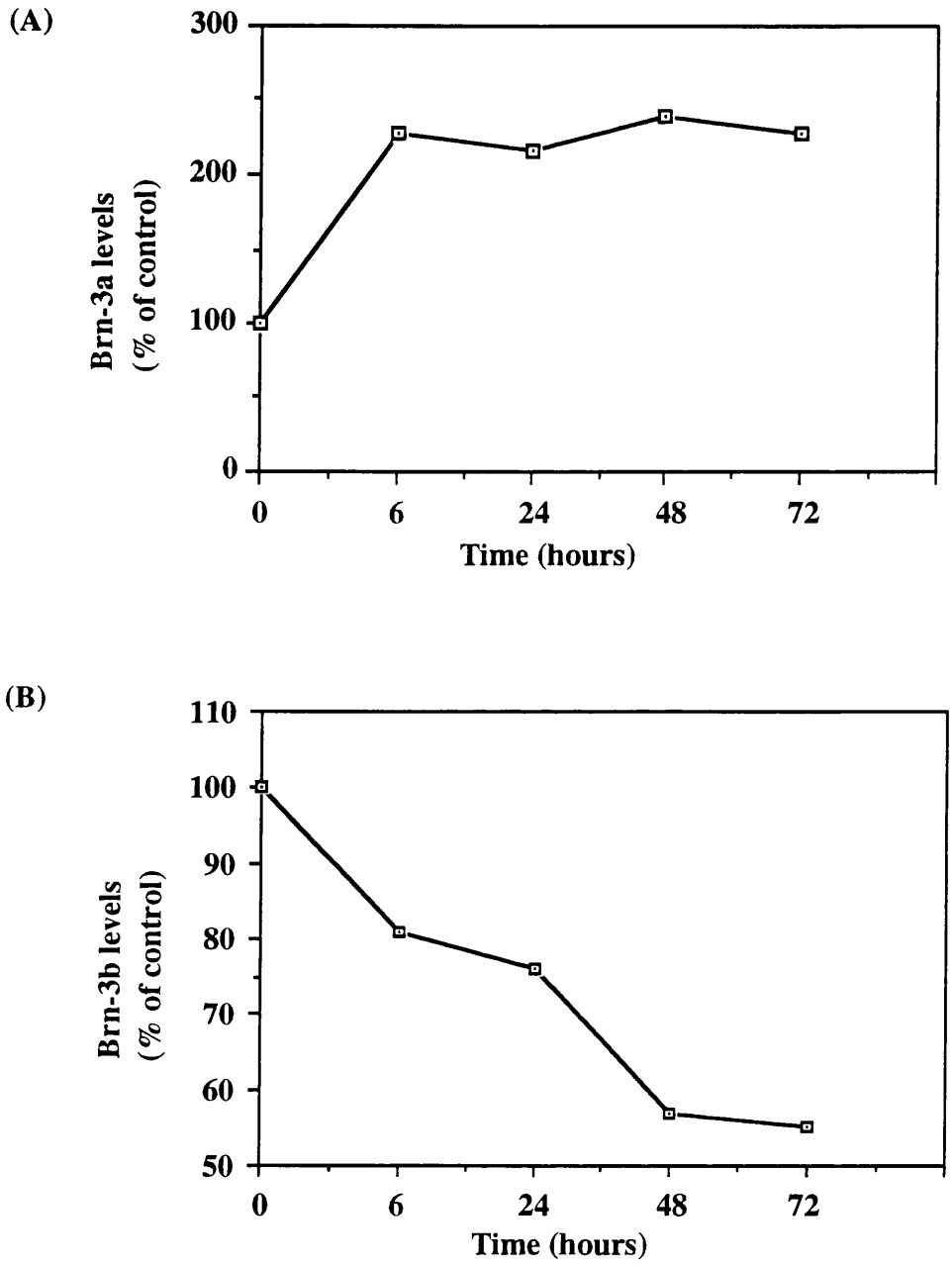


Figure 3.1.5: Typical changes observed in the levels of Brn-3a and Brn-3b mRNA at the times indicated following transfer of ND7 cells from full serum medium to serum free medium. Levels were measured by RT-PCR and are expressed as a percentage of the values obtained for untreated control cells at times indicated. Similar changes in the expression of the Brn-3a and Brn-3b mRNA has been observed in at least five independent experiments.

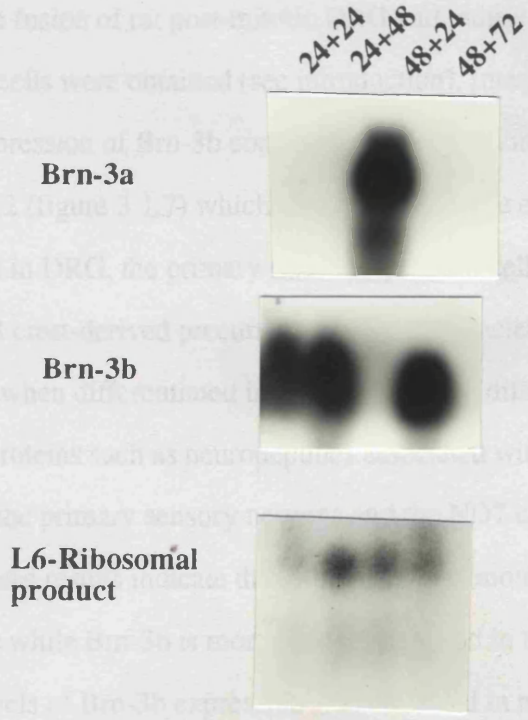


Figure 3.1.6: Levels of Brn-3a and Brn-3b mRNA in ND7 cells which were grown in serum free medium then transferred to medium containing 10% serum for the times indicated. In track 1 and 2 serum was withdrawn for 24 hours and replaced for 24 hours and 48 hours respectively while in track 3 and 4 cells were in serum-free medium for 48 hours followed by serum replacement for 24 and 72 hours respectively. The mRNA levels were measured by RT-PCR. The observation that cells grown for longer periods in serum free medium required an extended period in full serum medium for significant reversal of the Brn-3 levels was observed in other independent experiments.

3.1.4 Brn-3a and Brn-3b expression in other ND cell lines

The expression of these transcription factors was also studied in some of the other ND cells derived from the fusion of rat post-mitotic DRG and mouse neuroblastoma (N18 Tg2) from which the ND7 cells were obtained (see introduction). Interestingly, it was found that there was a higher expression of Brn-3b compared with Brn-3a in the parental neuroblastoma cell line, N18Tg2 (figure 3.1.7) which is in contrast to the expression pattern of these two factors observed in DRG, the primary neuronal parental cells. Neuroblastoma cell lines which are neural crest-derived precursors are more characteristic of neurons of the central nervous system when differentiated in vitro and show a different pattern of expression and localization of proteins such as neuropeptides associated with differentiation of these cells compared with the primary sensory neurons and the ND7 cell line (Prashad, 1975, Suboro *et al.*, 1992). These results indicate that Brn-3a may be more characteristic of differentiated sensory neurons while Brn-3b is more highly expressed in the proliferating neuroblastoma cells. Higher levels of Brn-3b expression was observed in most of these cell lines compared with Brn-3a with the exception of ND3. These differences in the expression seen may be related to the characteristics of the different cell lines reflecting properties of the parental cells. For instance ND3 cell line, like ND7 cells, was shown to express high amounts of cell surface markers which are characteristic of rat DRG and could also be induced to differentiate in defined medium to sensory neuronal like cells.

While many of these cells show some characteristics of the parental cells such as expression of the surface markers and neuropeptides, differentiated ND7 cells were closest in behaviour and characteristics to the terminally differentiated sensory neurons, exhibiting similar electrophysiological and morphological characteristics upon differentiatiation. Since we were interested in studying gene regulation in sensory neurons most of the later studies were carried out in ND7 cells.

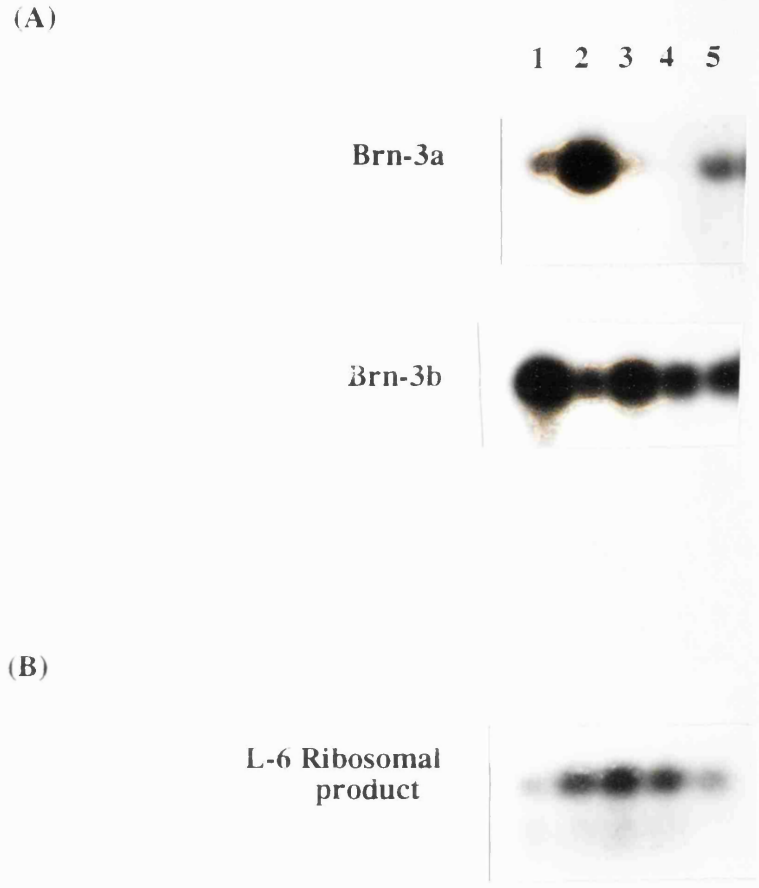


Figure 3.1.7 : (A) PCR analysis of the expression of Brn-3a and Brn-3b in cell lines derived from the fusion of the mouse neuroblastoma cell line, N18Tg2, and rat primary sensory neuronal cell obtained from neonatal dorsal root ganglion. Track 1 represent the levels in the parental cell line, N18Tg2; track 2- ND3; track 3- ND7; track 4-ND11 and track 5, ND21. **(B)** Amplification of the L6 ribosomal RNA provided an estimation of the level of the mRNA in each sample.

3.1.5 Expression of Brn-3a and Brn-3b in PC12 cell lines

To establish the expression of these factors in other neuronal cells, the sympathetic neuronal-like cell line, rat pheochromocytoma cell line, PC12, was studied. When cultured in serum containing media, PC12 cells replicate and possess noradrenergic properties of the non-neoplastic medullary chromaffin cells. In the presence of NGF, these cells cease proliferation and assume flattened sympathetic neuron-like morphology with extensive neurite outgrowth and increased adhesiveness to the cell substratum within three to seven days [Guroff, 1985].

Therefore, RNA obtained from proliferating or differentiated PC12 cells were used for RT-PCR as previously described, using Brn3a, Brn-3b and the L6 ribosomal primers. As seen in figure 3.1.8 neither Brn-3a or Brn-3b were expressed in undifferentiated, proliferating PC12 cells. However upon differentiation low levels of both Brn-3a and Brn-3b proteins were detected. The low levels observed when the cells develop characteristics of sympathetic neuronal cells may indicate a switch in their expression as the morphology of the cells changes and may thus be dependent on the expression of other factors specific to the neuronal cells which may be absent in the undifferentiated adrenal medulla chrommafin-like cells. However, it appears that Brn-3a and Brn-3b are more characteristic of sensory neuronal cells as observed by the much the lower expression in the differentiated sympathetic-like cells compared with sensory neurons and ND7 cells.

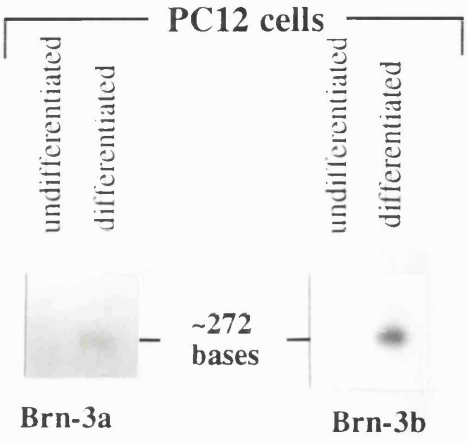


Figure 3.1.8: Expression of Brn-3a and Brn-3b in the sympathoadrenal derived cell line, PC12, in the proliferative, undifferentiated state and upon differentiated to sympathetic neuronal like cells. Levels were measured by RT-PCR.

3.1.6 Brn-3a and Brn-3b expression in non-neuronal cell lines and tissues

The level of mRNA of Brn-3a and Brn-3b was next studied in different tissues. The previously identified Brn-3a had been shown to be highly expressed during development of the brain and nervous system as well as being present in adult brain, sensory neurons of dorsal root ganglia and trigeminal ganglia (He *et al.*, 1989; Ninkina *et al.*, 1993; Gerrero *et al.*, 1993).

Initial experiments to study the distribution of Brn-3a and Brn-3b in tissues were carried out on RNA extracted from rat brain, DRG, liver and spleen, the mouse fibroblast cell line, 3T3 and human cervix. After 25 cycles of PCR Brn-3a was readily detected in DRG when no Brn-3b mRNA was detectable (figure 3.1.9 A). At higher cycle numbers, Brn-3b was also detected. Assuming that the efficiency of amplification was similar, these results suggested that Brn-3a mRNA was more abundant than Brn-3b in the neurons of the DRG. There was no significant expression of either factors in liver, spleen or the 3T3 cell line. The lack of amplification from brain cDNA resulted from very low levels of RNA observed during amplification of the invarient control, L6 ribosomal RNA. Later experiments demonstrated the expression in adult and embryonic brain (figure 3.1.9B).

However both Brn-3a and Brn-3b were detected in the human cervical tissue. Subsequent experiments using rat cervical tissue confirmed the presence of Brn-3b but not Brn-3a mRNA. This may either indicate that slight differences in the sequences in the human Brn-3a cDNA to which the primers annealed allowed the amplification of Brn-3b or that Brn-3a may be present in the human cervix and may also depend on cyclic changes (see later). Consequent to this Dr C.Ring of our laboratory, isolated, cloned and sequenced the human Brn-3b cDNA from a human testis cDNA library (Ring and Latchman, 1993).

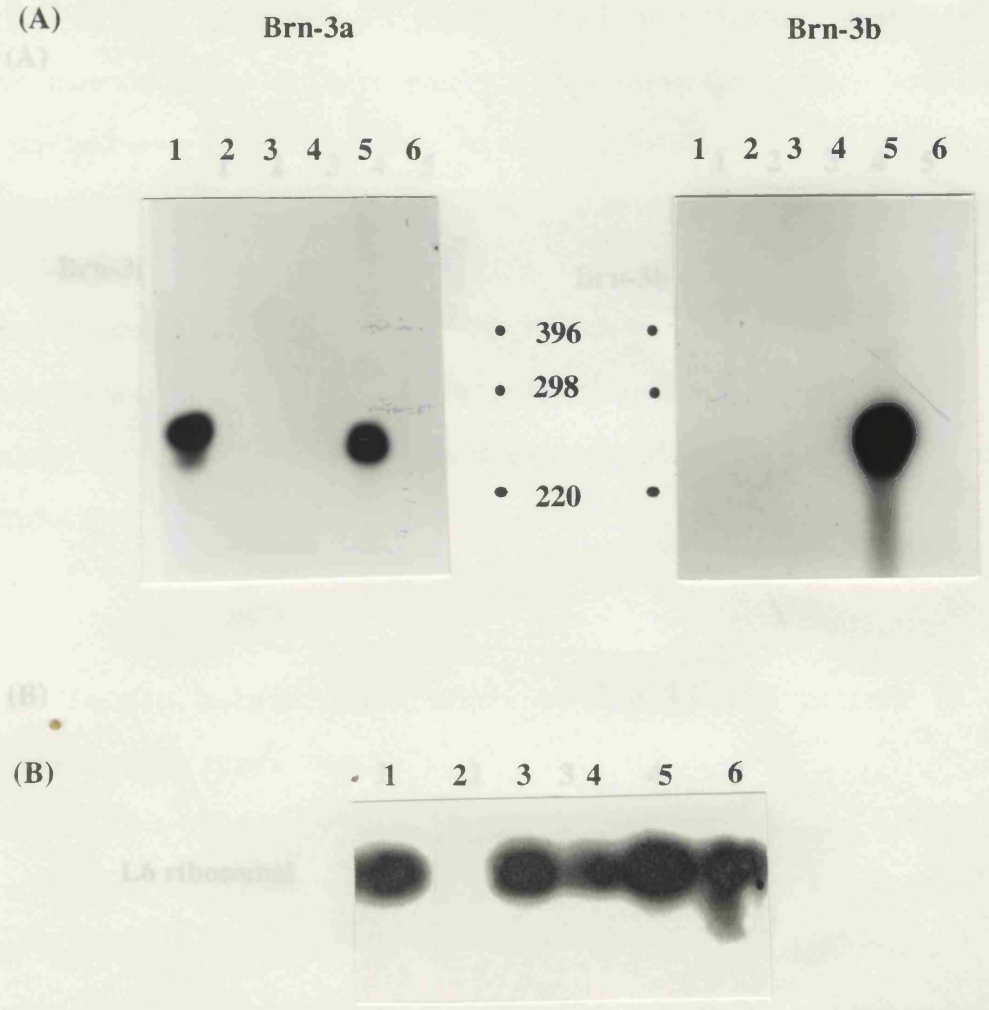
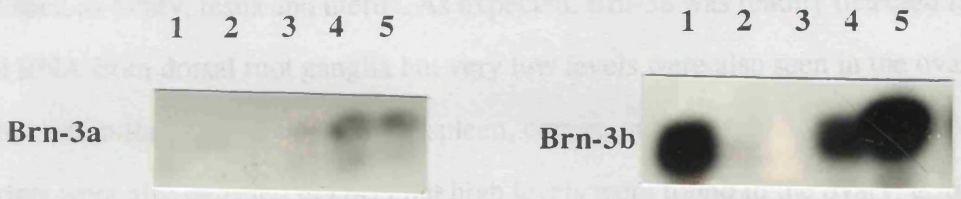


Figure 3.1.9 (i): **(A)** Brn-3a and Brn-3b mRNA expression in tissues compared with the positive control DRG. Levels were measured by RT-PCR using RNA obtained from different rat dorsal root ganglia (DRG) (track 1), brain (track 2), liver (track 3), spleen (track 4), mouse 3T3 cell line (track 5) and human cervix (track 6). These results represent the signal from product obtained following 25 cycles of amplification. **(B)** Amplification of the L6 ribosomal mRNA to check for variations in the RNA samples obtained from the different tissues (the lack of signal from Brn-3a or Brn-3b amplification of cDNA from brain tissue was reflected by the poor control signals indicating low yield of RNA from this tissue).

(A)



(B)

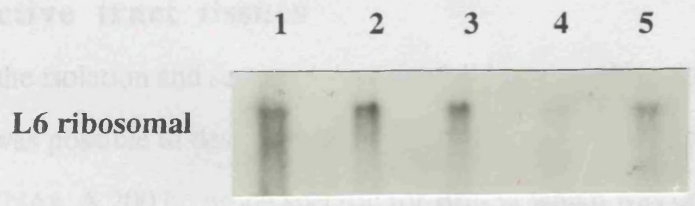


Figure 3.1.9 (ii): **(A)** Levels of Brn-3a and Brn-3b mRNA in rat cervix (track 1), heart (track 2), spleen (track 3), DRG (track 4) and brain (track 5) as measured by RT-PCR. The results show the amplification for 25 cycles. **(B)** L6 ribosomal PCR indicating the relative levels of RNA present in each sample.

3.1.7 Brn-3a and Brn-3b distribution in non-neuronal tissues

This study was repeated in a larger number of rat tissues including other reproductive tract organs such as ovary, testis and uterus. As expected, Brn-3a was readily detected in the control RNA from dorsal root ganglia but very low levels were also seen in the ovary and testis but not in the heart, kidney, liver, spleen, cervix, or uterus (figure 3.1.10). Brn-3b transcripts were also detected in DRG but high levels were found in the ovary, testis and uterus but not in heart, kidney, liver, lung or spleen (figure 3.1.10). Some of these findings were confirmed by results published by others during the period of this study (see introduction) (He *et al.*,1989; Gerrero *et al.*,1993; Nikina *et al.*,1993; Turner *et al.*,1994).

3.2.0 In-situ hybridization study of Brn-3a and Brn-3b in reproductive tract tissues

Following the isolation and sequencing of the full length cDNA clones of both Brn-3a and Brn-3b, it was possible to design probes which could distinguish between the Brn-3a and Brn-3b mRNAs. A 200 bp probe specific for Brn-3a which was designed by Dr N. Ninkina (Ninkina *et al.*, 1993) was obtained by restriction enzyme digestion of sequences in the non-coding region in the 3' end of the mRNA and should hybridize to both the long and short isoforms of Brn-3a. This sequence was cloned into the Not1/Sal1 sites of pGEM-5Zf vector (Promega) which facilitated riboprobe synthesis. Linearization with Not 1 enzyme and transcription with Sp6 RNA ploymerase yielded the antisense probe for the detection of the Brn-3a mRNA while Sac1 linearization and transcription with the T7 RNA polymerase gave rise to the control sense transcripts.

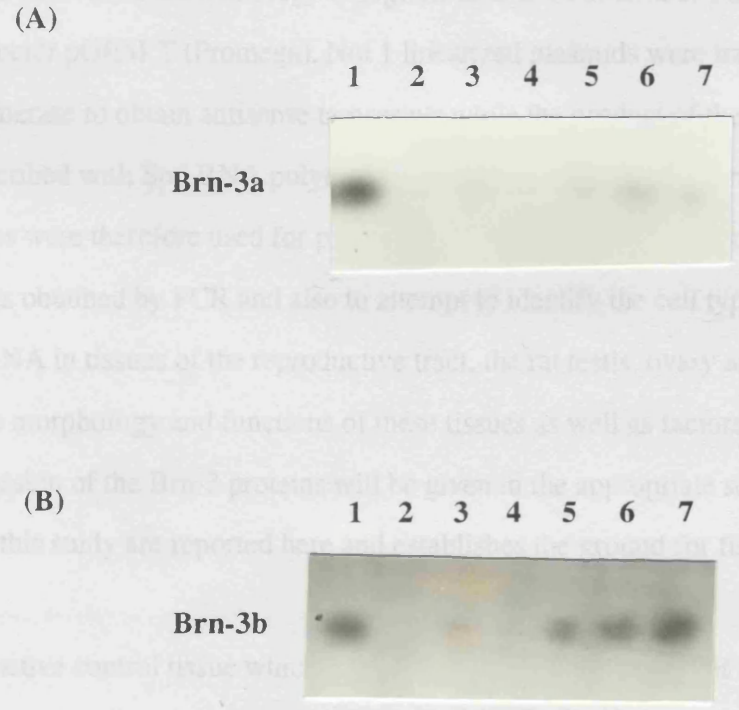


Figure 3.1.10: Expression of Brn-3a (A) and Brn-3b (B) mRNA as measured by RT--PCR in non-neuronal rat tissues compared with the levels in the positive control rat DRG (track 1). RNA for the experiment was obtained from heart (track 2), kidney (track 3), liver (track 4), uterus (track 5), ovary (track 6) and testis (track 7). Low signal was detected in the kidney with both Brn-3a and Brn-3b primers but this was not seen in subsequent experiments with different RNA sample so was therefore not considered significant.

The Brn-3b specific probe was made by Ms.J. Begbie of our laboratory who used specific primers to amplify the region of the cDNA immediately upstream of the POU domain which had very little homology to regions in Brn-3a or Brn3c. This region was cloned into the vector pGEM T (Promega). Not 1 linearized plasmids were transcribed with T7 RNA polymerase to obtain antisense transcripts while the product of the Nco1 digested clone transcribed with Sp6 RNA polymerase provided the sense transcripts. These specific probes were therefore used for preliminary in-situ hybridization studies to confirm the results obtained by PCR and also to attempt to identify the cell types which expressed Brn-3 mRNA in tissues of the reproductive tract, the rat testis, ovary and uterus. A brief outline of the morphology and functions of these tissues as well as factors which may affect the expression of the Brn-3 proteins will be given in the appropriate section. The initial results of of this study are reported here and establishes the ground for future studies.

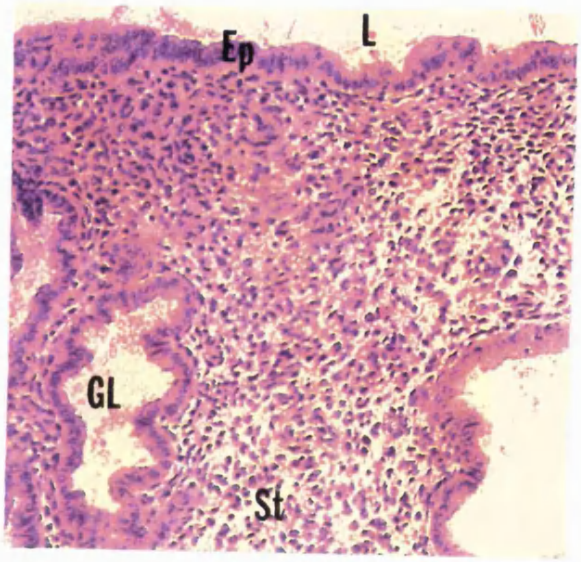
A positive control tissue which was used to test the specificity of the probe was carried out on sections of the brain and expression of both Brn-3a and Brn-3b were detected in the superior colliculus of the mesencephalon of the brain, a site shown to express both these factors in the adult murine brain. In addition, concurrent studies of the expression of these factors in rat DRG and trigeminal ganglia was also carried out by Ms. J. Begbie using these probes thus confirming that the riboprobes were specific and therefore suitable for further experiment.

3.2.1(i) Morphology and function of the uterus

Since it is beyond the scope of this report to discuss in much detail, the structure and function of the uterus during the estrous cycle, a brief outline of the gross changes which are characteristic of different stages of the cycle will be discussed. For a more extensive overview, please refer to Finn and Porter, 1975; Wynn, 1981, Clarke and Sutherland, 1990, Murphy and Ghahary, 1990 and references therein.

Analysis of the uterine structure reveals two distinct regions, the endometrium and the muscular layers made up of the myometrium and mesometrium (figure 3.2.1). The size and composition of the uterine cellular layers vary with species as well as with the physiological state of the animal. The endometrium which consists of three main tissues, the luminal epithelium, the glandular epithelium and the stroma, undergo considerable alterations in response to the accompanying cyclic hormonal changes during the reproductive cycle, with the response being much more pronounced in the epithelial layers. During the quiescent period or diestrus in the rat, the epithelial cells consist of a single layer of cells resting on a basement membrane. During the follicular or proliferative phase prior to ovulation there is accompanying proliferation in the endometrium under the influence of ovarian hormones such as oestrogen and mitosis is frequently observed in the luminal epithelial layer. The epithelial cells increase in size and complexity in preparation for implantation of a fertilized ovum. Later, under the influence of progesterone, the oestrogen primed glandular epithelium and stromal cells also undergo proliferation followed by differentiation. During the luteal or secretory phase which follows ovulation there is closure of the uterine lumen which changes from a large, irregular fluid-filled shape containing fingerlike projections seen during the proliferative stage to a slit-shaped lumen. This occurs under the control of the luteal-phase oestrogen secretion and progesterone. At the end of the cycle if no fertilization or implantation occurs considerable cell death occurs in the endometrium and the cycle repeats.

(A)



Ep - epithelium
L - lumen
GL - glands
St - stroma

(B)

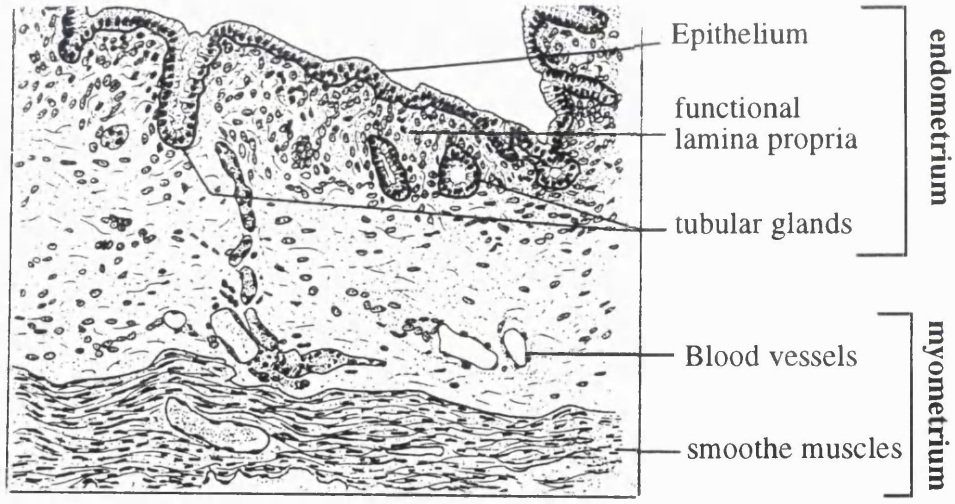


Figure 3.2.1: (A) Cross-section through the uterus showing the morphology of the different layers. (B) Schematic diagram of the uterus showing the cells of the endometrium and the myometrium. The endometrium contains tubular or coiled glands which are seen as invaginations of the surface epithelium. (Taken from Ratcliffe - Practical Illustrated Histology and Gartner and Hiatt-Colour atlas of Histology).

Thus the ovarian hormones under the influence of pituitary gonadotropins profoundly affect the responses of the uterine cells with oestrogen generally stimulating cellular proliferation while progesterone induces differentiation of cells and preparation of the uterus for implantation while modulating the effect of oestrogen on the uterus (Clarke and Sutherland, 1990). Early responses to oestrogen include increased metabolism and increased transcriptional activity while later responses result in enhanced expression of growth factors and their receptors (Murphy and Ghahary, 1990). Many of these growth factors profoundly express gene expression in tissues.

3.2.1(ii) Brn-3a and Brn-3b expression in the uterus

Thus to establish the function of the Brn-3 protein it was necessary to establish the cells which express the mRNA.The precise stage of the uterine cycle at which the uterus was taken for sections in this preliminary in-situ hybridization study was not clearly established but the multiple layers of epithelial cells observed as well as the convoluted nature of the lumen was suggestive of the proliferative or follicular stage. Initial results suggest that there was no significant expression of Brn-3a in the uterus since signals of similar intensity were observed upon hybridization with the sense and antisense probe of Brn-3a (figure 3.2.2).

However, hybridization of the Brn-3b probes resulted in strong signals localized mainly to epithelial cells of the endometrium (figure 3.2.3). There was some signals with the sense probe indicating some non-specific binding in this region. However signals in sections probed with the Brn-3b antisense probe were much higher at the base of the epithelial layer compared with the levels obtained with sense probe. This is a layer of cells which undergo extensive proliferation and may thus suggest that Brn-3b expression may be associated with this process. These results will need to be re-confirmed and investigated more thoroughly in later studies.

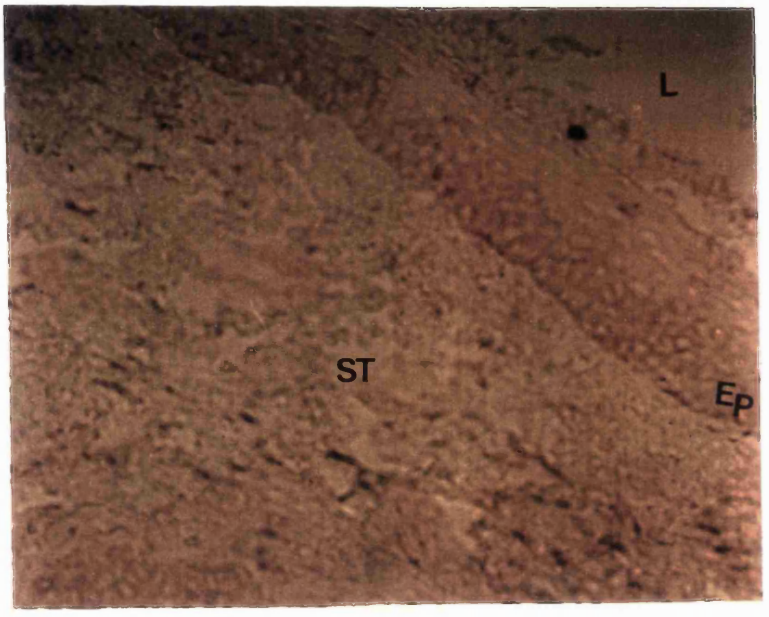
Figure 3.2.2 : Expression of Brn-3a in the rat uterus as assessed by in-situ hybridization using Brn-3a antisense probe (A) and compared with the signals obtained with the corresponding sense probes (B). There is no signal above background indicating Brn-3a is not expressed in the uterus.

L - lumen

ST - Stroma

Ep - epithelial cell

(A) **Brn-3a**
(antisense)



(B) **sense**

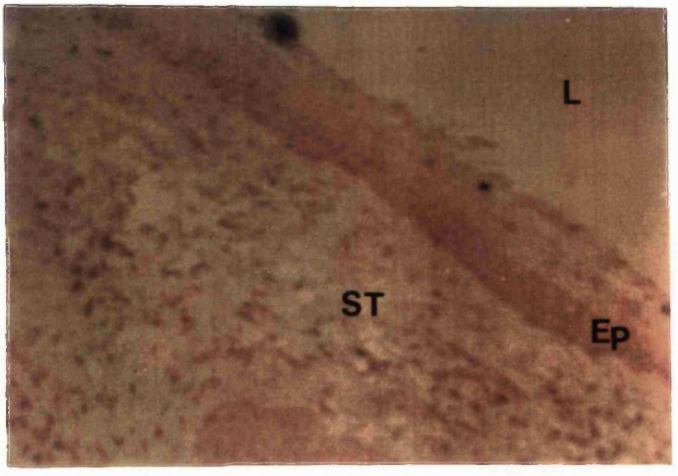


Figure 3.2.3 : In situ hybridization results obtained when the rat uterus was probed with the Brn-3b antisense (A) and sense (B) probes. There was some background signal with the sense probe, but a specific pattern of much stronger signal was obtained in the sections hybridized with the antisense probe localized mainly at the base of the epithelial cells where rapid proliferation takes place during the follicular phase of the estrous cycle. There was less signals detected in the cells which are nearer the lumen which may represent an area undergoing less cell division.

The signal observed in the region of the stroma is thought to be non-specific since the same pattern and intensity are observed in the sections probed with the sense probe.

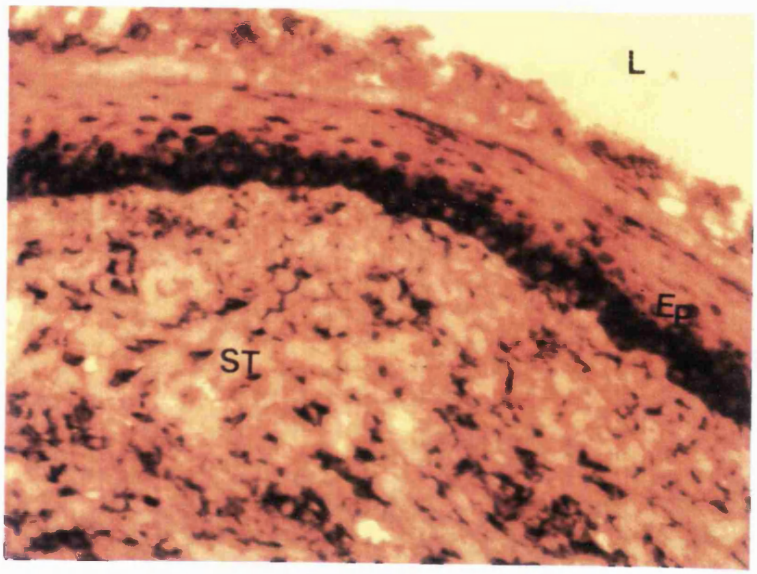
L - lumen

ST - Stroma

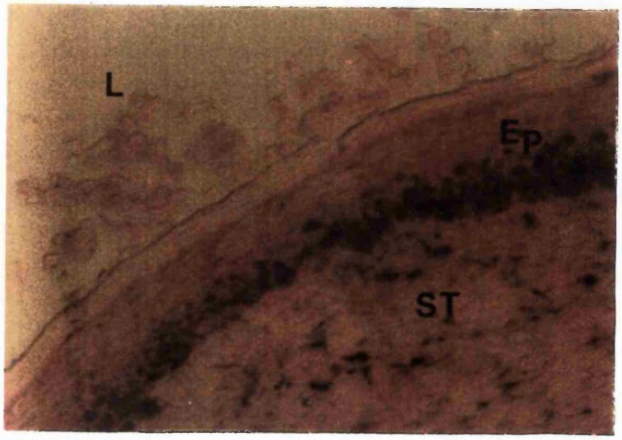
Ep - epithelial cell

Brn-3b probe

(A) antisense



(B) sense



These preliminary studies supported the PCR data showing Brn-3b but not Brn-3a in the uterus. It would be interesting to study the expression of both of these factors at different stages of the estrous cycle since changes in the expression in response to the changing cellular environment which accompanies this process may not have been detected in this study. Furthermore, co-expression studies to identify other factors with overlapping expression may give some clues as to the function of the proteins in the uterus as well as possible candidate genes which may be regulated by this transcription factor during cellular proliferation.

3.2.2(i) Morphology, regulation of function and gene expression in the ovary

A cross-section of the adult ovary at any time during the estrous cycle will reveal follicles at all stages of development [figure 3.2.4 (i)]. During the estrous cycle the premordial follicle(s) mature and at ovulation the mature oocyte is released. The ruptured follicle forms a corpus luteum which secretes hormones and persists for a period of time characteristic of the species, regressing during the nonpregnant cycle. A brief, simplified outline of the processes which accompany each stage will be given. For more detailed information please refer to Greep and Astwood, 1973; Greep, 1980; Tonetta and di-Zerega, 1989; and Richards, 1994 and references therein.

As the immature primordial follicle, which consists of an ovum surrounded by a single layer of cells, matures to a preovulatory one there is concomitant proliferation and maturation of the follicular constituents such as the granulosa cells. The ovum enlarges and becomes surrounded by a thick membrane, the zona pellucida, while the granulosa proliferates into many layers of cells and matures from undifferentiated fibroblastic cell type to differentiated steroid secreting cells with receptors for gonadotropins as well increased levels of adenylate cyclase and protein kinases, processes which may regulate gene activity.

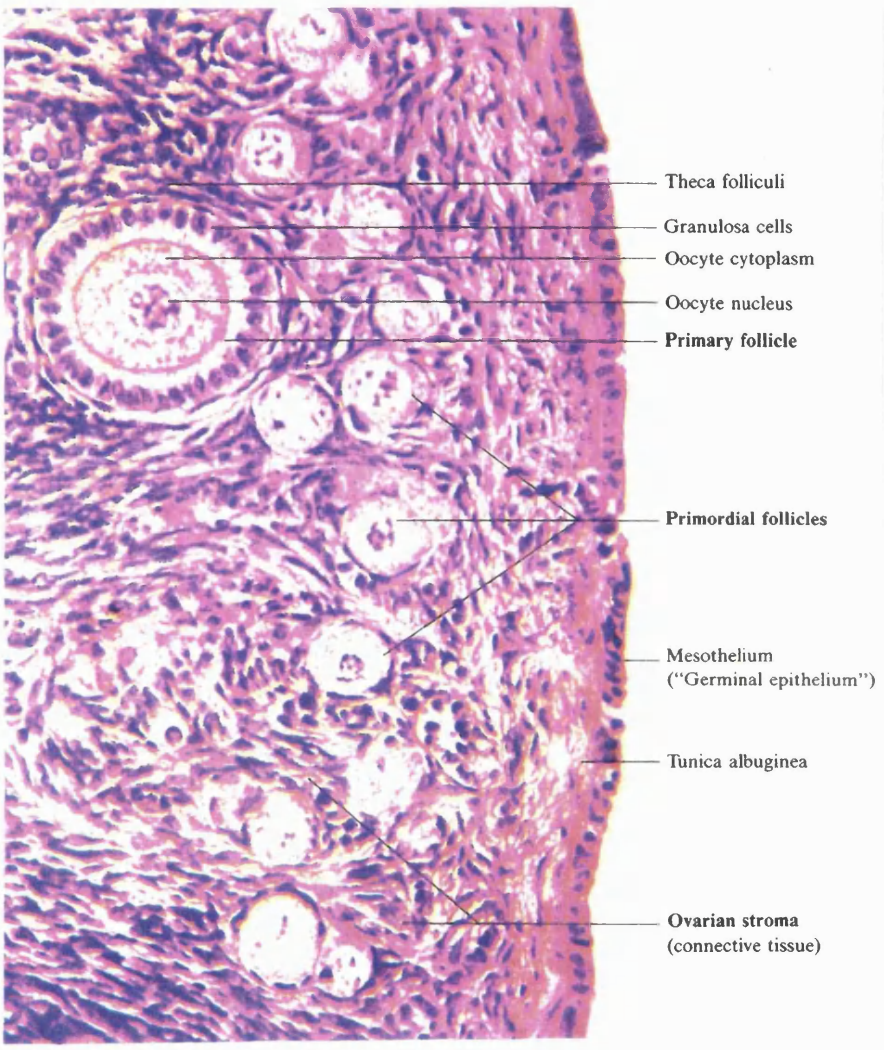


Figure 3.2.4 (i): A cross section of murine ovary showing the follicles at different stages of development. Morphological changes characterizing follicular development from the immature primary follicle to maturing follicles include changes in the size, shape and complexity of the follicle and surrounding cells.

Fluid accumulates in the cavity and a thin vascular theca interna supported by an outer connective tissue layer surrounds the follicle. Meiotic division occurs prior to ovulation, halving the number of chromosomes. This is followed by follicular rupture at ovulation and formation of the corpus luteum which matures and continues to secrete hormones. If no pregnancy results then the cells eventually die following occlusion of blood supply and are replaced by an avascular scar. There is extensive cellular proliferation and cell death during each cycle associated with changes in hormonal levels.

Follicular selection and maturation process is highly sensitive to gonadotropin hormones such as follicle stimulating hormone (FSH) and lutinizing hormone (LH) [figure 3.2.4 (ii)]. The androgens and female steroid sex hormones, oestrogen and progesterone, which are subjected to control by FSH and LH are also critical for controlling the events of follicle development and maturation. The theca cells are thought to produce these androgens which are converted to oestrogens by the granulosa cells. Increasing amounts of FSH and its receptors are associated with oocyte maturation, accumulation of cyclic AMP, activation of the aromatase system (which converts androgens to oestrogen) as well as induction of LH receptors for the LH response which occurs at ovulation. A preovulation surge of LH is required for meiosis and changes from oestrogen to progesterone secretion. Following ovulation, the corpus luteum continues to secrete hormones and if no pregnancy occurs, this structure dies and the cycle starts again.

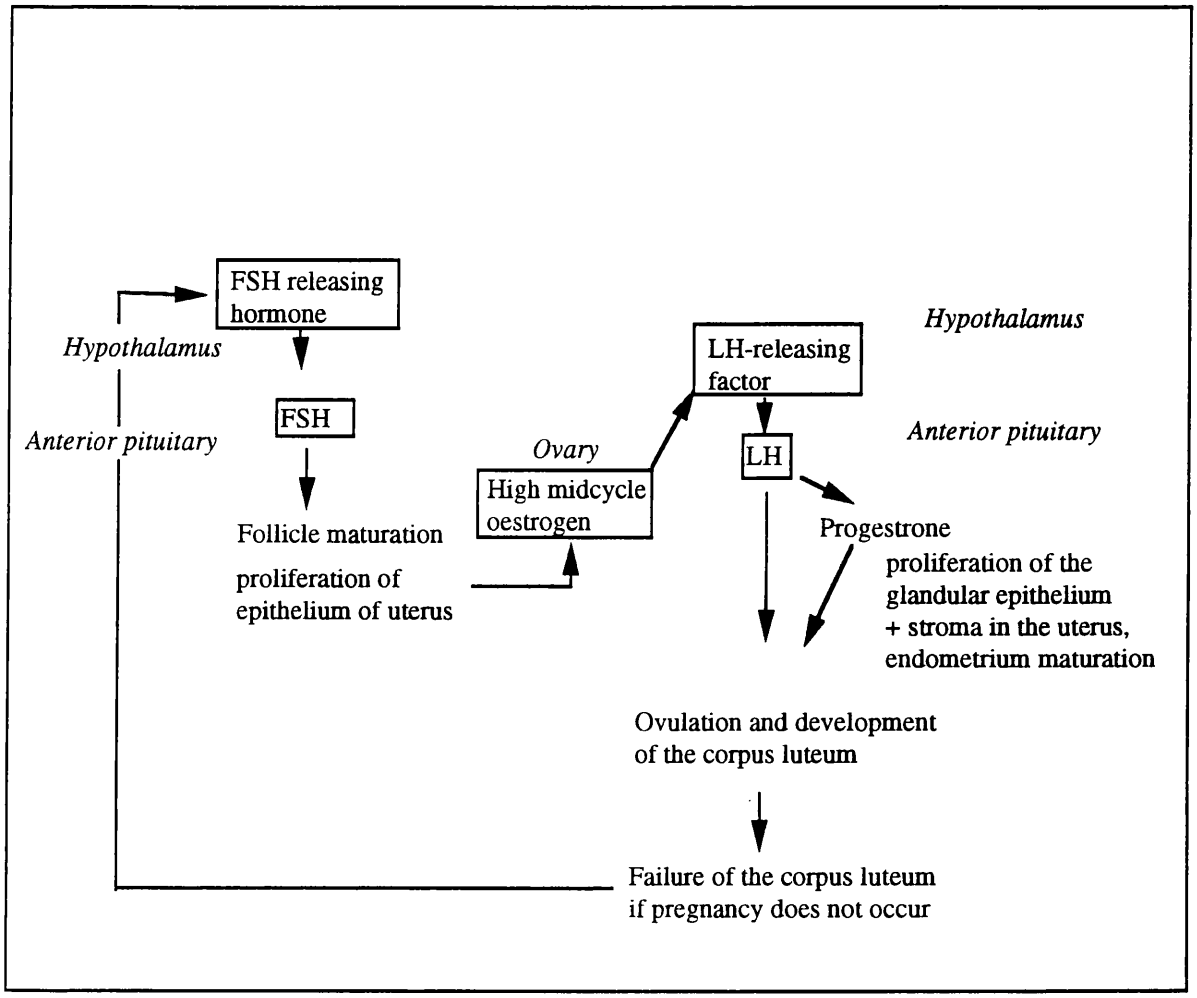


Figure 3.2.4 (ii): Schematic diagram showing the effect of pituitary hormones on the morphology and function of the ovary and uterus. (Taken and modified from Functional Histology - A core text- Borysenko *et al.*,1989)

3.2.2(ii) Brn-3a and Brn-3b in the ovary

Thus, there is extensive proliferation as well as differentiation and cell death which occurs at a number of stages in the ovarian cycle. To establish the cell types expressing the Brn-3 mRNAs, preliminary studies were carried out on sections of the rat ovary. Sections of ovary were probed with both the sense and antisense probes of Brn-3a and Brn-3b. There was a low level of diffuse hybridization over the follicles with the Brn-3a antisense probe compared with the sections probed with the sense probe (figure 3.2.5A and B). Although hybridization was localized to all the follicles but not to surrounding cells, it was difficult to specify the cells that express this factor. This result therefore indicates that there may be a low level of Brn-3a message in the ovary but speculation on the significance will be premature at this stage. It will be necessary to repeat this study using thinner serial sections and tissues from different stages of the ovarian cycle and to carry out other immuno-cytochemical studies to try and identify the specific cells which express Brn-3a.

Signals obtained using the Brn-3b antisense probe appeared to be localized to areas around the developing ova of some of the follicles (figure 3.2.6 A) compared with the corresponding sense control or other cells in the ovary including follicles at other stages of development. In contrast, the Brn-3b probe did not hybridize to retrogressing corpus luteum or immature follicles suggesting that the Brn-3b mRNA may be expressed in proliferating granulosa cells surrounding maturing ova. The signals were specific since incubation with the corresponding sense probe under similar conditions showed very little signal (figure 3.2.6 B). The stage of the cycle at which the ovary was taken was not clear but since the ovary and uterus were taken from the same animal it can be speculated that it was in the follicular phase. The significance of the restricted expression to developing follicles may pertain to cyclic changes in proliferation associated with secretion of pituitary hormone, FSH or ovarian hormones oestrogen which affect follicular development in the ovary.

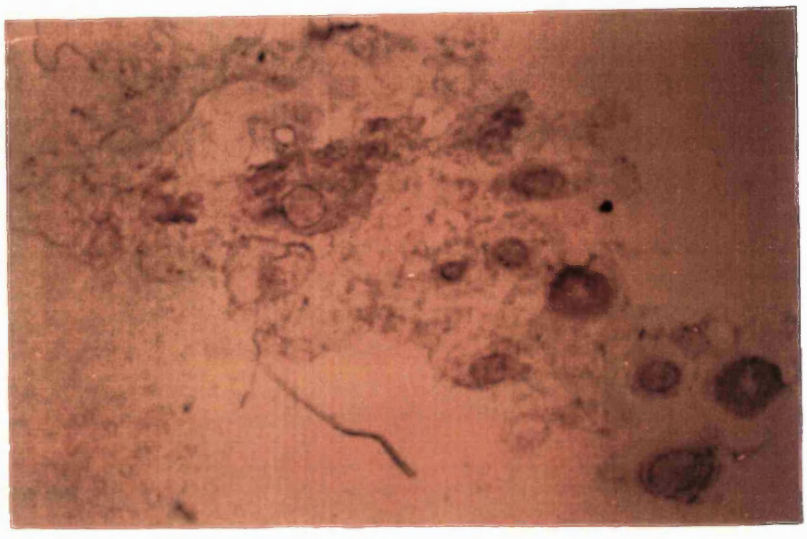
Figure 3.2.5 (i) : In situ hybridization results obtained when sections of rat ovary was probed with Brn-3a antisense probe. The ovarian follicles at different stages of maturation can be seen at low (A) and higher (B) magnification. There was a low, diffuse staining around the follicles compared with the section probed with labelled sense transcripts [figure 3.2.6 (ii)] but on higher magnification it was difficult to identify the specific cells hybridizing to the probe.

PMF – primary follicle (maturing)

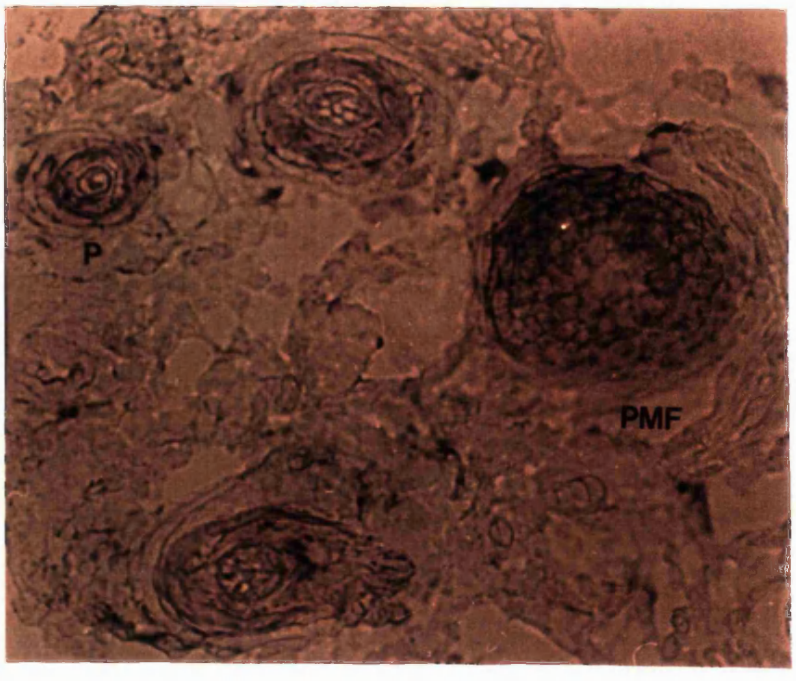
P – primordial follicle

Brn-3a probe
antisense

(A) X 10 magnification



(B) X 40 magnification



Brn-3a
(sense)

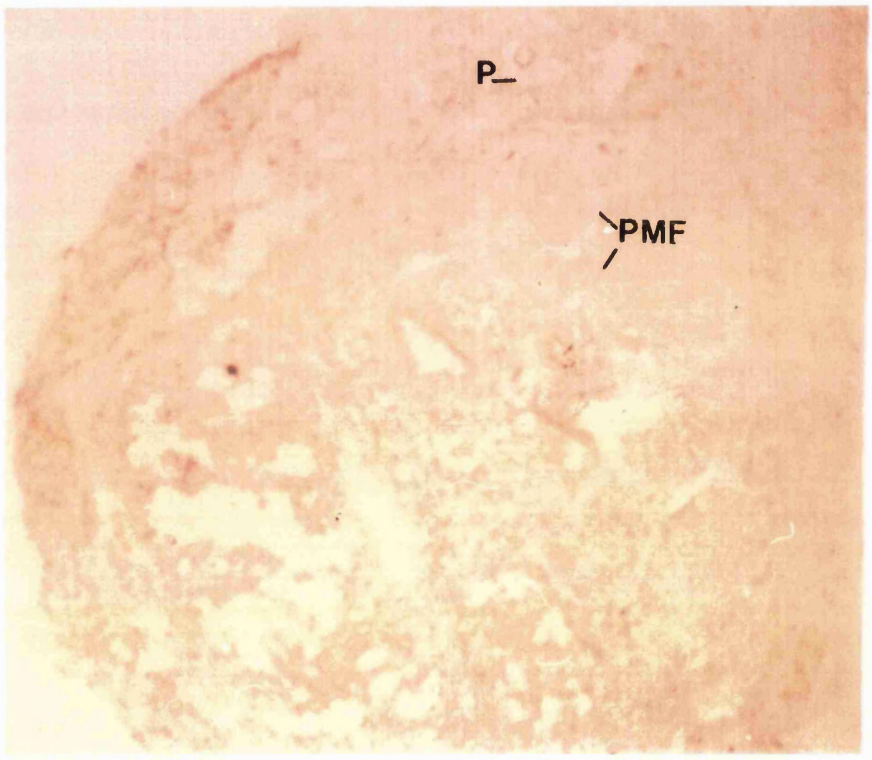


Figure 3.2.5 (ii) : Rat ovarian sections probed with the corresponding Brn-3a sense probe. There was no significant signals seen with this probe compared with the weak but diffuse patterns observed with the antisense probe.

Figure 3.2.6 (i) : In situ hybridization results obtained when the rat ovary was probed with the Brn-3b antisense probe showing follicles at different stages of maturation at low (A) and higher (B) magnification. High Brn-3b signals were observed in the granulosa cells of primary follicles undergoing proliferation around the maturing oocyte. No significant signals were observed in the corpus luteum or in the primary follicles with no cellular proliferation.

PMF - primary follicle (maturing)

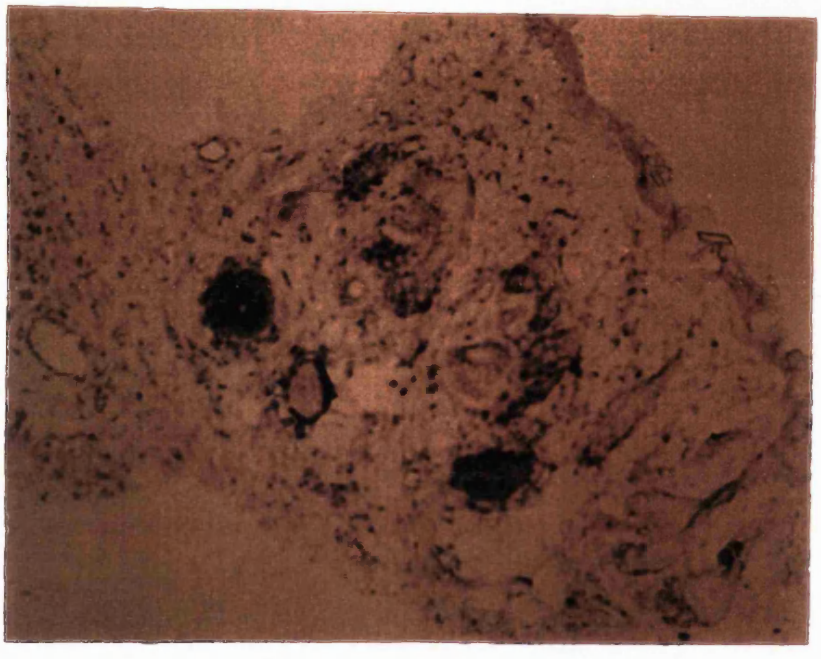
G - proliferating granulosa cells

P - primordial follicle

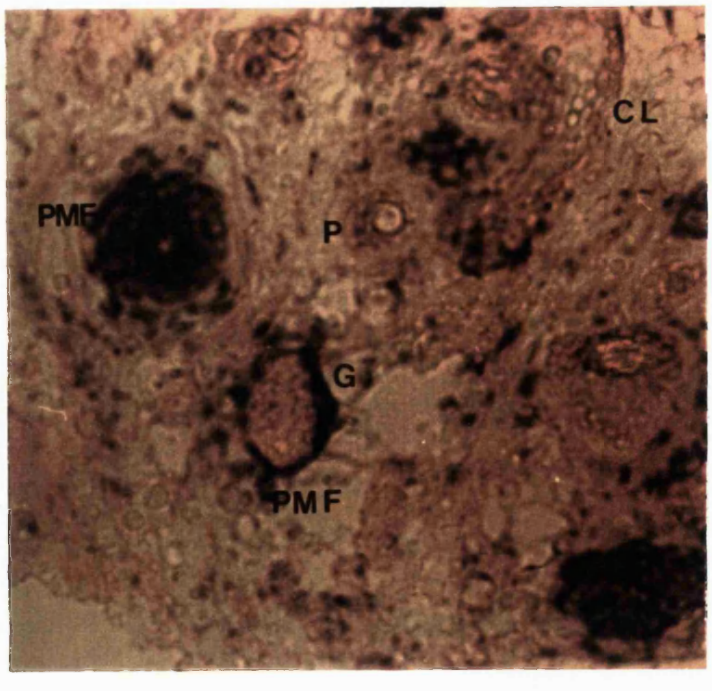
CL - corpus luteum

Brn-3b probe
antisense

(A) X 10 magnification



(B) X 40 magnification



Brn-3b
(sense)

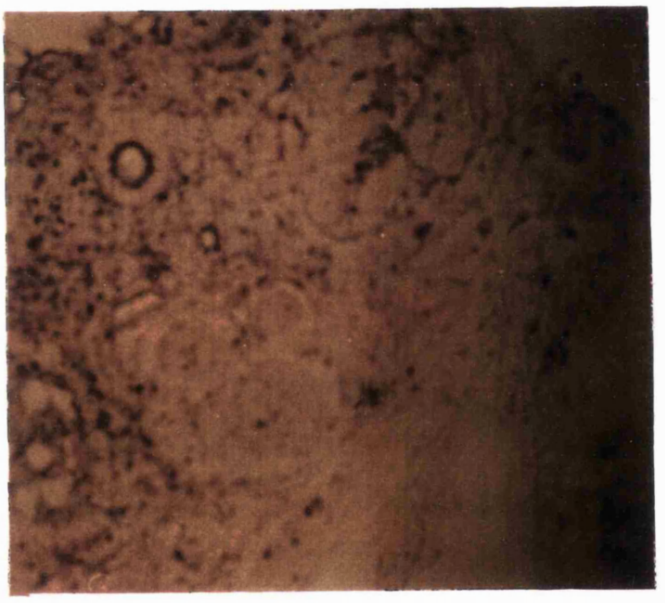


Figure 3.2.6 (ii) : Rat ovarian sections probed with the correspondingBrn-3b sense probe. There was no significant signals seen with this probe compared with the strong and specific hybridization obtained with the antisense probe.

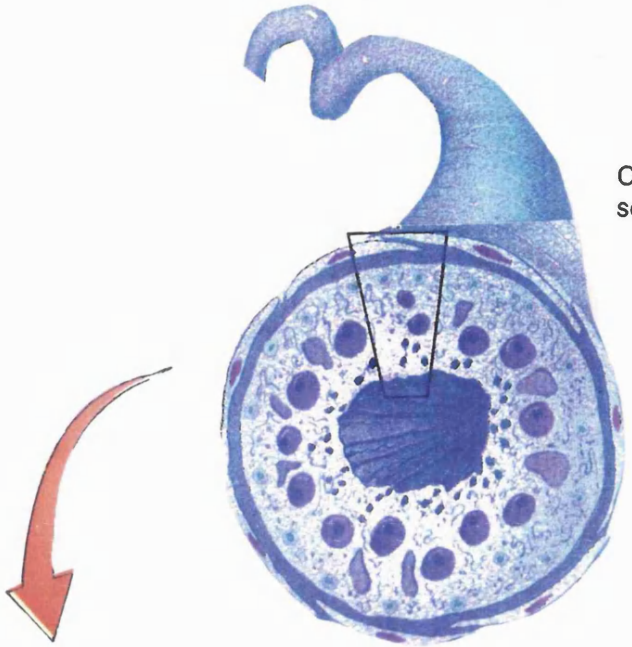
This will need to be analyzed in more detail by establishing whether the expression changes during the ovarian cycle as well as the conditions which influence this process. In addition the distinct cell types in which the transcripts are present will have to be studied further, perhaps by looking at co-expression of other factors known to be localized to specific cell types.

3.2.3(i) Morphology and function of cells in the testis

Because of the complex nature of the testis and of spermatogenesis a brief description will be given to clarify the results of the preliminary studies reported here. For a more detailed account please refer to Greep and Astwood, 1975; Greep, 1980, Skinner, 1991, Kierszenbaum, 1994.

The testis is comprised of two major compartments, the seminiferous tubule and the interstitum. The seminiferous tubule contains germ cells and the non-proliferating Sertoli cells while the interstitum contains supporting tissues such as capillaries and lymphatic vessels, fibroblastic cells, macrophages and Leydig cells. The primary function of the seminiferous tubule is to produce viable spermatozoa which is achieved by spermatogenesis, a highly regulated process which follows a rigid, species-specific sequence of temporal events. These include the proliferation of undifferentiated spermatogonial cells by mitosis, to produce type A spermatogonia (which are retained as stem cells that can reinitiate the process at a later date) or type B spermatogonia which differentiate to form spermatogonia that undergo, the reductive division (meiosis) that give rise to haploid spermatids which undergo final differentiation into mature spermatozoa (spermatogenic phase) (figure 3.2.7A)

Figure 3.2.7 (i): (A) Schematic diagram to show the main cellular processes occuring during spermatogenesis and the position of the developing spermatogonia within the seminiferous tubule. The immature spermatogonia are present in the basal compartments near the outer surface while the mature spermatozoa are in the lumen of the seminiferous tubule while spermatocytes and spermatids at intermediate stages are present within this region. (Taken fromGartner and Hiatt, Colour atlas of Histology).


Cross section of
seminiferous tubule

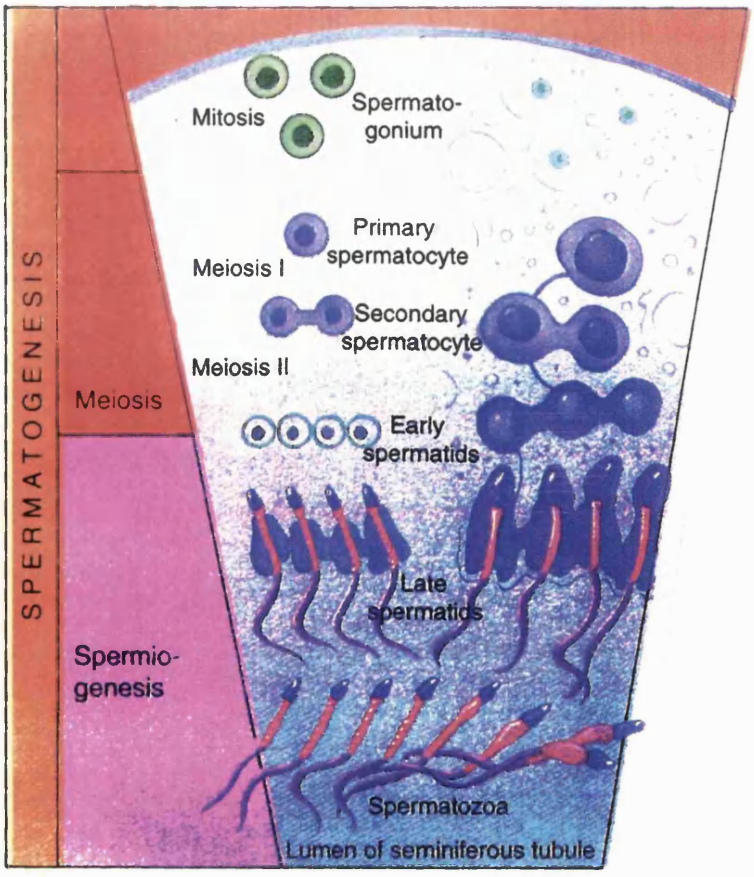
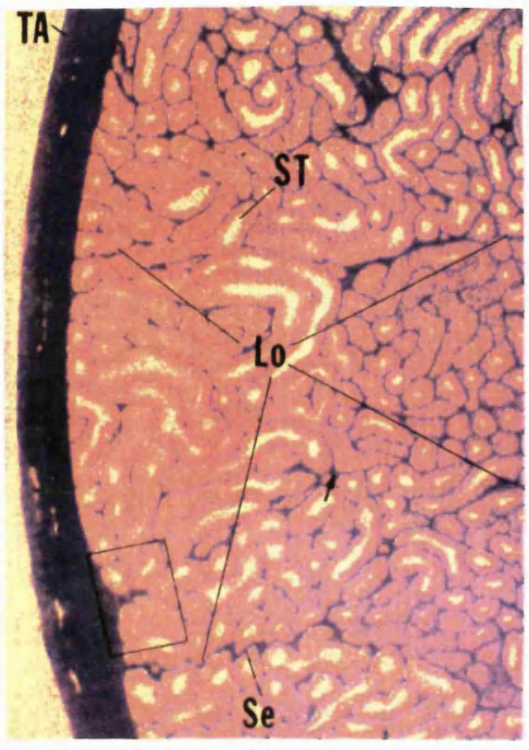
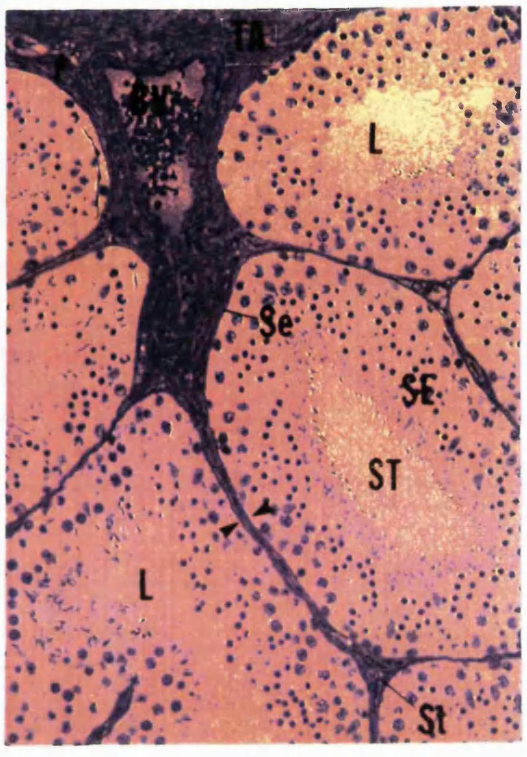

SPERMATOGENESIS
Meiosis
Spermio-
genesis
Mitosis
Spermato-
gonium
Primary
spermatocyte
Meiosis I
Secondary
spermatocyte
Meiosis II
Early
spermatids
Late
spermatids
Spermatozoa
Lumen of seminiferous tubule

Figure 3.2.7 (ii): (A) Cross section of the testis showing the morphology of a cross-section through the seminiferous tubules with increasing magnification (1-4). Note the different tubules showing cells at different stages of the spermatogenic cycle from the immature cells to the mature spermatids.(Taken from Gartner and Hiatt, Colour atlas of Histology, 1994).

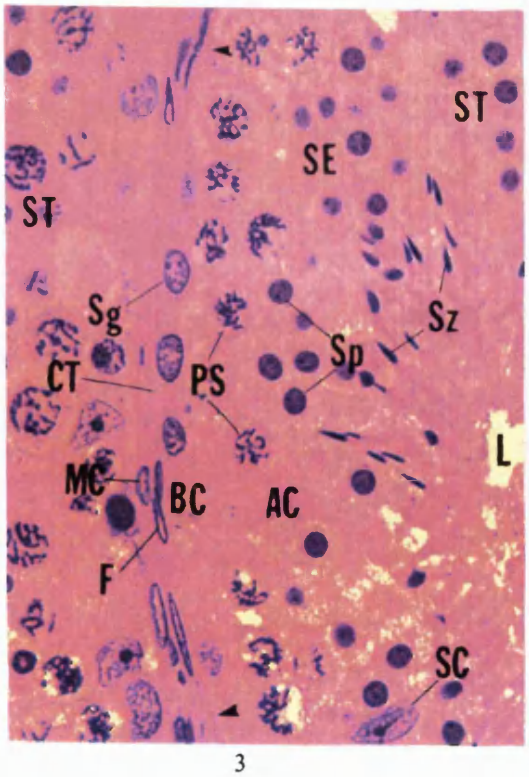
A	arterieoles	PS	primary spermatocyte
AC	adluminal compartment	SC	Sertoli cells
Ad /Ap	Type A spermatogonia	SE	seminiferous epithelium
B	Type B spermatogonia	Sg	spermatogonia
BV	Blood vessels	Sp	spermatid
CT	Connective tissues	ST	seminiferous tubule
F	Fibroblast	St	Stroma
		Sz	spermatozoa
		TA/TV	Tunica albuginea/ vasculosa (capsule)


TA
ST
Lo
Se

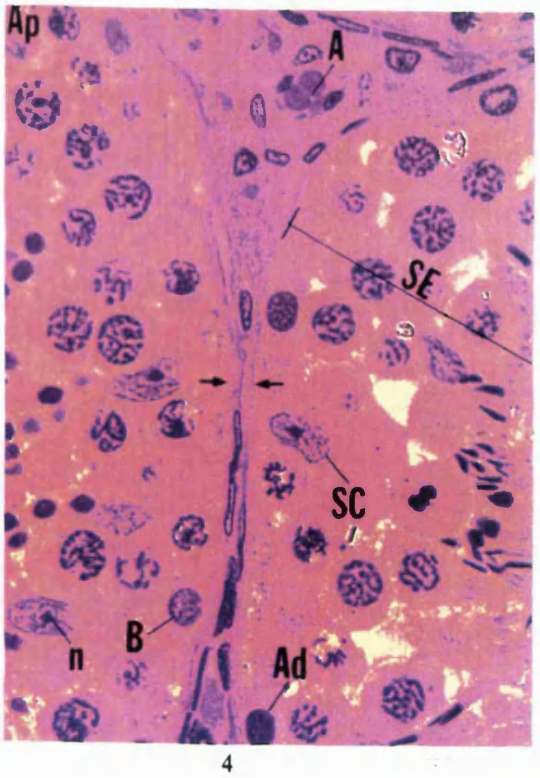
1


TA
L
Se
SE
ST
L
St

2


ST
SE
ST
Sg
Sz
Sp
CT
PS
L
MC
BC
AC
F
SC

3


Ap
A
SE
SC
n
B
Ad

4

In mammals, the process of spermatogenesis is a cyclic event in which rounds of cellular proliferation and differentiation occur. Prior to completion of one cycle of spermatogenesis, a new cycle is initiated to accomplish the daily production of billions of spermatazoa. Thus spermatogonial proliferation, meiosis and spermatogenesis occurs simultaneously in the adult testis so that any given generation of spermatogenic cells overlap with an earlier or later generation to create a constant combination of spermatogenic cells known as cell associations (figure 3.2.7B). These distinct cellular associations are observed in different compartments of the seminiferous epithelium since the proliferating population of germ cells move slowly upwards in the epithelium as they progress from immature spermatogonia at the base to mature spermatocytes and ultimately to spermatozoa at the free surface. In the rat, 14 specific cellular associations or stages have been described (Greep, 1980). Thus in a cross-section of the seminiferous tubules, germ cells at different stages of this process may be observed.

The process of spermatogenesis depends upon the interactions between the major cell types of the testis, the germinal cells, Sertoli cells, Leydig cells and the peri-tubular cells (Skinner, 1991) since the seminiferous tubule provides an effective blood/testis barrier which restricts the interaction of the germ cells with external factors. These interactions are mediated via the Sertoli cells which acts as a functional and structural bridge linking blood and lymphatic tubular space with the seminiferous luminal compartment and hence play a critical role in spermatogenesis. The interactions between germinal cells and Sertoli cell has been demonstrated by the findings that *in-vitro* cultures require the presence of both cell types to support the spermatogenic process (Skinner, 1991).

The Sertoli cells produce several secretory proteins, such as the androgen binding protein (ABP), transferrin, proteases and protease inhibitors, extracellular matrix components utilized by the dividing or differentiating germ cells, growth factors such as EGF, TGF-α (Mullaney and Skinner, 1992), IGF I (Ceilleau *et al.*, 1990), basic FGF and NGF (Persson *et al.*, 1990) which affect cellular proliferation or differentiation, regulatory proteins such as inhibin which inhibits the secretion of follicle stimulating hormone (FSH) as well as metabolites such as lactate / pyruvate which provides the energy for the spermatogenic cells and oestrogen which regulates the process (Skinner, 1991).These cells exhibit cyclical changes that are related to the types of cells they contact and are influenced by the Leydig cells which produce steroidal sex hormones such as androgens and the pituitary hormone, FSH.

Spermatogenesis is thus subjected to hormonal influences of the gonadotropins, FSH and LH as well as androgens. The exact role of FSH is not known but has been shown to be critical for the initiation of spermatogenesis, maintainance of the germinal epithelium and proliferation of the spermatogonial population in rats. Increased FSH correlates with increasing steroidgenesis in the Leydig cells and altered cellular concentration of cyclic AMP (Delmas *et al.*, 1993, Kierszenbaum, 1994). In addition it regulates the production of inhibin in Sertoli cells, thus providing a negative feedback mechanism which control its own expression.

The different effects of FSH in both the testis and ovary may be mediated via the specific signal transduction pathway which it activates. FSH receptors are localized mainly in Sertoil cells in the testis and interaction of the hormone with its receptors on testicular target cells result in activation of adenylate cyclase, accumulation of cyclic AMP and activation of cyclic AMP dependent protein kinase (PKA). This is thought to then activate RNA transcription and protein biosynthesis. Recent work by Foulkes *et al.*, (1993) showed that

FSH regulated the expression of cyclic AMP-responsive element modulator (CREM) which in turn converts from an antagonist (repressor form of CREM) which is highly expressed in the pre-meiotic spermatocyte stage to an activator of gene transcription in more mature spermatids. LH facilitates completion of spermatogenesis by stimulating testicular steroidogenesis by the Leydig cells. The precise role of these androgens are still not fully understood but have been shown to be required for meiosis to occur at the completion of the spermatogenic process.

3.2.3(ii) Brn-3a and Brn-3b in testis

Thus the expression of the Brn-3 mRNAs in the testis may affect one of a number of stages and so establishing the specific cell types expressing the transcripts and the phase of the cycle of these cells will enable us to elucidate the role of this factor in the testis. The testis sections used in these preliminary studies were cut from frozen testis samples. The resolution at a cellular level was not very high since it was difficult to obtain thin sections. The loosely connected seminiferous tubules of the testis and the surrounding interstitial cells such as the Leydig cells are contained by a tough capsule consisting of an outer serous membrane, a middle layer of collagen and smooth muscle fibres and an inner layer of connective tissue. The tough capsule and the poorly anchored tubules made it difficult to obtain thin sections from the frozen samples. In future experiments it may be necessary to try alternative methods of fixing and mounting this tissue for better resolution.

Brn-3a appear to be expressed at low levels in the testis as observed by the hybridization signal of the antisense which were localized to specific cells not seen in the sections probed with the sense transcript (figure 3.2.9). The rounded shape of the cells and their localization to the outer surface of the seminiferous tubules suggest that these may be primary spermatocytes or Sertoli cells.

Figure 3.2.8: In situ hybridization results of rat testis probed with Brn-3a antisense (A) and sense (B) probes. The low signals obtained were localized to specific cells, the identity of which are still to be established.

Brn-3a probe

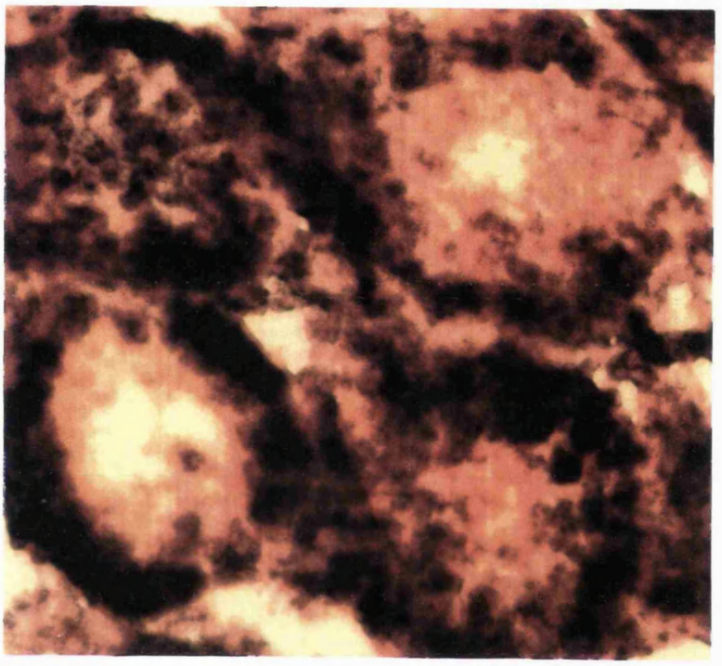
(A) antisense

(B) sense

Figure 3.2.9: In situ hybridization results of rat testis probed with Brn-3b antisense (A) and sense (B) probes. The high signal obtained with the antisense probe appeared to be localized to cells at the edges of the seminiferous tubules where primary spermatocytes are usually found. However, the poor resolution resulting from the thickness of the sections and hence the superimposition of the signals makes it difficult to precisely identify the cell types in these preliminary studies.

Brn-3b probe

(A) antisense



(B) sense

However, the stage of spermatogenesis cycle of the positive cells could not be established as better sections and further co-expression studies will be required to achieve this.

There was more abundant expression of Brn-3b mRNA (fig 3.2.10). It was difficult to readily identify the specific stage at which the cells expressing Brn-3b mRNA were in the spermatogenic cycle since a cross-section of the seminiferous tubule will show spermatids at different stages of development while the thickness of the sections limited the resolution. To establish the cell and stage specific localization may therefore require repeated experiments including immunocytochemical staining or hybridization of the cells with factors known to be localized in tubules at different stages of the cycle. However, these preliminary results suggested that Brn-3b may be localized to the base of the tubules with intense signals detected in areas around the edges of the tubules suggesting that they may be expressed in the immature spermatogonia cells undergoing mitosis. If this can be confirmed then it may indicate that the Brn-3b is present in proliferating cells of the uterus and ovary and testis.

These preliminary in-situ results support the PCR data showing that while Brn-3a was highly expressed in the brain and DRG, lower level were detected in the testis and ovary. However, Brn-3b mRNA appeared to be abundant in the tissues of the reproductive tract. However, much more work will be required to precisely locate the cells which express Brn-3a and Brn-3b mRNA as well as trying to identify the role in these cells. For a summary of the distribution of Brn-3a and Brn-3b in different tissues and cell lines see table 3.2.1.

Tissue / cell line	Brn-3a	Brn-3b
ND7 - undifferentiated	+	++++
ND7 - differentiated	++++	++
PC 12 - undifferentiated	-	-
PC 12 - differentiated	+	+
ND3	++	-
ND11	++	+++
ND21	+	++
N18	-	++++
Brain	+++	++
DRG	++++	++
Heart	-	-
Liver	-	-
Lung	-	-
Kidney	-	-
Spleen	-	-
Cervix	-	++++
Uterus	-	++++
Testis	-/+	++++
Ovary	-/+	+++

Table: 3.2.1: Summary of the distribution of Brn-3a and Brn-3b in cell lines and tissues found in this study.

3.3.0 Discussion

The isolation of the cDNA encoding the novel protein, Brn-3b, from sensory neuronal derived cell line ND7 cells showed it to be highly homologous to Brn-3a in the POU domain. The presence of a family of Brn-3 proteins was confirmed by the subsequent cloning of the highly related Brn-3c factor from dorsal root ganglion.

Comparison of the sequences of Brn-3a and Brn-3b reveal that most of the resultant amino acid changes are in the poorly conserved linker region. However there is a valine to isoleucine conversion in the first helix of the POU-homeodomain, a region which is generally highly conserved among all POU domain proteins. This may have implications on protein-protein interactions of these proteins as observed with the *Drosophila* POU proteins, I-POU and tI-POU which are also members of the POU-IV subclass. Differences in two amino acids in the POU-homeo domain of these proteins result in changes in the DNA binding characteristics and protein-protein interactions of two otherwise identical proteins. I-POU which lacks two amino acids in the amino terminus of the POU-homeo domain is incapable of binding a DNA motif bound by the tI-POU but interacts with Cf1a protein and modulate its activity (Treacy *et al.*, 1991; Treacy *et al.*, 1992). However, tI-POU cannot interact with the Cf1a or I-POU proteins (see introduction).

The implications of the single amino acid change within this region of Brn-3a and Brn-3b remains to be elucidated but the result of functional studies by Theil and Moroy (1994) as well as those reported in chapter 4 and 5 indicate that these two proteins may have different effect on the activity of target promoters which may reflect differences in the ability of these two proteins to interact with each other or different proteins.

The results of our studies suggested that Brn-3a may be more characteristic of cell lines and tissue with neuronal like properties, such as differentiated ND7 cells and sensory neurons of the dorsal root ganglia and trigeminal ganglia and brain. Low levels of Brn-3a was also detected in the testis and ovary but not in heart, lung, liver, kidney, spleen and uterus or cervix (table 3.2.1).

In contrast, while Brn-3b was expressed at lower levels in the differentiated ND7 cells and sensory neurons the mRNA was found to be highly expressed in proliferating ND7 cells as well as in some tissues of the reproductive tract such as the rat uterus, ovary, testis and cervix. Like Brn-3a, Brn-3b transcript was undectable in tissues such as heart, lung, liver, kidney and spleen (table 3.2.1).

Some of this data is supported by the results of studies undertaken by different groups which have reported on the expression of these factors in various murine tissues. For instance, in the adult mouse Brn-3a has been detected in specific regions of the brain as well as in sensory neurons of the dorsal root ganglia, the trigeminal ganglia and the retina. A much lower level of Brn-3a is also found in the pituitary and pituitary derived corticotroph cell lines and in B-cell lines but not in non-neuronal tissues such as liver, spleen, kidney, heart lung or adrenal glands (Ninkina *et al.*, 1992; Bhargava *et al.*, 1993; Gerrero *et al.*, 1993). Similarly, Brn-3b mRNA has been identified in various structures of the brain, the sensory neurons of the DRG and trigeminal ganglia, the retina and the spinal cord in the adult mouse (Xiang *et al.*, 1993; Turner *et al.*, 1994). However no Brn-3b was detectable in a number of other tissues including liver, kidney, heart, lung and pituitary.

Preliminary results of the expression of these two factors in the tissues of the reproductive tract by in-situ hybridization studies confirmed the results obtained by PCR since high levels of Brn-3b were detected in the uterus, ovary and testis while there may be lower expression of Brn-3a only in the ovary and testis. It appears that in these tissues Brn-3b transcripts may be associated with regions undergoing rapid cellular proliferation under the influence of FSH suggesting that its expression may also be regulated by this hormone or factors downstream of its activity such as the cyclic AMP pathway. The effect of FSH can be mimicked by cyclic AMP indicating a central role for the adenylate cyclase pathway in the cyclic processes occuring in these tissues. This may be modulated by the differential expression of the isoforms of either adenylate cyclase or in the cyclic AMP response element binding proteins (CREB). For instance the repressor form of CREM is expressed during early spermatogenesis cycle with the activator form of CREB predominating later and this expression is modulated by FSH also (Greep, 1980). Brn-3a and Brn-3b expression are regulated by cyclic AMP in ND7 cells (chapter 4 and 5) so it is possible that in the ovary and testis cyclic AMP may also affect the function of these proteins. This remains to be established. FSH also appears to stimulate the production of growth factors such as IGF I in the granulosa cells during proliferation of the ovary (Hatey *et al.*,1992, 1995) and the expression of a number of other growth factors (EGF / TGF-α, basic FGF, NGF) in the testis which are regulated at various stages (Kerszenbaum, 1994). The expression of both Brn-3a and Brn-3b have been shown to be modulated by combinations of growth factors (chapter 4). Thus these signalling systems may provide a pathway for regulation of the expression of these factors in these tissues.

This regulation of expression of a number of transcription factor has been demonstrated in the ovary and testis which may be stage or tissue specific (Morales *et al.*, 1989, Skinner, 1991, Kierszenbaum, 1994). These include members of the protein kinase family such as *c-kit*, which is co-expressed with its ligand *steel* (*stl*) gene product in different cells of both

tissues and are critical for proliferation of the primordial germ cells and primary spermatocytes. In addition a number of other transcription factors including a number of homeodomain and POU domain genes have also been detected in the testis and ovary which shows stage and cell specific expression. Genes encoding the homeobox Hox-1.4 (Wolgemuth *et al.*, 1993) or the POU domain factors Sperm 1 (Andersen, 1993), Oct-6/ Tst-1 (He *et al.*, 1989; Mokuni *et al.*, 1990; Wegner *et al.*, 1993), and Brn-5 (Andersen *et al.*,1993) have been detected in the testis. Sperm1 is the only POU factor to date which has restricted expression to the testis since like Brn-3a and Brn-3b the other factors were also found to be expressed in neuronal tissues during development of the embryo (Suzuki *et al.*, 1990; Wolgemuth *et al.*, 1992). Sperm1 mRNA is expresses in the period preceeding meiosis and may thus exert a regulatory function in events leading to terminal differentiation. In the ovary, mRNA of Oct 3/4 POU protein (which belong to the same subclass as Sperm1) has been detected in primordial germ cells, unfertilized oocytes and is downregulated upon differentiation (Scholer *et al.*,1989; Rosner *et al.*, 1991). The Oct-6 mRNA detected in adult rat testis is distinct from the transcript detected in the brain (Suzuki *et al.*,1990) and the precise role in the testis is still unclear. Similarly, *Hox-1.4* gene product in the germ cells and the embryo are different transcripts of the same gene. It is only expressed in the testis of adult mice undergoing meiosis during spermatogenesis but not in embryonic or neonatal testis. The precise function is still to be elucidated but the restricted expression following meiosis suggested a role during spermatid maturation (Wolgemuth *et al.*, 1992). The existence of different isoforms of Brn-3a and Brn-3b may indicate a similar pattern of differential expression and this also remains to be established.

The function of the Brn-3 proteins in these tissues are still to be elucidated. However, the finding that the mRNA of the opioid peptide gene precursor molecule, POMC, shows differentially regulated expression in the testis (Kilpatrick *et al.*, 1987, Skinner, 1991) may provide a possible target gene regulated by these factors since previous studies have shown

that the POMC promoter is regulated by Brn-3a (Gerrero *et al.*, 1993) and Brn-3b (see chapter 5). It is therefore possible that this gene or others which are still unidentified may be regulated by Brn-3 proteins. Therefore further work will be needed to identify the isoforms of Brn-3 proteins expressed, as well as their spatial and temporal expressions to help elucidate the role of the Brn-3 proteins in these tissues. In addition to repeating these in-situ hybridization experiments to confirm these findings, serial studies will be carried out to establish whether the expression is stage specific or influenced by the cyclic nature of these processes and immuno-cytochemical studies may be carried out to identify factors that co-localize in the cells which may help to identify the precise cell types that express the Brn-3 mRNA.

Thus the results of our experiment suggest that Brn-3a expression in adult neuronal cells may be associated with the appearance and maintainance of differentiated cell phenotypes but its presence in ovarian and testicular tissues will need to be studied more exhaustively for a firm conclusion to be made. Brn-3b appear to be more characteristic of proliferating cells both in ND7 cells and in the tissues of the reproductive tract but its precise function is not clear. However, since it is also expressed in the non-dividing cells such as in sensory neurons it may interact with and modulate the expression of other proteins such as Brn-3a. This will be discussed in the next two chapters.

Chapter 4
Results

Modulation of Brn-3a and Brn-3b expression in ND7 cells

4.0.0 Background

Transcription factor activity may be regulated at a number of levels such as its own transcription, the stability of the mRNA and protein products, post-transcriptional modifications, post-translational processing, and regulation of the expression or function of proteins which may interact with this factor to modulate its activity. For instance, phosphorylation provides a rapid and readily reversible mechanism commonly used to modulate the activity of transcription factors in response to environmental changes by modifying the DNA binding and/or the protein-protein interaction required for transcriptional activity (reviewed in Berridge, 1988; He and Rosenfeld, 1991; Struhl, 1991; Jackson, 1992; Hunter and Karin, 1992, Hill and Treisman, 1995). These effects may be produced by the integration of information carried by multiple signal transduction pathways so providing flexibility and versitility for gene regulation.

In the previous chapter the differential expression of the Brn-3a and Brn-3b transcription factors in various tissues and cell types provided the evidence for regulation at the mRNA level. We were therefore interested in looking at factors which may affect the expression and activity of these factors. As previously discussed, the expression of both Brn-3a and Brn-3b were regulated upon differentiation of ND7 cells in the absence of serum or in low serum medium containing the cyclic AMP analog, dibutyryl cyclic AMP (chapter 3). The precise effect of serum factors or cyclic AMP on the Brn-3 promoters are yet to be established since the promoter sequences are still to be identified and studied. While cyclic AMP may have a direct effect on changing the phosphorylation states within the cells by acting as the second messenger in the protein kinase A pathway, serum factors may provide the primary extracellular signals which trigger receptor mediated signalling cascade and culminate in changes in the program and rate of gene expression.

Diverse signalling cascades may be initiated by ligand binding to cell surface receptors such as the seven-pass trans-membrane receptors coupled to G -proteins; the cell surface spanning tyrosine kinases and the heterodimeric receptors associated with Src-type tyrosine kinases (for reviews see Hunter and Karin, 1992; Karin, 1992; Hill and Triesman, 1995; Heldin, 1995; Marshall, 1995; Neer, 1995). Receptor stimulation which results in the transient production of second messengers such as cyclic AMP, calcium, diacylglycerol and inositol triphosphate and the subsequent activation of down-stream kinases provide points of convergence of these pathways. These pathways may culminate in the activation or repression of transcription factor activity and hence gene expression in the cells.

4.0.1 Cyclic AMP effects on cell growth and differentiation.

An example of the mechanism by which these factors mediate transcriptional regulation can be observed by the effect of the ubiquitous signal transduction molecule, cyclic AMP. Genes regulated by cyclic AMP contain specific conserved sites, the cyclic AMP response elements, (CRE), a highly conserved DNA sequence which may be present as single or multiple copies in the promoter of responsive genes. The CRE is bound by specific CRE binding proteins such as CREB, CREBP1, ATF-2, ATF-3 and ATF-4 (Activating Transcription Factors) and CRE modulators, CREM and ICER upon phosphorylation of specific residues (Gonzalez and Montminy, 1989; Kageyama *et al.*, 1991; reviewed in Meyer and Habener, 1993; Lalli and Sassone-Corsi, 1994). This interaction may affect transcriptional activity either negatively or positively (Lamph *et al.*,1990; Ziff, 1990).

While activation of the cyclic AMP pathway was shown to promote cellular growth and proliferation in many cells it was also shown to enhance the survival of non-dividing post-mitotic sympathetic and sensory neurons (Rydel and Greene, 1988; Deckworth and Johnson, 1993; Buckmaster and Tolkovsky, 1994) and the sensory neuronal cell line, ND7

(Howard *et al.*,1993) where it appears to inhibit apoptosis and enhance neurite outgrowth. In these neurons, cyclic AMP mimics the trophic effect of NGF which is required for target field innervation and neuronal survival during development and protects against programmed cell death in a manner and time course which is similar to NGF (Rydel and Greene, 1988; Deckworth and Johnson, 1993). However these two factors may act via different signalling pathways with the cyclic AMP mediated effect being dependent on protein kinase A activation and which is blocked by specific inhibitors which have no effect on the NGF mediated effect (Rydel and Greene, 1988).

4.0.2 Serum factors in cell proliferation and neuronal differentiation

Neuronal survival, growth and differentiation are also influenced by factors present in serum (reviewed in Rozengurt, 1986; Walaas and Greengard, 1991). Studies with neuroblastoma cell lines which can be induced to differentiate by serum deprivation showed decreased PKC activity in response to serum withdrawal (Schuch *et al.*, 1989). Upon differentiation in the presence of PKC inhibitors these cells did not differentiate fully but developed shorter neurites indicating that other pathways may also be involved in this process.

Growth factors present in serum have potent effects on cellular proliferation as well as survival and differentiation depending on the cell type and interactions with other factors (Heath, 1993; Herschman, 1991). For instance, the multifunctional fibroblast growth factors (FGFs) are potent mitogens on fibroblast cells but promote neuronal survival and neurite outgrowth (Walicke *et al.*, 1986; Anderson *et al.*, 1988; Walicke and Baird, 1988) and may contribute to neurotransmitter function in various regions of the brain during development (McKinnon *et al.*, 1990; Rukenstein *et al.*, 1991; Collarini *et al.*, 1991; Zurn, 1992).

Growth factors interact with specific high affinity receptors located in the plasma membrane of target cells which can then activate intracellular signalling pathways, culminating in a multiplicity of intracellular events (reviewed in Heldin, 1995). Many of these molecules can interact with more than one type of receptor so conferring diversity of action by some growth factors.

4.0.3 Families of Growth Factors

Some growth factors which were known to promote survival of neuronal populations and were also expressed in various parts of the developing nervous system and in mature neuronal tissues were selected to try and identify the factors modulating the Brn-3a and Brn-3b mRNA in the ND7 cells. The results are reported later in this chapter. Of these, the acidic and basic fibroblast growth factors (FGF) and insulin-like growth factors (IGF I and II) were found to affect the expression of these proteins and will thus be dissused in some detail. Others factors such as PDGF and NGF (McKinnon *et al.*,1990; Collarini *et al.*, 1991; Rukenstein *et al.*, 1991; Deckworth *et al.*, 1994; Ginty *et al.*, 1994) were used but demonstrated no significant effect on the levels of Brn-3 proteins in the ND7 cells used for these studies. Although NGF is important for survival and neurite outgrowth during sensory neuronal development (Ruit *et al.*, 1992), its role in the mature neuron is less well defined and may be related to the production of functional proteins such as Substance P (Johnson *et al.*, 1986; Lindsay, 1988). ND7 cells were found to be unresponsive to NGF, possibly a property resulting from the neuroblastoma parental cells which were also unresponsive to this factor (Howard *et al.*, 1993). However, studies carried out using primary sensory neurons from DRG cultures had shown that NGF treatment had no effect upon the mRNA expression of either Brn-3 or Oct-1. In contrast, Oct-2 mRNA was upregulated in NGF treated sensory neurons (Wood *et al.*,1992). As such treatment with NGF was used as a control factor.

Two members of the Fibroblast Growth Factor (FGF) family studied were acidic FGF and basic FGF (reviewed in Burgess and Maciag, 1989). These are different molecular entities identifiable by their distinct isoelectric points but with similarity in biological functions. Whereas both proteins were detected in embryonic brain, acidic FGF was restricted principally to cells of central and peripheral nervous system in adult tissues while basic FGF was more widely distributed and was found in most adult and foetal tissues, in normal as well as tumorigenic cells . Acidic and basic FGFs have been shown to be mitogenic for the same cell types possibly interacting with the same cell surface tyrosine kinase receptors thus eliciting similar effects on the cells (Neufeld and Gospodarowicz, 1986; Wanaka *et al.*, 1990).

In addition to inducing proliferation in fibroblasts, both FGFs may also act as neurotrophic factors, since they increase the survival and neurite outgrowth of neurons from multiple brain regions as well as supporting the differentiation of a wide variety of neuroectoderm and mesoderm- derived cells (Rydel and Greene, 1987; Anderson *et al.*, 1988; Walicke, 1988; Rukenstein *et al.*, 1991). *In vitro*, the FGFs can also induce neurite outgrowth from the sympathetic neuronal cell line, PC12 and can act synergistically with NGF to promote neuronal survival in these cells (Rukenstein *et al.*, 1991). Synergistic interaction with PDGF has also been observed to promote the development and differentiation of oligodendrocytes (McKinnon *et al.*, 1990). Furthermore, Zurn (1992) reported that the FGFs may be involved in the regulation of neurotransmitter expression of cholinergic and noradrenergic neurons in sympathetic neurons *in-vitro* without affecting the survival of the cells.

The Insulin-like Growth Factors, IGF I and IGF II, demonstrate similar structure and biological function but have distinct cell receptors as well as different tissue specificity. IGF I predominantes during early life, declining after puberty while IGF II is found at high levels during embryonic and foetal development in a wide variety of tissues and declines soon after birth (Heath, 1993). In adults, expression persists only in epithelial cells lining the surface of the brain.

Of functional relevance to neuronal cells was the finding that IGF I but not IGF II was able to protect cerebellar granule neurons against apoptosis under specific conditions, in a manner similar to protection seen by cyclic AMP induction, NGF (D'Mello *et al.,* 1993). In addition, IGF I was demonstrated to have potent effects on cultured neural tissues including stimulation of mitosis in sympathetic neuroblasts, promotion of neurite outgrowth in cortical, sensory and sympathetic neurons and induction of oligodendrocyte differentiation (Bondy,1991).

The mechanisms by which many growth factors mediate transcriptional changes are not entirely clear since many common effects are elicited by diverse growth factors binding their receptors which culminate in different cellular effects. For instance, while Nerve Growth Factor (NGF) and Epidermal Growth Factor (EGF) both activate tyrosine kinase receptors in PC12 cells and trigger events such as the phosphorylation of cellular proteins, membrane ruffling and activation of protein kinases. However, stimulation with EGF ultimately leads to DNA replication and cell division while NGF acts to promote neuronal outgrowth, survival and differentiation (Chao, 1992).

Recent studies in PC12 cells have also demonstrated that the strength or duration (or both) of the signals can influence the cells response in terms of differentiation or proliferation (Traverse *et al.,* 1994; Hill and Triesman, 1995; Marshall, 1995). This effect coupled with

the synergistic interactions of various factors which bind to specific sites on cellular promoters to activate transcription may provide some specificity of the transcriptional response required for growth and proliferation in mitogenesis and for cellular differentiation (Rozengurt and Sinnett-Smith, 1988; McKinnon *et al.*, 1990; Collarini *et al.*, 1991; Rukenstein *et al.*, 1991; reviewed in Struhl, 1991; He and Rosenfeld, 1991; Hill and Treisman, 1995).

We were thus interested in looking at the effect of cyclic AMP and factors in serum which may activate different signalling systems on the expression of the Brn-3 mRNA in ND7 cells as well as looking at the functional effect of the changes in the Brn-3 protein levels on promoter activity. The results of those studies are reported in this chapter.

Results:

4.1.0 Identification of factors regulating Brn-3a and Brn-3b expression

It was previously demonstrated that Brn-3a and Brn-3b mRNAs exhibited distinct but overlapping expression pattern in ND7 cells such that differentiated cells contained higher levels of Brn-3a transcripts compared proliferating cells while the reverse pattern was observed for Brn-3b. In order to investigate the underlying factors engendering these changes, the components of the differentiation media were studied to try and isolate specific factor/s responsible for the changes in mRNA levels observed when used in combination.

4.1.1 Effect of cyclic AMP on Brn-3a and Brn-3b mRNA expression

In the initial experiments an increase in Brn-3a mRNA was observed in ND7 cells differentiated either by transfer to medium containing 0.5% FCS and supplemented with 1mM dibutyryl cyclic AMP or to a serum free medium (50% DMEM/ 50% HAMS supplemented with insulin, transferrin and sodium selinite). To study the effect of cyclic AMP on Brn-3 mRNA expression, independently of decreased serum concentration, ND7 cells were grown in medium containing 10% FCS + 1mM cyclic AMP or in serum-free medium with or without 1mM cyclic AMP. Untreated control cells were grown in full growth medium containing 10% foetal calf serum. After 72 hours, the cells were harvested, RNA extracted and used in RT-PCR to quantify the mRNA levels of Brn-3a and Brn-3b as previously described. Any variation in the total RNA between different samples or in the efficiency of reverse transcription were equalized by the amplification of the constituitively expressed L6 ribosomal mRNA. Figure 4.1.1 demonstrates the results of a typical experiment and is representitive of the changes in the levels of Brn-3a and Brn-3b observed upon transfer of ND7 cells to serum free medium or full serum medium supplemented with cyclic AMP.

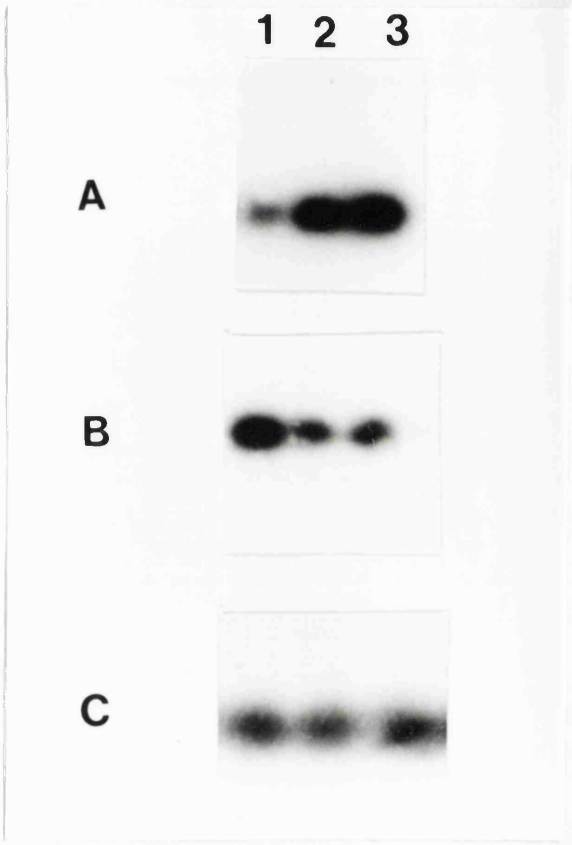


Figure 4 .1.1 : RT-PCR assay of transcripts encoding Brn 3a (panel A), Brn 3b (panel B) and the ribosomal L6 protein control (panel C). cDNA used for the reactions were synthesized from RNA prepared from cells grown in full growth media (FGM) (track 1), in serum free media (track 2) or in FGM with 1mM cAMP added (track 3).

As expected, cells which were grown in serum free medium were differentiated by 72 hours and this was accompanied by an increase in Brn-3a mRNA levels and a concomitant decrease in Brn-3b transcripts seen before. Similar changes were also observed in cells grown in the cyclic AMP supplemented full serum media such that an increase in the Brn-3a mRNA and a clear decrease in Brn-3b transcripts were obesrved compared with the levels seen in undifferentiated ND7 cells. A similar pattern was observed in a number of independent experiments.

The combined treatment of adding cyclic AMP to the serum free medium did not appear to have a synergistic effect since there was a relatively small increase in Brn-3a with both the addition of cAMP and low serum compared to that obtained with each treatment individually while the level of Brn-3b in the combined treatment was similar to that observed upon serum removal (figure 4.1.2). Therefore it appears that the expression of these two factors are also oppositely regulated by cyclic AMP and serum factors. Interestingly, the addition of 1mM cyclic AMP to full growth medium, which resulted in changes in levels of Brn-3a and Brn-3b mRNA also caused morphological changes in the ND7 cells such that by 72 hours there were extensive process formation characteristics of differentiated ND7 cells (see introduction, figure 1.4.1) but without significant reduction of total cell numbers which accompanies differentiation in serum free media (Howard *et al.*,1992). The significance of this observation will be discussed later.

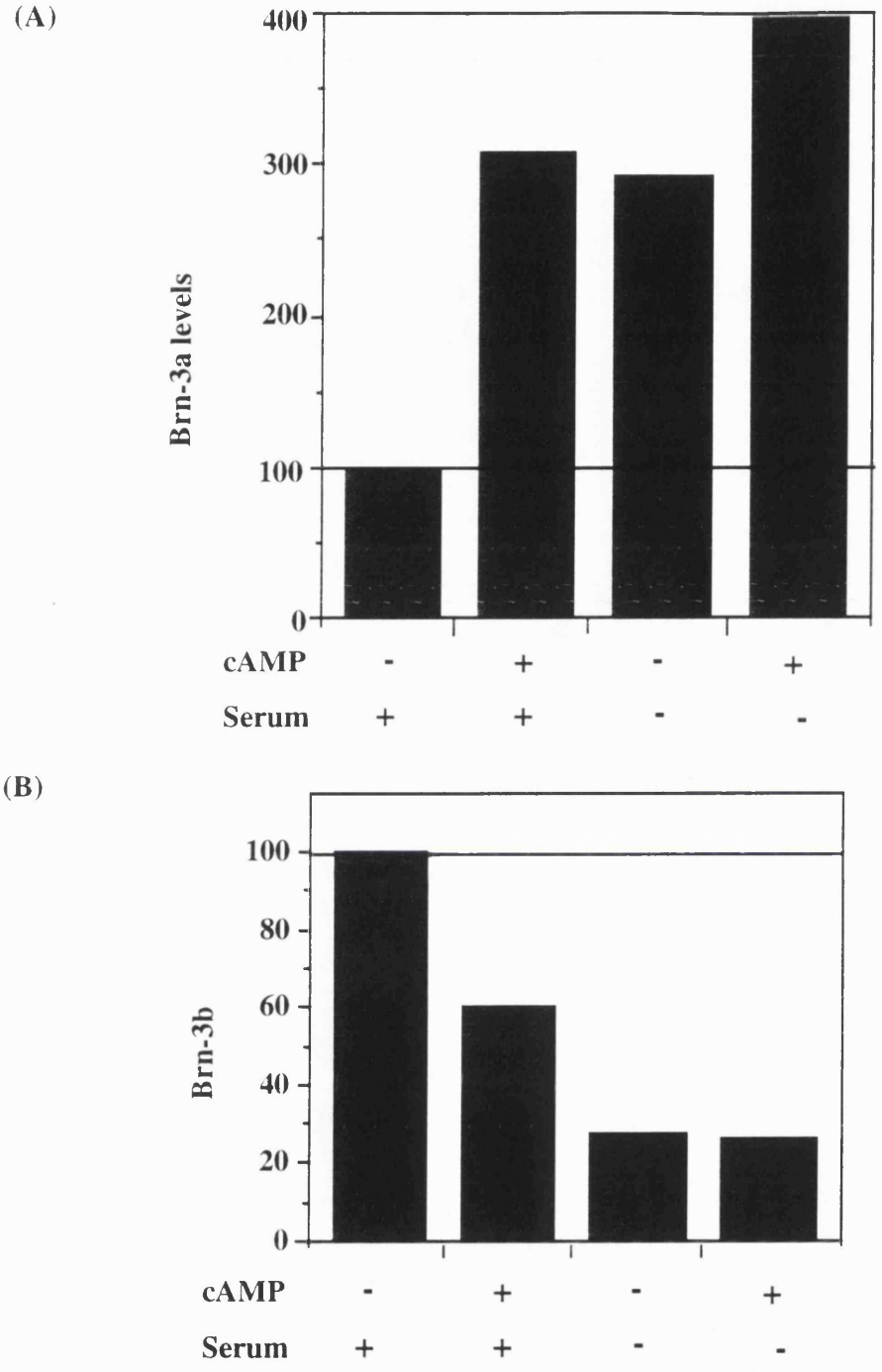


Figure 4.1.2 : Representitive levels of expression of Brn-3a and Brn-3b in a typical experiment in which ND7 cells were grown for 72 hours in media with or without serum or cyclic AMP, as indicated. The values are expressed as a percentage of the level in untreated ND7 cells (100%) and have been equalized on the basis of the levels of the L6 ribosomal protein RNA.

4.1.2 Competitive PCR

Most of the experiments done to study the levels of Brn-3a and Brn-3b in the cell culture and tissues was done using a semi-quantitative PCR protocol (described in methods) where duplicates of the reactions were carried out and samples were taken after different numbers of cycles to ensure the reaction was in the exponential phase. Differences in the RNA concentration or cDNA synthesis were corrected for by the amplification of the constituitively expressed, invariable mRNA species such as the L6 ribosomal RNA. Previous attempts to quantify the PCR products by co-amplification of control mRNA species in the same tube as the target sequence resulted in competition with the target sequence in a manner which did not reflect the ratio of differences seen between various samples when the reactions were carried out with individual sets of primers.

Competitive templates which were designed according to the method described by Celi *et al.*, (1993) were found to be more effective for co-amplification with the target sequences. The competitor was designed to be approximately one hundred bases shorter than the target sequences with regions at the ends being complementary to the primers used for the PCR with the target sequence hence allowing co-amplification of the competitor with the target sequences. Co-amplification of an equal amount of the competitor templates with the experimental samples therefore provided an internal control for the efficiency of the PCR reaction. The competitor template product was distinguished from the product of the natural mRNA on the basis of its smaller size upon resolution on agarose gel .

Some of the PCR experiments using Brn-3a and Brn-3b primers were repeated using the cDNA previously amplified and the competitive templates to check for any differences that may be associated with amplification differences in different reaction tubes. An example of this is shown in figure 4.1.3.

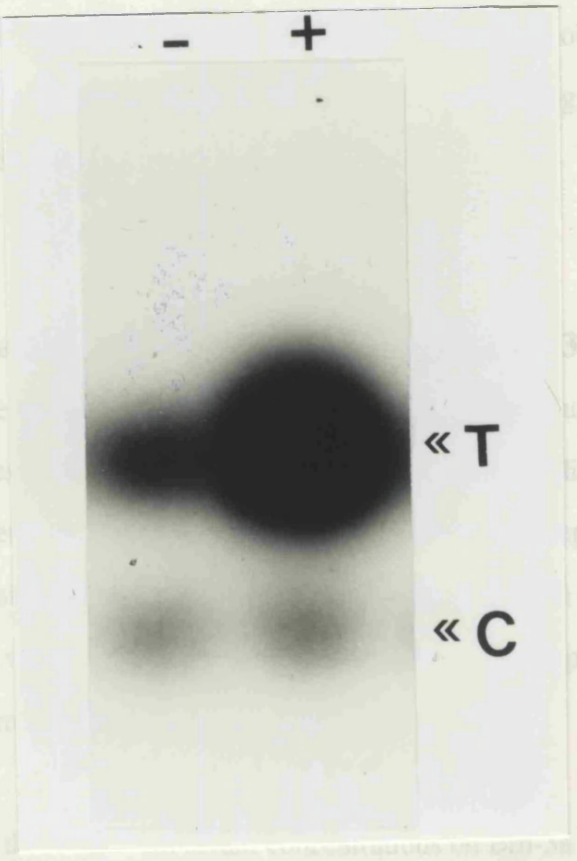


Figure 4.1.3: RT-PCR using competitor template to assay for differences such as tube-to-tube or sampling variations during the amplification of Brn-3a. cDNA samples used for PCR wereobtained from ND7 cells grown in the absence (-) and presence (+) of 1mM cyclic AMP. The competitor template (C) was amplified using the same primers as the target sequences and the product was approximately 100 bases shorter than the target product (T). The increase in Brn-3a mRNA in the presence of cyclic AMP was similar to that observed previously while the competitor remained constant.

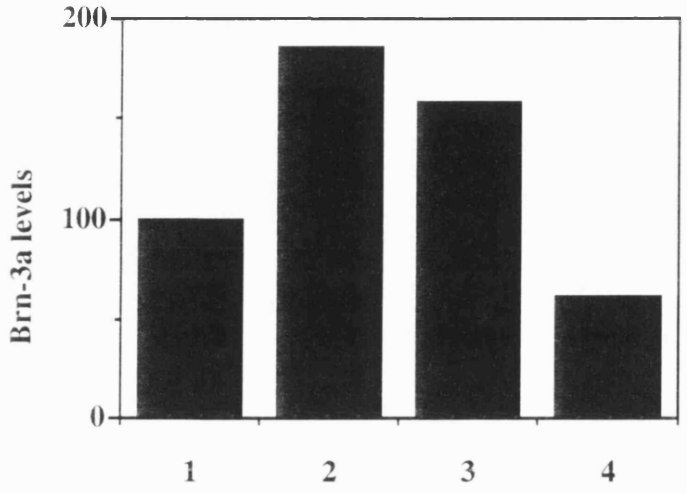
RT-PCR amplification of cDNA from ND7 cells grown in the full serum medium with or without dibutyryl cyclic AMP showed a similar increase in Brn-3a mRNA and a decrease in the Brn-3b levels when compared with levels obtained previously (figure 4.1.1) while the competitor template product remains relatively constant. The results obtained in these experiments therefore validated the results which were obtained using the semi-quantitative strategy previously described.

4.1.3 Serum Factor effects on Brn-3a and Brn-3b expression

A reduction or complete removal of the serum from the growth medium resulted in morphological changes in ND7 cells which was associated with the differentiation of these cells to a neuronal phenotype. The effect which was observed was dependent on the level of serum deprivation since lowering the serum concentration to 0.5% induced growth arrest and neurite formation which appeared to be dissociated from the apoptosis observed upon differentiation in serum free medium (see chapter 6).

To study the effect of these different serum concentrations on Brn-3a and Brn-3b, ND7 cells were transferred to media containing different serum concentration and harvested after 72 hours. The expression of Brn-3a and Brn-3b transcripts were then analyzed by RT-PCR. The results shown in figure 4.1.4 are representative of the changes observed in Brn-3a and Brn-3b levels in a number of independent experiments carried out using different RNA preparations.

(A)



(B)

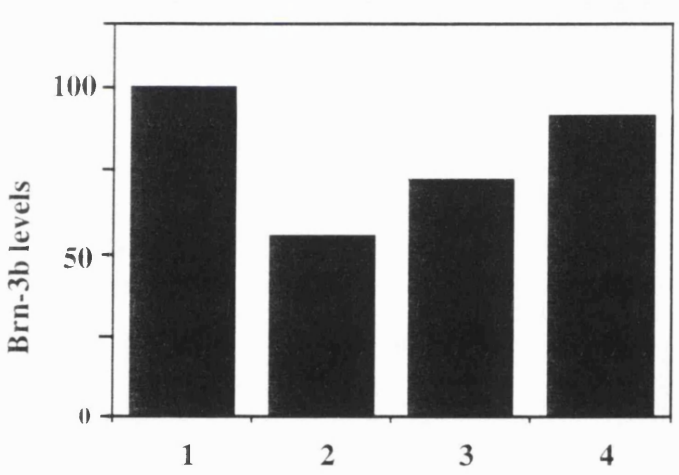


Figure 4.1.4 : Representative levels of Brn-3a (A) and Brn-3b (B) expressed in ND7 cells following 72 hours of transfer to 10% serum media (full growth media, FGM) (track 1), serum free media (track 2), 0.5% serum (track 3) and 1% serum (track 4). Results were obtained by RT-PCR of cDNA synthesized from RNA from the treated cells using Brn-3a and Brn-3b primers and variation in the RNA levels were equalized using the level of L6 ribosomal amplification. Values are expressed as a percentage of the control untreated ND7 (track 1) which is represented as 100%.

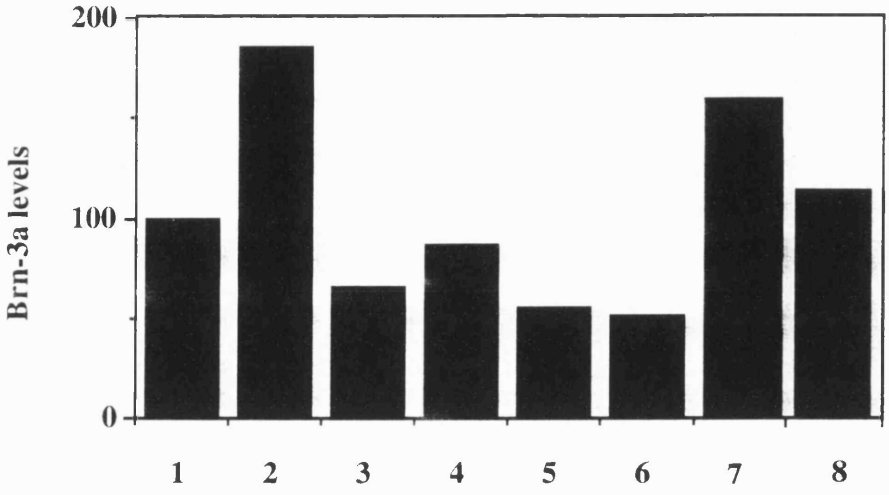
An increase in the Brn-3a mRNA expression was observed upon transfer of the cells to serum free medium or medium containing 0.5% FCS although the increase was more marked in the cells maintained in serum free medium. However in medium containing 1% serum there was no increase in Brn-3a levels. In this medium, cellular proliferation was maintained and there was neurite outgrowth and cellular differentiation was comparable with the cells in 10% FCS. Thus, it appears that Brn-3a levels correlated with the cessation of cellular proliferation and extension of neurite processes which is associated with the differentiated state of the cells.

Brn-3b transcripts showed a decrease following transfer of the cells to serum free media. which is in accordance with the our previous results (figure 4.1.4B). However, in contrast to Brn-3a, the decrease in Brn-3b levels was observed in cells grown in media containing both 0.5% and 1% serum, albeit to different levels, thus indicating that the change in the Brn-3b mRNA is much more sensitive to variation in the serum concentration and perhaps specific serum factors regardless of the effect on cellular proliferation or neurite outgrowth.

4.1.4 Growth Factors regulating Brn-3a and Brn-3b expression

In an attempt to identify factors in serum which may regulate the expression of Brn-3a and Brn-3b, growth factors were added individually or in combination to ND7 cell cultures grown in serum free media or in medium containing 0.5% FCS. The treated cells were harvested after 72 hours and the levels of Brn-3a and Brn-3b were measured by RT-PCR and quantified as described previously. The levels were expressed as a percentage of the untreated controls (figure 4.1.5).

(A)



(B)

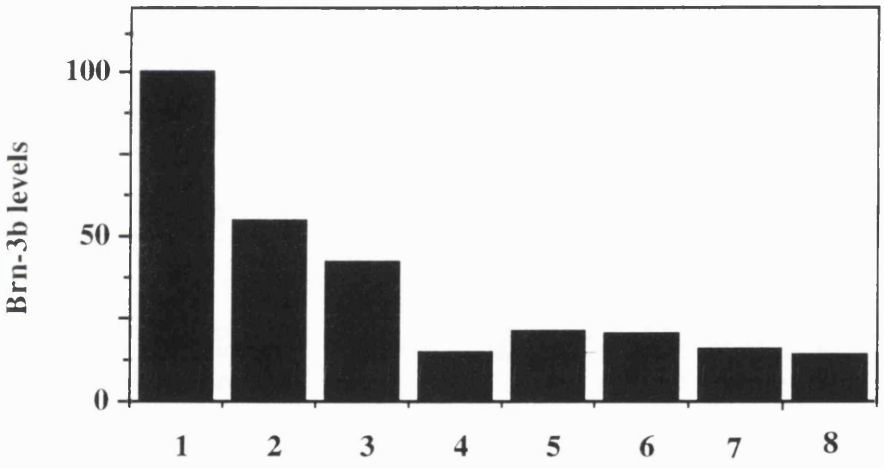
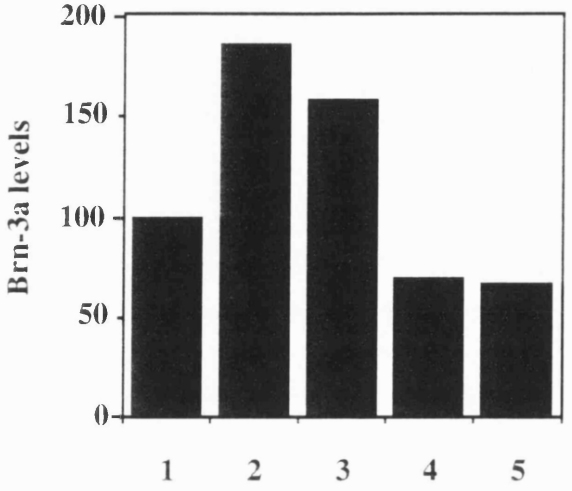


Figure 4.1.5 : Representitive levels of Brn-3a (A) and Brn-3b (B) in ND7 cells grown in full serum medium and transferred to the same medium (track 1), serum-free medium (track 2) or serum free media supplemented with acidic FGF (track 3), basicFGF (tracks 4), IGF I (track 5), IGF II (tracks 6) NGF (track 7) and PDGF (track 8). The values were normalized relative to the level of these factors measured in the control, untreated cells grown in full serum media. A similar pattern of expression was observed in at least three independent experiments.

NGF had no effect on the mRNA of either Brn-3a or Brn-3b. This was expected since Wood and collegues (1992) had previously shown that NGF had no effect on Brn-3 expression. However PDGF had no significant effect on the expression of either factor when compared with the levels in the undifferentiated control. In contrast, the addition of acidic FGF, basic FGF, IGF I and IGF II were capable of reducing the upregulation of Brn-3a transcripts which was observed on transfer to serum free medium (figure 4.1.5A) but these individual factors had no effect on increasing the expression of Brn-3b mRNA to the level observed in full serum medium (figure 4.1.5B). However, the addition of these growth factors to the medium in combination also abolished the rise in Brn-3a associated with transfer of the cells to serum free media but was also capable of reversing the decrease in Brn-3b levels observed in serum-free media [figure 4.1.6 (i)]. The pairwise combinations of the acidic and basic FGF or IGF I and II could also reduce Brn-3a mRNA to near the levels found in undifferentiated ND7 cells but could not reverse the change in Brn-3b mRNA level observed when these were added in combination [figure 4.1.6 (ii)].

A number of these RT-PCR experiments were repeated using primers which were outside the POU domain of both Brn-3a and Brn-3b and amplified a region in the 3' non-coding sequences of Brn-3a and sequences 5' to the Brn-3b POU domain. Results showed very similar changes in the pattern of expression of these factors under given conditions so further substantiating the results obtained in the experiments using the primers to the POU domain.

(A)



(B)

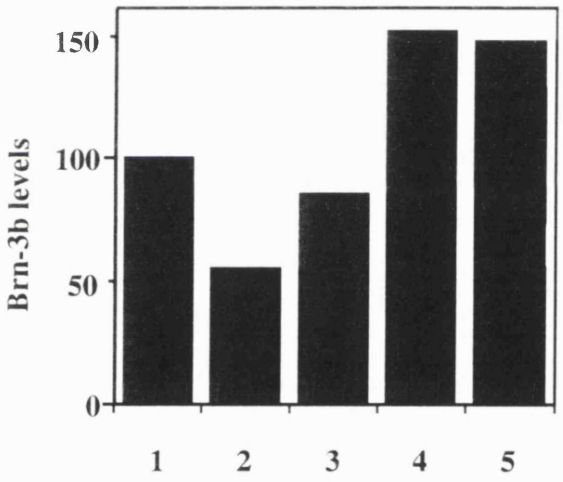


Figure 4.1.6 (i) : Representitive levels of Brn-3a (A) and Brn-3b (B) mRNA expressed in ND7 cells seventy two hours after transfer from growth medium containing 10% foetal calf serum to the same medium (control) (track 1), to serum-free medium (track 2), to medium containing 0.5% serum (FCS) (track 3), serum free medium with a combination of acidic FGF, basic FGF and IGF I and II added (track 4) or medium containing 0.5% serum and the same combination of growth factors (track 5). The levels are expressed as a percentage of that value obtained for the control cells.

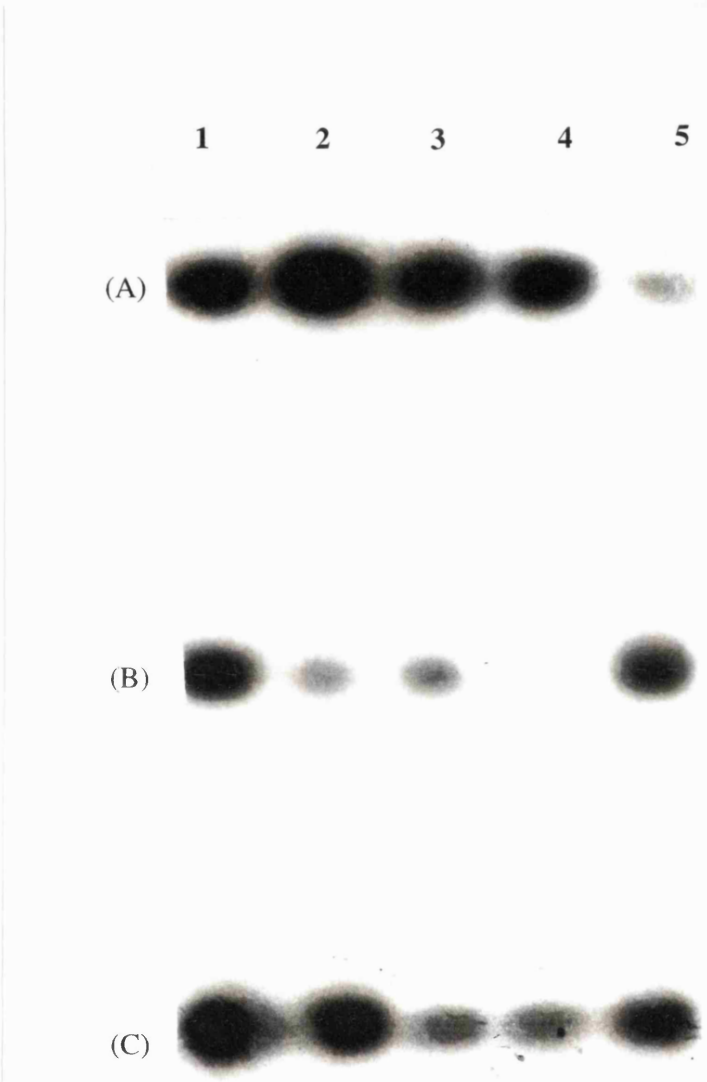


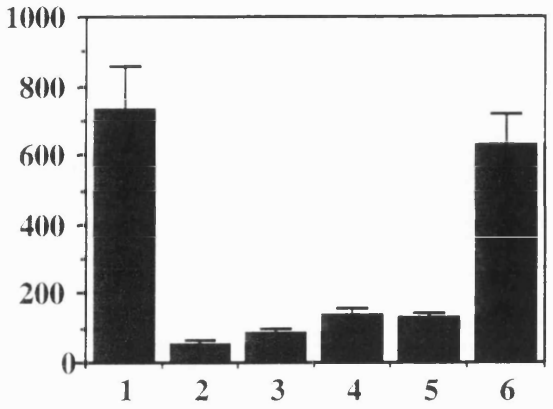
Figure 4.1.6 (ii) Expression of Brn-3a (A) and Brn-3b (B) in ND7 cells grown either in full growth medium (10% FCS); track 1, in serum-free medium, track 2; in serum free medium with acidic and basic FGF added, track 3; with IGF I and II added, track 4 or with both FGFs and IGFs added in combination, track 5 as measured by RT-PCR. The variation of the RNA levels are indicated by the differences in the L6 ribosomal PCR product (C).

4.1.5 Effects of serum removal and growth factors on cell proliferation, survival and morphology

To correlate the changes in Brn-3 expression with changes in the ND7 cells the effect of serum removal and addition of individual or combination of growth factors on cellular proliferation, survival and process formation were investigated. The ability of these cells to exclude trypan blue was taken as a measure of cell survival (Howard *et al.*,1993). In these experiments addition of growth factors had no effect on the cell survival (figure 4.1.7A). There was no effect on cell proliferation either since the total cell number remained unchanged indicating that the growth factors on their own was not sufficient to induce cells to divide in reduced serum or serum free media (figure 4.1.7B). However the growth factors in combination, but not individually, appear to affect neurite outgrowth in these cells since a decrease in the number of cells with processes was observed when growth factors were added to low serum (0.5%) or serum free media compared with those without the growth factors (figure 4.1.7C).

These results therefore suggest that serum removal had opposite and antagonistic effects on the expression of Brn-3a and Brn-3b which is not associated with cell survival or proliferation. However, conditions which resulted in changes to neurite outgrowth on these cells correlated with changes in Brn-3a expression. Although the addition of the FGFs and IGFs did not result in gross morphological changes it is possible that the may trigger pathways involved in the regulation of Brn-3a transcription which are not yet clear. The regulation of Brn-3b mRNA does not appear to correlate with any specific changes in the ND7 cells.

(A)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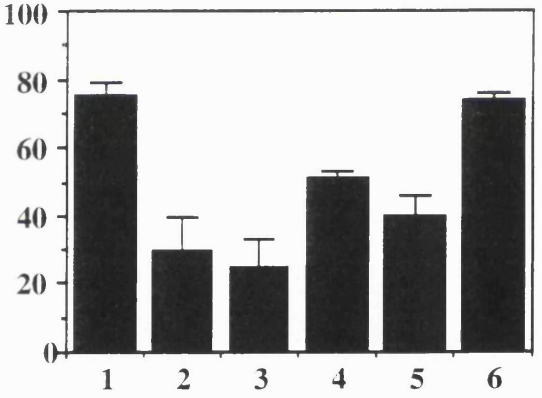
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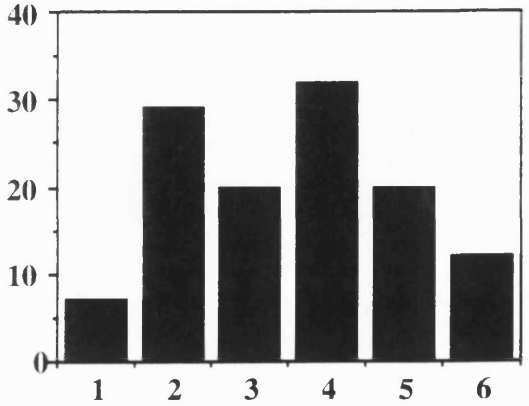
(C)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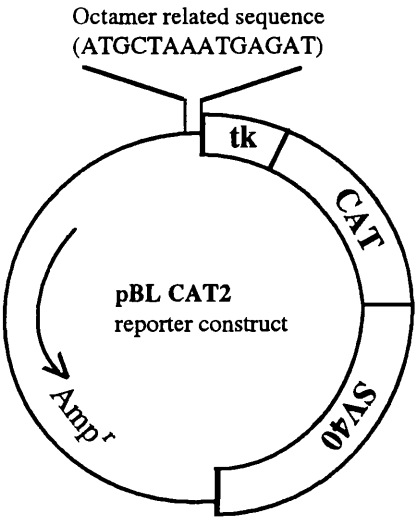
4.2.0 Functional activity of Brn-3a and Brn-3b: effect on gene activity via an octamer motif

To understand the significance of the changes in the Brn-3a and Brn-3b mRNA expression observed in ND7 cells, the effect of these transcription factors on the promoter activity was studied. An artificial promoter under the control of the octamer-related DNA motif, ATGCTAATGAGAT, which was shown to be recognised and bound by the POU domain of Brn-3a and Brn-3b (Theil *et al.,* 1993) was used for initial studies. The reporter construct which contained this synthetic octamer related motif cloned upstream of the herpes simplex virus thymidine kinase promoter in the vector pBLCAT 2 (figure 4.2.1A) was co-transfected with expression vectors containing the full length cDNA of Brn-3a and Brn-3b (figure 4.2.1B) into BHK-21 fibroblast cells which expressed little endogenous Brn-3 proteins (Lillycrop, personal communication). CAT assays were performed and normalized using the results of measurements of the DNA uptake carried out as described in the methods.

The results shown in figure 4.2.2 are representitive of the promoter activity observed in a number of similar, independent experiments. When the Brn-3a expression plasmid was co-transfected with this promoter construct, a high level of CAT activity was observed compared with the control suggesting strong stimulation of the promoter. This increased activity was dependent on the octamer motif since no activity was detected upon co-transfected of the empty reporter construct (pBL CAT2) the Brn-3a expression vector. In contrast, Brn-3b appeared to result in some repression of activity to a level below that obtained on transfection with the vector only.

(A)



(B)

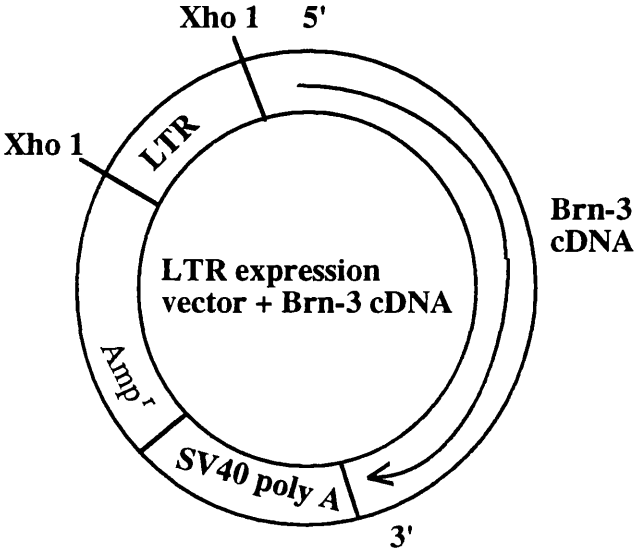


Figure 4.2.1: Schematic representation of the plasmids used for transfection assays. (A) represent the reporter construct containing the binding site for the Brn-3 proteins cloned upstream of the thymidine kinase promoter that drives the expression of the CAT gene. (B) represent the LTR poly A expression plasmid into which the Brn-3 cDNA were cloned.

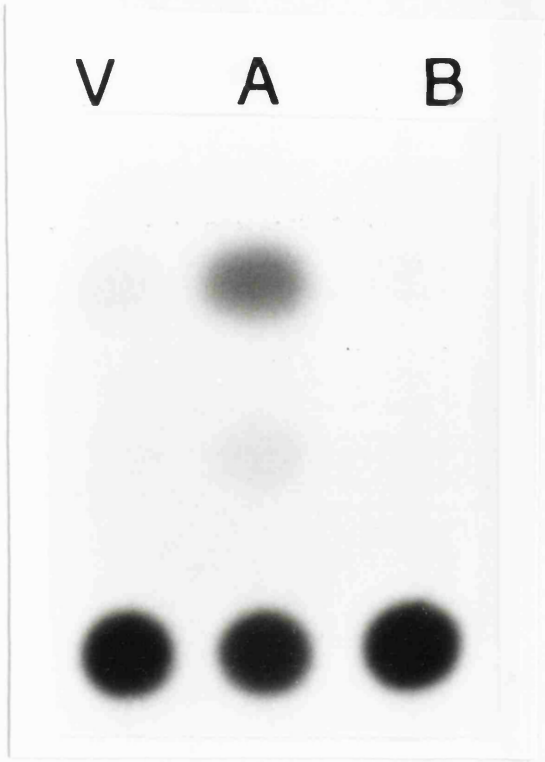


Fig. 4.2.2 : Assay of chloramphenicol acetyl transferase (CAT) activity in BHK cell line with a reporter construct containing the motif ATGCTAATGAGAT shown to bind Brn 3 with either the empty vector (V), or the vector containing Brn 3a cDNA (A) or Brn 3b(s) gene (B). The values indicate the percentage of chloramphenicol acetylated in each transfection and hence the promoter activity..

4.2.1 Effect of Brn-3a and Brn-3b on promoter activity in ND7 cells

These co-transfection experiments were repeated in the ND7 cells which express constituitive levels of the Brn-3 proteins. The results of the CAT assays demonstrated a similar but more pronounced effect to that observed in the BHK cells upon co-transfection with the Brn-3a expression construct resulting in an increase in the promoter activity and the Brn-3b protein causing a decrease in the promoter activity (figure 4.2.3). The similarity in the effect of the Brn-3 proteins on promoter activity in these cell lines (which differ in endogenous expression of Brn-3 proteins) indicate that the response is likely to result from direct interaction of the Brn-3 proteins with the promoter sequence and did not require cell specific factors to initiate the transcriptional effect.

Interestingly, co-transfection of both Brn-3a and Brn-3b with the target promoter resulted in a loss of the stimulation seen with Brn-3a only (figure 4.2.4). These results confirmed that the effect observed upon transfection with Brn-3b expression plasmid was associated with functional Brn-3b protein expression since at present there are no antibody to Brn-3a or Brn-3b factors available to facilitate the measurement of functional proteins expressed in the transfected cells. However, this finding also suggested that there is interaction between the Brn-3a and Brn-3b proteins on this octamer motif which modulates promoter activity.

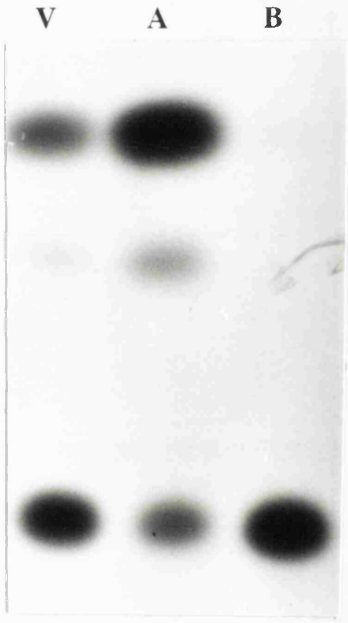


Fig. 4.2.3 : CAT assay showing the promoter activity in extracts obtained from ND7 cell transfected with the reporter construct containing the octamer motif, ATGCTAATGAGAT, (oligo A) shown to bind the Brn 3 proteins with either the empty vector (V), or the vector containing Brn 3a cDNA (A) or Brn 3b(s) gene (B). The values indicate the percentage of chloramphenicol acetylated in each case.

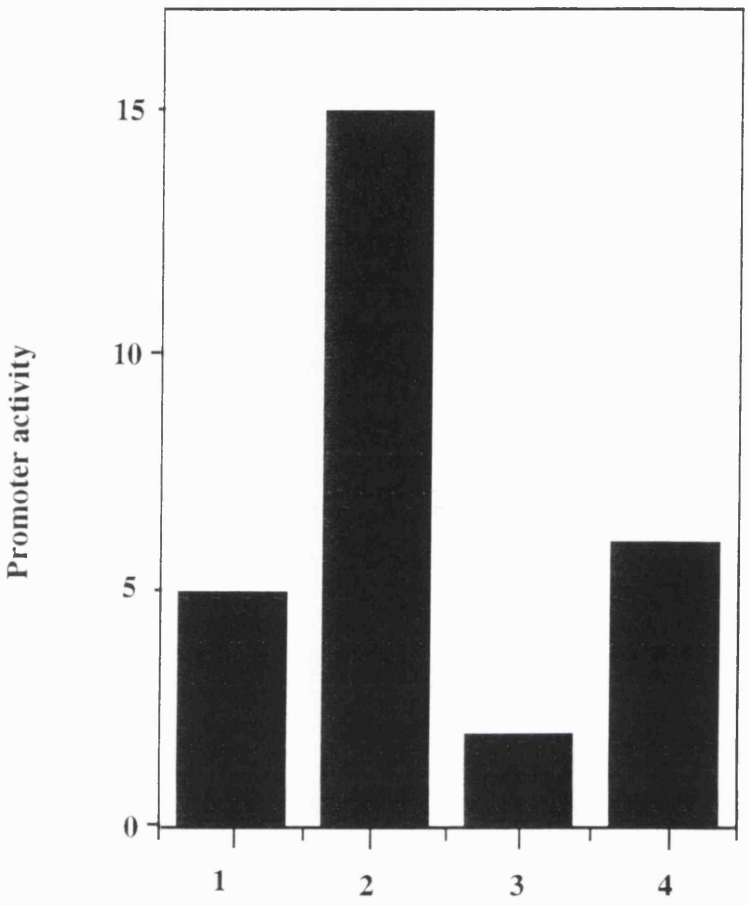


Figure 4.2.4: Quantitation of a typical CAT assay in which the reporter construct containing the Brn-3 binding site (oligo A) was transfected with either empty vector (track 1), Brn-3a (track 2), Brn-3b (track 3) or with both Brn-3a and Brn-3b proteins together (track 4) into ND7 cells. The values represent the percentage of chloremphenicol converted in the extracts. The different samples were equalized for protein levels and DNA uptake.

4.2.2 Increased Brn-3a expression increases CAT activity via the octamer motif in ND7 cells

The observation that co-expression of the Brn-3a protein increased the activity of the promoter through the octamer site suggested that conditions which changed the ratio of Brn-3a and Brn-3b in ND7 cells should also cause a change in the promoter activity. As shown previously, cells grown in serum-free medium or growth medium supplemented with cyclic AMP showed increased Brn-3a mRNA expression and should therefore also modulate the promoter activity. To test this, the reporter construct containing the octamer motif was transfected into ND7 cells which were either grown in FGM containing 10% FCS, in serum-free (differentiation) medium or in FGM containing 10% FCS and 1mM cyclic AMP. Control cultures which were grown in similar conditions were co-transfected with the unmodified vector, pBLCAT 2.

Assay for CAT activity showed a clear stimulation of the promoter activity in the cells grown in serum-free medium (figure 4.2.5) or in the FGM containing cyclic AMP (figure 4.2.6). This activity was dependent on the presence of the octamer motif since no activity was observed when the vector control, pBL CAT2 was transfected in similar conditions. The effect paralleled the activity observed upon co-transfection of this promoter with the Brn-3a expression construct.

When this reporter construct was transfected into BHK cells which were grown in the absence or presence of cyclic AMP there was no basal promoter activity and no increase on cyclic AMP treatment. These findings therefore support the observation that BHK cells expressed no endogenous Brn-3 proteins, and that the effects observed in ND7 cells in the absence of transfected Brn-3 proteins must be attributed to factors expressed in ND7 cells and which were modulated by cyclic AMP or the serum-free conditions.

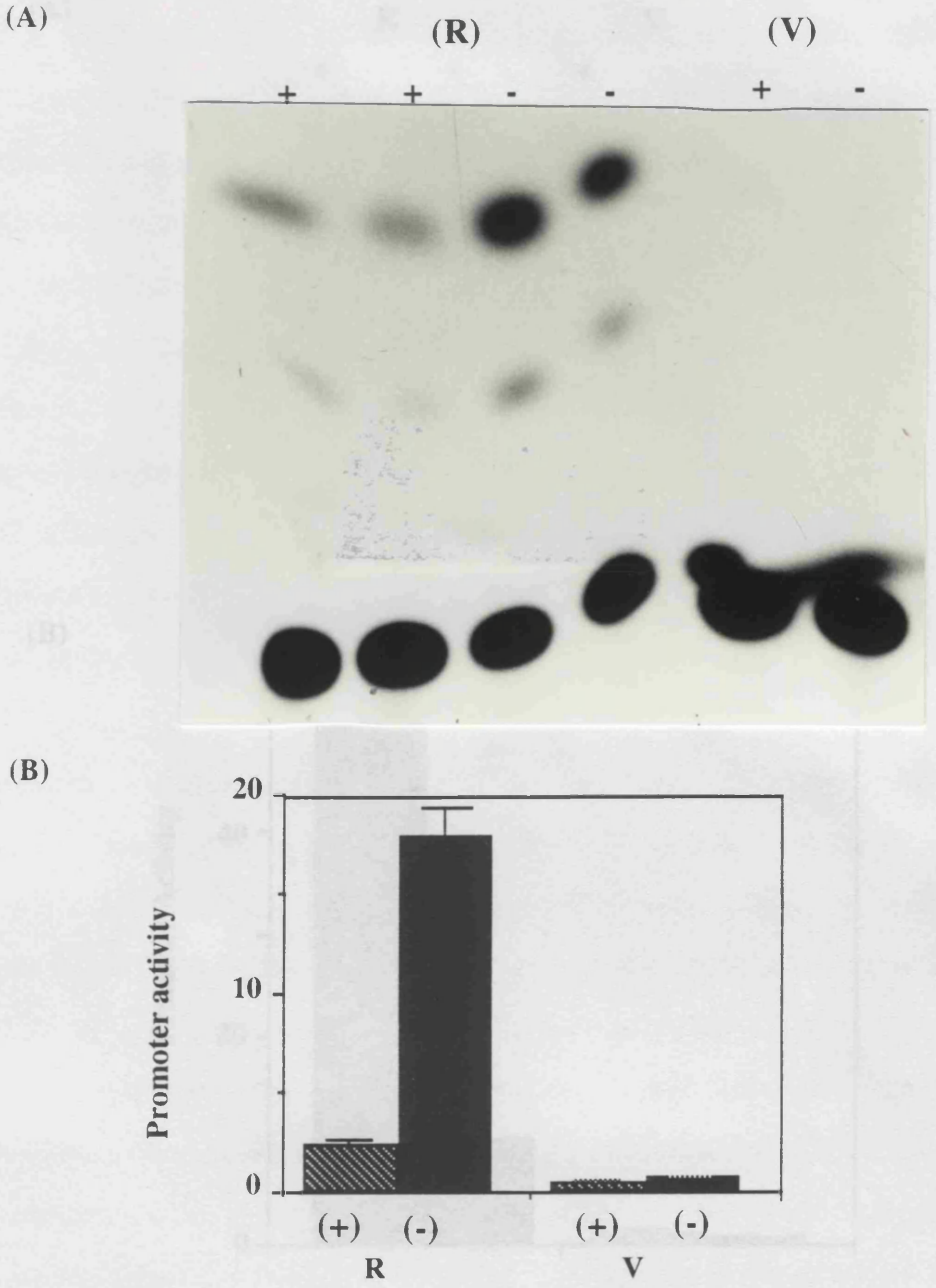


Figure 4.2.5: (A) Assay of CAT activity of ND7 cells grown in the presence (+) or absence (-) of 10% foetal calf serum and transfected with either the plasmid vector pBLCAT2 (V) or this plasmid containing the Brn-3 binding site, oligo A.
(B) Levels of CAT activity in replicate experiments using the empty vector (V) or the reporter plasmid containing the Brn-3 binding site (R) in the presence (+) or absence (-) of 10% serum.

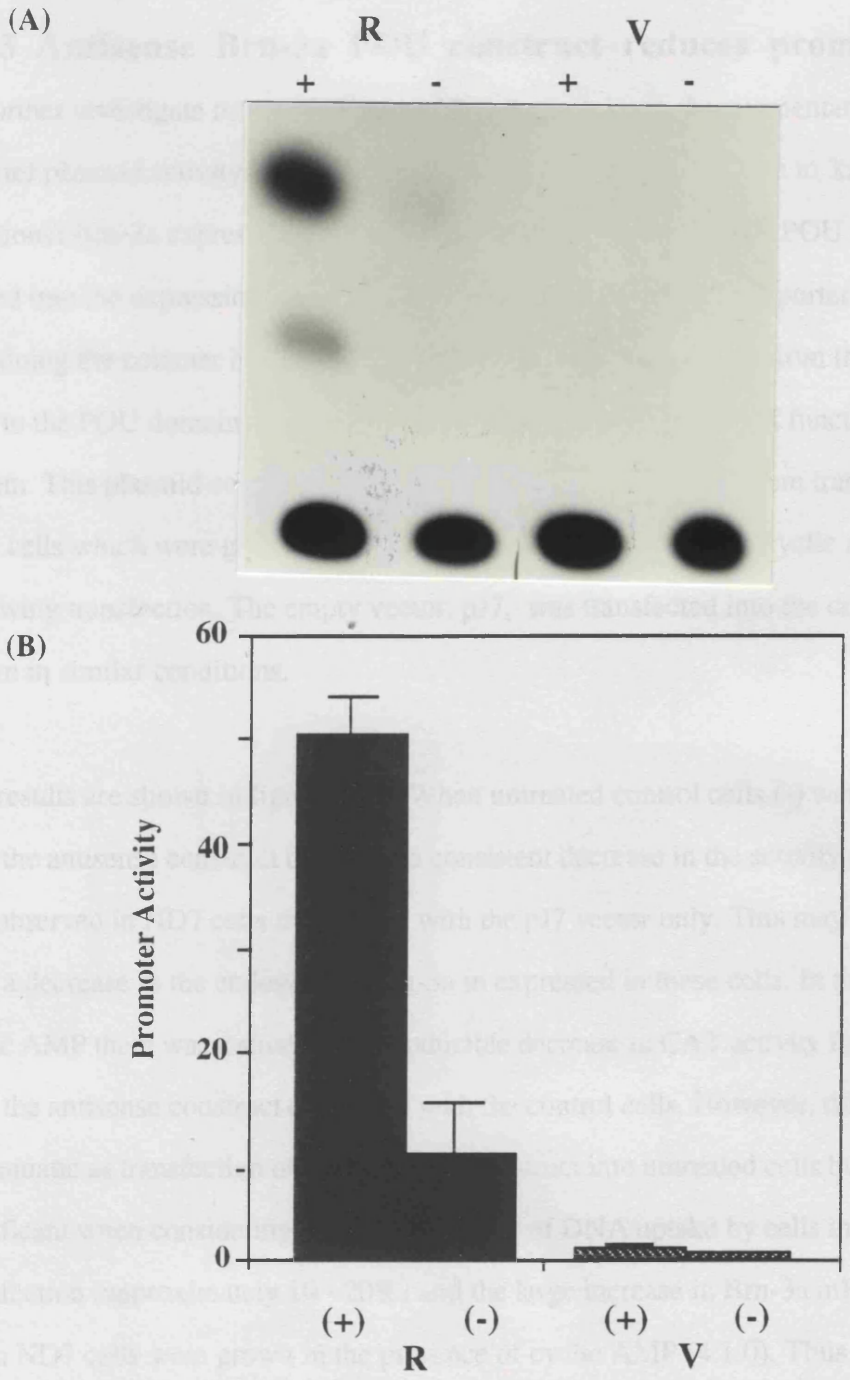


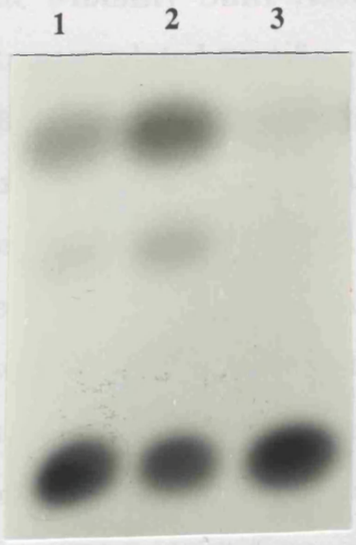
Figure 4.2.6: (A) Assay of CAT activity of ND7 cells grown in the presence (+) or absence (-) of 1mM cAMP and transfected with either the empty vector (V) or the reporter plasmid containing the Brn 3 binding site (oligo A). (B) Promoter activity from four experiments in which ND7 cells were transfected with vector (V) or reporter construct (R) in the presence (+) or absence (-) of 1mM cAMP.

4.2.3 Antisense Brn-3a POU construct reduces promoter activity

To further investigate the involvement of Brn-3a proteins in the augmentation of the reporter plasmid activity via the octamer motif, an attempt was made to 'knock-out' functional Brn-3a expression. The antisense sequence to the Brn-3a POU domain was cloned into the expression vector, pJ7 and co-transfected with the reporter construct containing the octamer binding site. Antisense transcripts resulting from this vector should bind to the POU domain of the mRNA and prevent the translation of functional Brn-3a protein. This plasmid containing this antisense sequence was therefore transfected into ND7 cells which were grown in full growth media with or without cyclic AMP added following transfection. The empty vector, pJ7, was transfected into the control cells and grown in similar conditions.

The results are shown in figure 4.2.7. When untreated control cells (-) were co-transfected with the antisense construct there was a consistent decrease in the activity compared with that observed in ND7 cells transfected with the pJ7 vector only. This may be associated with a decrease in the endogenous Brn-3a in expressed in these cells. In the presence of cyclic AMP there was a small but reproducible decrease in CAT activity in cells transfected with the antisense construct compared with the control cells. However, this effect was not as dramatic as transfection of the antisense construct into untreated cells but may be significant when considering the low efficiency of DNA uptake by cells in transient transfection (approximately 10 - 20%) and the large increase in Brn-3a mRNA observed when ND7 cells were grown in the presence of cyclic AMP (4.1.0). Thus the uptake of the antisense Brn-3a POU construct may not be sufficient to eliminate all of the proteins resulting from the large increase in the Brn-3a mRNA in response to cyclic AMP. These results thus support the previous observations that Brn-3a may be directly involved in the activation of promoter via the octamer related sequence.

(A)



(B)

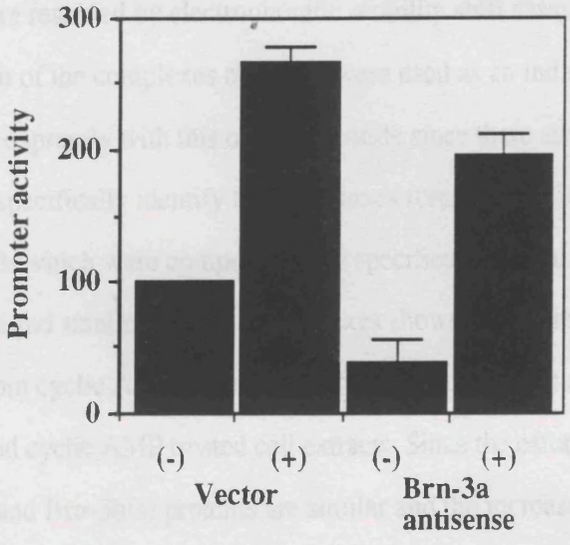


Figure 4.2.7: (A) Assay of CAT activity in ND7 cells transfected with the reporter construct containing the Brn 3 binding site and with expression vector, pJ7, alone in FGM (1), pJ7 in FGM + 1mMcAMP (2) or with pJ7 containing the Brn 3a POU domain sequence in the opposite orientation to produce antisense Brn 3a POU transcripts, (3). (B) The mean of three CAT assays using extracts from ND7 cells transfected with empty pJ7 vector in the absence (-), or presence of cAMP (+) and the construct expressing antisense Brn3a POU in the absence (-) or presence (+) of cyclic AMP.

4.3.0 Electrophoretic Mobility Shift Assay with ND7 cell extract and the octamer related motif

The ability of Brn-3a and Brn-3b to regulate the activity of a reporter plasmid via an octamer related motif in both ND7 cells and BHK cells suggested that these proteins must bind directly to this octamer related sequence. Theil *et al.*, (1993) showed that the Brn-3a and Brn-3b POU domain bind this octamer motif. To investigate if the changes in the levels of Brn-3a upon treatment with cyclic AMP could be detected by the changes in the binding of this octamer sequence, total cellular extracts were obtained from cells grown in the absence and presence of 1mM cyclic AMP and used in binding assays as described in the methods. Following incubation with end labelled oligonucleotide probe the complexes formed were resolved by electrophoretic mobility shift assay. Sequence specific competition of the complexes observed were used as an indication of the levels of proteins interacting expressly with this oligonucleotide since there are no antibodies available at present to specifically identify the complexes formed with Brn-3a and Brn-3b proteins. Three bands which were competed away specifically by unlabelled primers were observed. The largest and smallest of these complexes showed an increase in intensity in the cell extracts from cyclic AMP treated cells (figure 4.3.1). There may be a small decrease in the middle band cyclic AMP treated cell extracts. Since the estimated molecular weight of the Brn-3a(s) and Brn-3b(s) proteins are similar and the increase in Brn-3a levels is accompanied by the concomitant decrease in Brn-3b protein there will be little overall change in the total concentration of these two proteins. In addition, while both isoforms of both Brn-3b (35 kDa and 43 kDa) were detected in ND7 cells the short isoform of Brn-3a (35 kDa) predominate in these cells (Liu and Latchman- unpublished results). This may suggest that the middle band which showed a small decrease may correspond to the Brn-3b(l) complex, with the smaller complex resulting from binding of Brn-3a(s) and any Brn-3b(s) present. This identity of the largest band which appeared to be increased upon cyclic AMP treatment as well as these smaller complexes remains to be confirmed

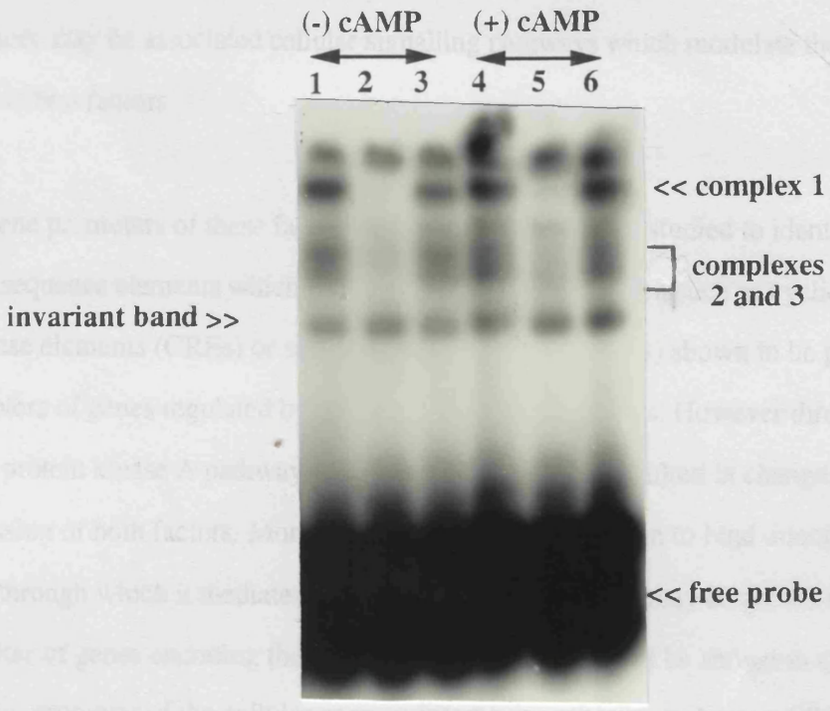


Figure 4.3.1 : Electrophoretic Mobility Shift Assay (EMSA) of the complexes formed when theBrn-3 binding octamer sequence (oligo A) was incubated with cellular extract obtained from ND7 cells in the absence (lanes 1-3) and presence of cyclic AMP (lanes 3 - 6). There appear to be three bands which are specifically competed away with the unlabelled 'cold' oligonucleotide while the smallest band remains unchanged.

4.4.0 Discussion

The expression of the mRNA of Brn-3a and Brn-3b are oppositely regulated in ND7 cells such that, in serum-free conditions which results in differentiation of the cells to a neuronal phenotype or in the presence of cyclic AMP, Brn-3a mRNA is upregulated while Brn-3b transcripts show a decrease compared with the proliferating cells. It is therefore possible that there may be associated cellular signalling pathways which modulate the transcription of these two factors.

The gene promoters of these factors are yet to be cloned and studied to identify specific DNA sequence elements which may modulate their expression such as cyclic AMP response elements (CREs) or serum response element (SREs) shown to be present in the promoters of genes regulated by cyclic AMP or serum factors. However direct stimulation of the protein kinase A pathway by dibutyryl cyclic AMP resulted in changes in the expression of both factors. Moreover, the ability of the protein to bind directly to the DNA motif through which it mediates this effect suggest that there may be a CRE site in the promoter of genes encoding these factors. In addition, as will be shown in chapter 5, the minimal promoter of the cellular gene, α-internexin, contains no known CRE but it is strongly activated in the presence of cyclic AMP in ND7 cells but not BHK cells. A DNA element shown to bind Brn-3a is present in this promoter supporting the idea that cyclic AMP directly affects the transcription of Brn-3a and allows it to modulate the activity of its target gene promoters.

Serum factors also regulate the expression of Brn-3a and Brn-3b. However, Brn-3a mRNA levels appear to correlate more closely with changes in the morphology of the cells with high levels detectable when the cells are induced to put out processes while Brn-3b expression may be more sensitive to serum factors since the changes in serum concentration in the growth media resulted in changes in the Brn-3b mRNA level detected.

Growth factors such as acidic FGF, basic FGF, IGF I and II, which have been shown to act as trophic factors for some neuronal cells were found to modulate the expression of Brn-3a individually and in combination while Brn-3b requires the combinatorial effect of a number of these growth factors to synergistically modulate its expression. The ability of the individual FGFs and IGFs to regulate the expression of Brn-3a but not Brn-3b may indicate different requirements for the expression of these two factors under some conditions.

The addition of cyclic AMP to full serum media which result in changes in the levels of the Brn-3 mRNA, also induces process formation suggesting that the pathway mediating this effect overrides the factors proposed to inhibit neurite outgrowth in serum (Schwab *et al.*, 1993). This process occured without significant changes in total cell numbers which accompany differentiation in serum free media as a result of apoptosis. Howard *et al.*, (1993) had previously shown that transfer of ND7 cells to serum free media which was supplemented with 1mM cyclic AMP protected the cells from apoptosis which occured in cells transferred to serum-free media only. Cells differentiated in serum free media arrested in G_0 /G_1 phases of the cell cycle but in the presence of cyclic AMP there were a larger number of cells undergoing cell division. Cyclic AMP therefore appear to regulate pathways involved in cell proliferation, as well as differentiation and cell survival. The modulation of Brn-3a and Brn-3b by cyclic AMP and its specific role in this process will need to be examined further. However, Brn-3a is expressed at higher levels in differentiated neuronal cell while Brn-3b is more characteristic of the proliferating cell type and conditions which induce process formation in ND7 cells also resulted in changes in the expression of these two factors regardless of the pathway through which this is effected. It is therefore possible that Brn-3a expression may be associated with the mechanism that mediate neurite outgrowth from ND7 cells. The results of recent studies carried out by Lakin *et al.*, (1995) supported this theory. In these experiments a construct expressing the

antisense strand of Brn-3a POU domain under the control of a glucocorticoid - inducible MMTV promoter was transfected into ND7 cells. When cell lines stably expressing this construct were treated with dexamethasone the promoter was inducedto produce the antisense cRNA which effectively reduced the expression of Brn-3a and a clear decrease in the levels of the endogenous Brn-3a mRNA was observed. The cells which were subjected to this treatment showed deficient neurite outgrowth compared with control cells when differentiated by transfer to serum free media. Measurement of other parameters such as DNA synthesis and cell survival indicated that these cells were not dividing and were as viable as the control cells thus suggesting that inhibition of Brn-3a synthesis was closely associated with reduced neurite outgrowth. In addition the level of the neuronal protein SNAP-25, was also lower in these cells. The SNAP-25 promoter was shown to be activated by Brn-3a indicating that the changes in levels observed may be associated with the decreased Brn-3a expression in this cell line.

This overlapping expression of Brn-3a and Brn-3b in the neuronal cell line was paralleled by the ability of Brn-3a to activate the transcription of a synthetic promoter via an octamer-related motif while Brn-3b represses this activity. In addition, factors which increase the levels of the activator, Brn-3a, in ND7 cells such as differentiation or the presence of cyclic AMP also resulted in an increase in the promoter activity and this effect can be partially reversed by 'knockout' of Brn-3a using an antisense POU domain construct.

These two transcription factors also appear to interact to modulate the activity of the target promoter providing a possible strategy for regulating the promoter activity of target genes by these factors which are co-expressed in some tissues and cell types. This mechanism, which utilizes specific protein-protein interactions provides a powerful method of regulating gene activity since it requires combinatorial expression and interaction to specify the genes which are expressed in a cell. This has been demonstrated with a number of other

POU-domain transcription factors [Struhl, 1991; Verrijzer *et al.*,1992(i); Treacy and Rosenfeld, 1992; Verrijzer and van der Vilet, 1993].

Thus, it is possible that high expression of the repressor form of Brn-3b may interact with and downregulate the activator, Brn-3a. However, in response to extracellular signals which induces differentiation, for example, the changes in the relative levels of these two proteins will allow the predominant Brn-3a to activate transcription of target genes. This model is consistent with the effect of the long or short isoforms of Brn-3a, Brn-3a(l) or Brn-3a(s), and the short isoform of Brn-3b(s) observed on transcriptional activity by our group as well as on transformational capacity reported by Theil and Moroy, (1994). However while all reports to date have shown Brn-3b to be a repressor of promoter activity, the long isoform this gene, Brn-3b(l), has been shown to be an activator of the CRH promoter. Its expression pattern and interactions with the Brn-3a isoforms remains to be established. Thus, the existence of multiple forms of these proteins which may vary in their expression, their ability to interact and modulate the functions of each other may provide a powerful mechanism for their control of promoter activity in cells which co-express these factors.

Chapter 5

Results

Regulation of cellular gene promoters by Brn-3a and Brn-3b

5.0.0 Background

5.0.1 Promoters regulated by Brn-3a and Brn-b

Although the natural target gene promoters regulated by the Brn-3 proteins remain to be conclusively established, studies by a number of groups have identified known DNA motifs which are recognised and bound by the Brn-3 proteins with varying affinities. Result published by Theil *et al.,* (1993) demonstrated that the binding characteristics of Brn-3a, Brn-3b and Brn-3c are similar, with all three proteins showing high affinity binding to an IgHep oligonucleotide sequence (5'- GGTAATTTGCATTTCTAA-3') which consisted of an octamer motif flanked by a heptamer. Mutation of either the heptamer or octamer sequences resulted in diminished binding indicating that there may be cooperative binding on the two sites by the Brn-3 proteins. There was lower affinity binding to a single wild-type octamer sequence (5'-GGTAATTTGCATTTCTAA-3') or the HSV derived octamer motif (5'-GCATGCTAATGATATTCTTT-3').

Results of the experiments discussed in the previous chapter showed that Brn-3a and Brn-3b can bind to an octamer related sequence (oligo A), derived from the IgG promoter to modulate the activity of an artificial promoter in the reporter plasmid. In co-transfection studies, Brn-3a activated transcription of this promoter while Brn-3b(s) repressed basal promoter activity. In addition, Morris *et al.*, (1994) showed that these proteins could modulate the expression of a reporter construct via an octamer related motif, ATGCAATT, (referred to as PAP3/4), derived from the human papillomavirus promoter in a manner similar to that observed with the oligo A motif. However, Gerrero *et al.*, (1993) reported poor activity via octamer related sequences in the corticitroph cell line, AtT20, where low levels of Brn-3a mRNA was detected. It is therefore possible that cell specific factors also influence the affinity of these proteins for specific sites and hence affect the activity of these proteins. This group also observed that Brn-3a regulated the activity of the corticotrophin

releasing hormone (CRH) promoter in these cells. The putative DNA sequence element that was recognized by Brn-3a in this promoter was shown to be similar to the canonical sequence recognised by Brn-2, a member of the POU III subfamily (Li *et al.*, 1993). The DNA motif recognised by Brn-3a in the CRH promoter was shown to have a core of 5'-GCATAAATAAT-3'. Similar sequences were also present in the CE2 sites of the pro-opiomelanocortin (POMC) gene promoter which was also reported to be regulated by Brn-3a and Brn-3b proteins with Brn-3a(l) (Gerrero *et al.*, 1993) and Brn-3b(l) (Turner *et al.*, 1994) being capable of activating the transcription of reporter constructs via this motif.

Functional analysis of the different domains of the Brn-3a and Brn-3b indicated that the amino terminus of Brn-3a(l) was necessary for transformation of fibroblast cells *in vitro* while the short isoforms, Brn-3a(s) and Brn-3b(s) which lack amino acids in this region could not transform cells but interfered with the ability of long Brn-3a(l) to transform cells (Theil *et al.*,1993, Theil and Moroy, 1994). In addition, results of work in our laboratory showed that Brn-3b could antagonize the stimulatory effect of Brn-3a on an octamer related motif, PAP3/4, (Morris *et al.*, 1994). Using chimeric constructs in which the different domains of Brn-3a and Brn-3b were interchanged (see figure 5.2.1) it was shown that the difference in the transactivation capacity of these two factors via this synthetic octamer motif was dependent upon the carboxy terminal region which contain the POU domain of these two proteins. Changing of this domain from Brn-3a to Brn-3b and vice-versa converted the activator, Brn-3a into a repressor and Brn-3b into an activator.

We were interested in identifying cellular target genes regulated by these proteins as well as looking at the domains involved in the achieving the effects on the transcriptional activity. This would enable us to elucidate the function of the proteins *in-vivo* and to identify the binding site in these promoters. Moreover, identification of cellular promoters with the binding site for Brn-3 proteins would allow a greater understanding of the interactions

required for transcriptional modulation by these factors since the binding site cloned in isolation may provide information on the effect of these proteins on transcription but did not allow for effects resulting from interactions with cellular factors which also bind to sites in the cellular gene promoters to modulate transcription.

The initial strategy was to study a number of genes which showed overlapping expression with the Brn-3 proteins in neuronal cells and whose promoters were known to contain one or more octamer related motif. Some candidate promoters with octamer related sequences which were investigated in this study included the promoters of the intermediate filament protein, α-internexin, the neuronal protein, GAP-43, the L1 adhesion molecule and the pro-opiomelanocortin (POMC) gene promoters. In addition, the effect of the Brn -3a and Brn-3b protein on transcription of other genes whose promoters contained octamer related DNA binding site such as SNAP-25, calcitonin gene related peptide (CGRP) or tyrosine hydroxylase (TH) were also investigated by others. Neither Brn-3 proteins affected the activity of the promoters of genes encoding either CGRP or TH despite the presence of octamer related motifs (Dawson and Latchman, unpublished observations). There was weak activity on the promoters of L1 and GAP43 genes which will be discussed later. In contrast these proteins strongly modulated the activity of the α-internexin promoter (see below) and SNAP 25 promoter (Lakin *et al.*, 1995).

Much of the results presented in this chapter reports on the regulation of the α-internexin gene promoter by the Brn-3 proteins. As such a brief description of the α-internexin gene product and its expression will be given. In addition, the POMC, gene promoter was used to study the effect of the different Brn-3 proteins on a promoter with a recognition sequence that was distinct from the octamer related motif. The regions of the promoter of this gene which interact to mediate transcription will also be described briefly.

5.0.2 The α-internexin gene

The promoter of the the α-internexin gene was considered as a potential candidate gene because of the presence of three octamer related sequences upstream of the start site of transcription (figure 5.1.1) and its pattern of expression in neuronal cells. The α-internexin gene encodes a neuronal intermediate filament protein which was originally identified in the rat spinal cord and optic nerve (Pachter and Liem 1985). It is an axonal protein found in most neurons of the nervous system and is one of the earliest intermediate filament protein to be expressed in the developing neurons of the central and peripheral nervous system with early detection in the postmitotic outer layer of the neural tube.

In the rat nervous system the expression pattern of α-internexin mRNA was studied using in-situ hybridization. The mRNA was first detected by e10 in specific tisssues such as the ganglion of the eight cranial nerve and in the myelencephalon (Fliegner *et al.*, 1994), when the first neurons in this region are generated (Altman and Bayer, 1980). By e12, the mRNA was localized to the sensory and sympathetic ganglia, the brainstem, the basal forebrain and the spinal cord as well as in the telencephalon where neurogenesis is just beginning at this stage. By e16, α-internexin was abundant throughout most of the telencephalon, the retina, the basal and peripheral ganglia, the sympathetic ganglia, dorsal root ganglia and ventral horn of the spinal cord. In the adult rat there was lower expression of this protein in both the central and peripheral nervous systems. High levels were found in the granule layer of the cerebellum, the mylencephalon, the hindbrain, the red nucleus of the trigeminal ganglia and the spinal cord. A comparison of the expression patterns of this protein with that of Brn-3a and Brn-3b shows extensive overlap since both Brn-3a and Brn-3b were found in regions of the thalamus, mesencephalon, hindbrain and retina as well as in the sensory neurons of the trigeminal and dorsal root ganglia and the spinal cord during development and in adult tissues (see chapter 1).

5.0.3 Pro-Opiomelanocortin (POMC) gene promoter

The POMC gene product is the precursor molecule for pituitary hormones such as the adrenocorticotropin hormone (ACTH), melanocyte stimulating hormone, (MSH) and β-endorphine and is expressed early in pituitary development in corticotroph cells of the anterior pituitary (Droin *et al.*, 1991). The POMC mRNA was detected before the expression of many other pituitary hormones such as the growth hormone and prolactin and the Pit-1 transcription factor which regulates their expression (Lugo *et al.*, 1989). Furthermore Pit-1 has restricted distribution to lactotroph and thyrotroph cells and was not detected in the corticotroph or gonadotroph where POMC transcripts were identified suggesting that different factors may be involved in the regulating POMC expression. Low levels of Brn-3a were reported in the pituitary and in the pituitary derived cell line, AtT20, which expresses POMC transcripts (Gerrero *et al.*, 1993).

Study of the elements in the POMC promoter suggested that the synergistic interaction of different trans-acting factors which bind to multiple regulatory elements are required for transcriptional activation (Therrien and Drouin, 1991). Three distinct domains which contribute to transcriptional activity of this promoter have been identified as the proximal, central and distal domains containing the proximal elements, 1 - 4 (PE-1 to PE-4), the central elements, 1 and 2 (CE-1 and CE-2) and the distal elements, 1 - 3 (DE-1 to DE-3), respectively. Many of these elements bind distinct factors in cellular extract.The distal and central domains appear to act in synergy for transcriptional activity . Mutations in the CE-1 and CE-2 sites also resulted in decreased activity, thus emphasizing their requirement for transcriptional activity. Gerrero *et al.*, (1993) had also previously shown that this promoter was activated by Brn-3a(l) and that a heterologous promoter with multiple central elements (CE-2) sites were sufficient for the binding and activation by this transcription factor. This therefore suggested that the DNA binding site for Brn-3a may be localized to the CE-2 sites.

Results:

5.1.0 Regulation of cellular promoters by Brn-3 proteins

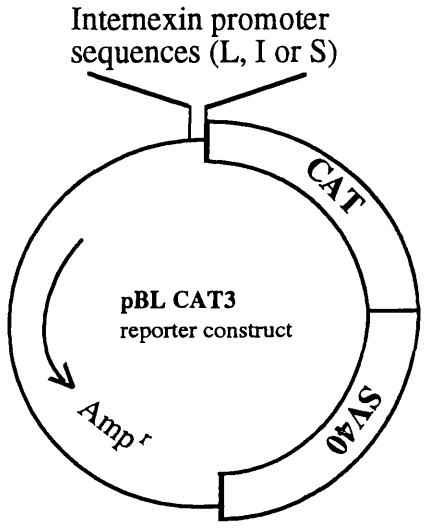
Preliminary study of the effect of the Brn-3a, Brn-3b and Brn-3c proteins on the full length α-internexin gene promoter containing the three octamer related motifs was carried out using the promoter construct (-1219 to +73) in which the promoter was cloned into the empty CAT vector, pBLCAT 3, upstream of the chloremphenicol acetyl transferase (CAT) gene (figure 5.1.1). Similar constructs containing the full length promoters of the L1 and GAP-43 genes were also used since these promoters also contained octamer related sequences. These constructs were co-transfected with the expression vectors containing full length cDNAs encoding the Brn-3 proteins. Transfections were carried out in BHK fibroblast cells which lack endogenous Brn-3 expression and in ND7 cells which constituitively express the Brn-3a, Brn-3b and Brn-3c proteins.

In these experiments Brn-3a and Brn-3c strong activated the α-internexin promoter while Brn-3b repressed basal promoter activity in both ND7 cells [figure 5.1.2(i)) and in BHK cells (figure 5.1.2(ii)] but the Brn-3 protein had little effect on the L1 or GAP-43 promoters [figure 5.2.2 (iii)]. This effect of Brn-3a and Brn-3b on transcriptional activity on the α-internexin promoter was similar to the effect observed upon co-transfection of the Brn-3 expression vectors with the synthetic octamer binding motif (oligo A) (chapter 4). As before, the effects appear much stronger in ND7 cells which may be associated with the presence of endogenous Brn-3 proteins and other neuronal specific factors that augment the expression and activity of these transcription factors. Thus, Brn-3a and Brn-3c can activate the transcription of this cellular promoter in the same way as a heterologous promoter containing their binding site in isolation while Brn-3b acted as a repressor of promoter activity, indicating that these proteins affect transcription in a similar manner regardless of the binding site being in isolation or within the context of a promoter which contains sites for the other proteins.

Figure 5.1.1: Schematic representation internexin constructs used for transfection assays. (A) represent the reporter construct containing the internexin promoters cloned into the pBLCAT3 plasmid so that it drives the expression of the CAT gene.

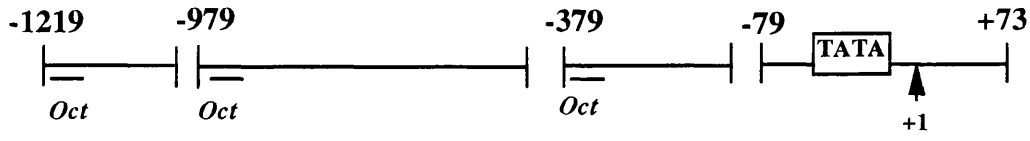
(B) (i) shows the positions of the octamer binding sites in the internexin promoters with relation to the TATA box and the transcription initiation site (+1), while (ii) indicates the lengths of the promoters used for the transfection assays to look at the effect of the Brn-3 proteins on activity and to identify the binding site for Brn-3 in the sequences. (L) represent the full length promoter, (I), the intermediate length and (S), the short, minimal promoter.

(A)

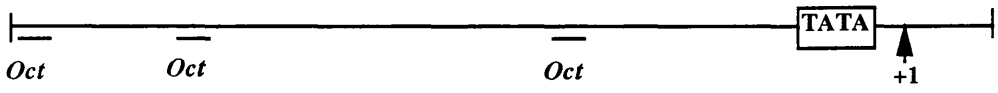


(B) α-Internexin promoter

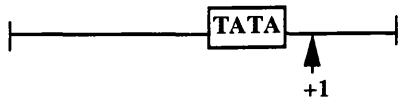
(i)



(ii) (L) (-1219 to +73)



(I) (-254 to +73)

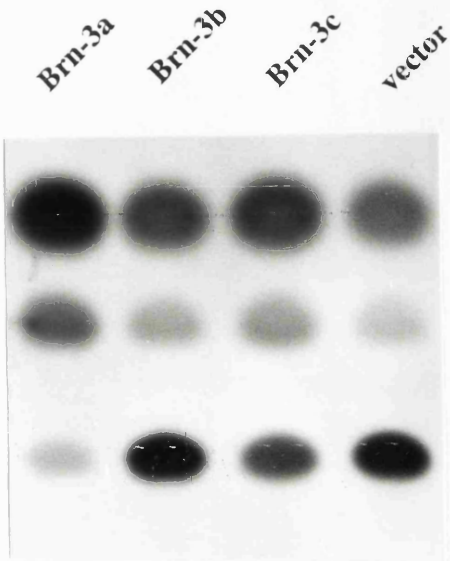


(S) (-79 to +73)

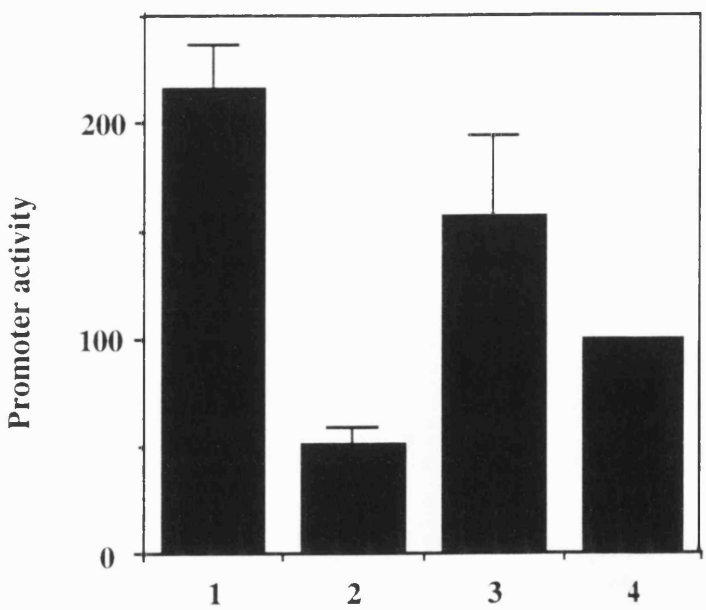
Figure 5.1.2 (i) : (A) Typical CAT assay showing the activity of a reporter construct containing the full length internexin promoter sequence (-1219 to +73 bases) co-transfected with expression vectors encoding Brn-3a, Brn-3b, Brn-3c and vector control.

(B) The result of a number of independent experiments showing the activity of this promoter when co-transfected with Brn-3a (track 1), Brn-3c (track 2) Brn-3b (track 3) and vector control (track 4). Values are expressed as a percentage of the vector control and the standard errors are indicated by the bars.

(A)



(B)



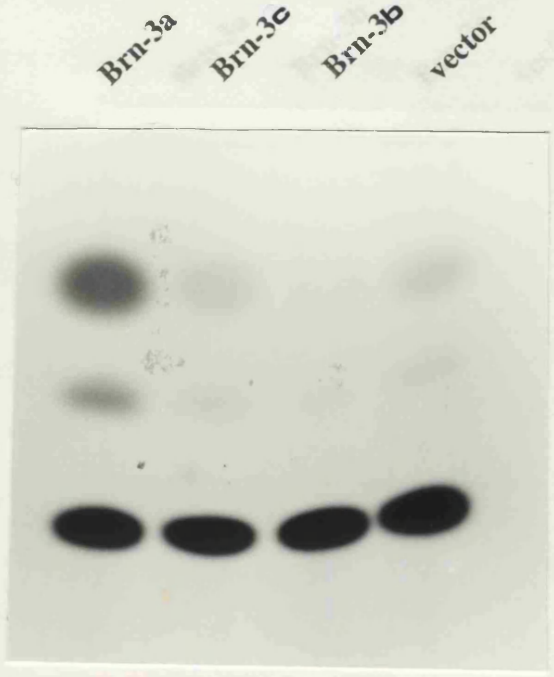
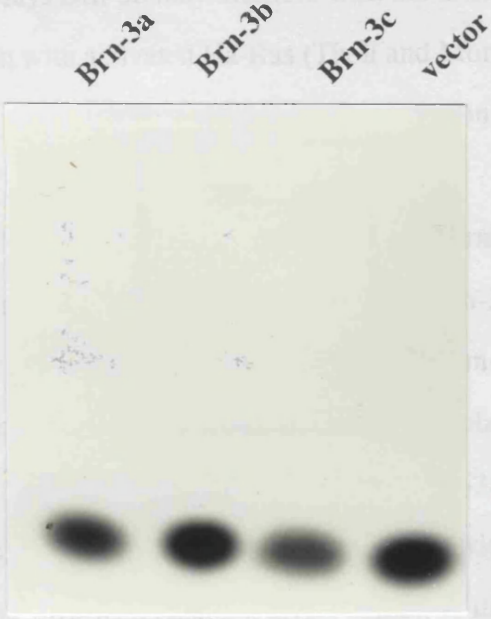


Figure 5.1.2 (ii) : Typical CAT assay showing the activity of a reporter construct containing the full length internexin promoter sequence (-1219 to +73 bases) co-transfected with expression vectors encoding Brn-3a, Brn-3b, Brn-3c and vector control in BHK cells. The lower basal activity in the BHK cells resulted from the lack of endogenous Brn-3 proteins in these cells.

(A)



(B)

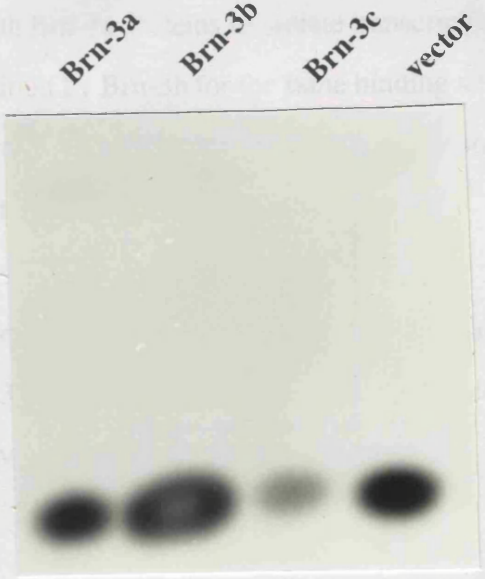


Figure 5.1.2 (iii) : Assay of CAT activity of a reporter construct containing the L1 (A) and GAP-43 (B) promoters when co-transfected with either the Brn-3a, Brn-3b, or Brn-3c expression constructs or the empty vector, into ND7 cells.

5.1.1 Brn-3a and Brn-3b interaction on α-internexin promoter

Previous reports suggested that Brn-3b binds to Brn-3a and prevents it the Brn-3a protein binding the DNA site on the octamer motif with high efficiency *in-vitro* (see chapter 4) whereas in transformation assays Brn-3b may interfere with the ability of Brn-3a to transform cells in conjunction with activated Ha-Ras (Theil and Moroy, 1994) suggesting that the interactions between these two factors modulate the function of the Brn-3a protein.

To study the interactions of these proteins which may affect gene transcription, co-transfection studies were carried out with the Brn-3a isoforms, Brn-3a(l) and Brn-3a(s) and the Brn-3b(s) expression vectors and the full length internexin promoter. The results indicated that Brn-3 proteins also interacted on this promoter to regulate the resultant transcriptional activity by these proteins (figure 5.1.3). When Brn-3a(l) or Brn-3a(s) were co-transfected with Brn-3b(s) there was a loss of the promoter activity mediated by either of the Brn-3a proteins on their own with resultant levels similar to that observed for Brn-3b. This effect was reproducible in a number of independent experiments, thus suggesting that Brn-3b may interact with Brn-3a proteins to initiate transcriptional activity. This may be achieved either by competition by Brn-3b for the same binding site thus preventing Brn-3a binding and stimulating promoter activity or by interactions of Brn-3a with Brn-3b protein forming a complex which represses the promoter activity.

Another interesting observation was that while Brn-3a(l) and Brn-3a(s) were capable of activating the promoter, Brn-3a(s) which lack some of the amino acids in this region of the protein (see introduction), gave rise to slightly higher activity.

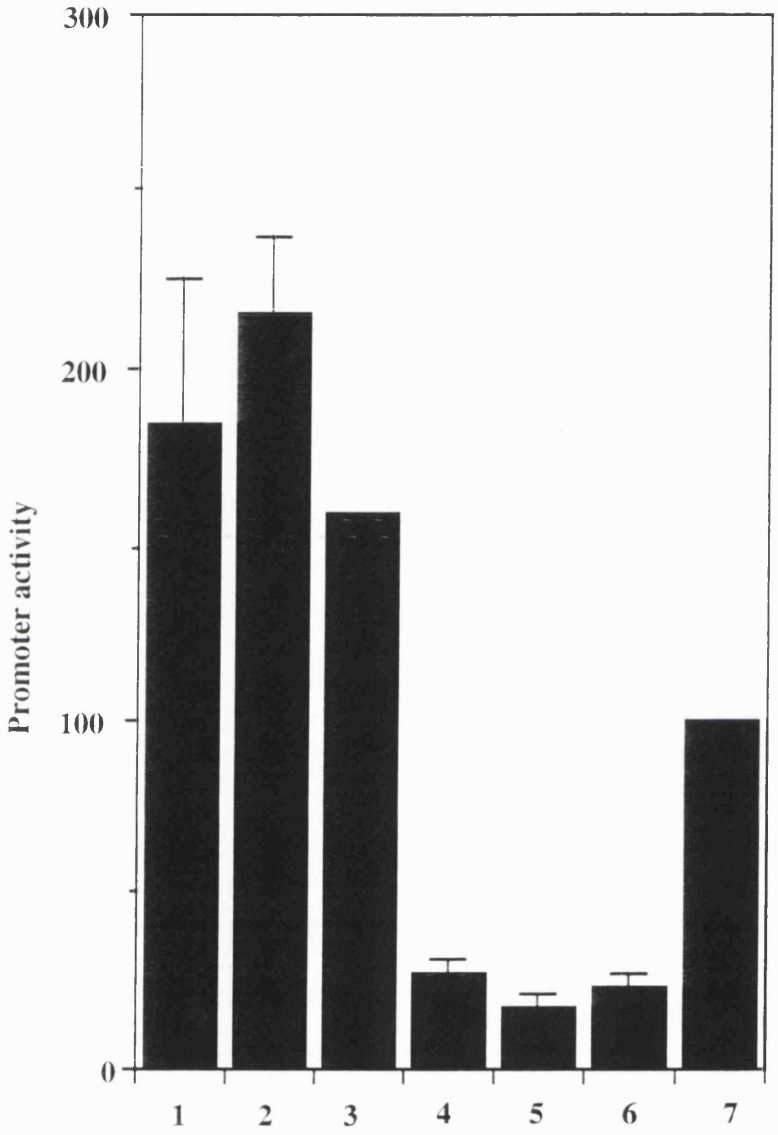


Figure 5.1.3: Results showing the interactions of Brn-3a and Brn-3b on the internexin promoter. Promoter activity was measured by assay of the CAT activity of a reporter construct containing the full length internexin promoter in ND7 cells co-transfected with Brn-3a(l) (track 1), Brn-3a(s) track 2, both Brn-3a isoforms in combination (track 3), Brn-3b(s) (track 4), Brn-3b(s) with Brn-3a(l) (track 5) or with Brn-3a(s) (track 6). Levels are expressed as a percentage of the vector control (track 7).

It may be speculated that in addition to an activation domain in this region there are sequences which may bind other cellular factors with resultant attenuated transactivation capacity of Brn-3a(l) protein on the promoter but which may be involved in the events required for transformation by this protein. This observation was further highlighted by the regulation of the POMC gene promoter by these proteins which will be discussed later.

In addition, co-transfection of the two isoforms of Brn-3a also resulted in a small decrease in the activity of either protein on its own. These results suggested that the interaction of these two proteins also modulate their ability to activate transcription perhaps by changing their affinity for the binding site or by preventing complexes with other factors which are required to bind these proteins to mediate the transcriptional changes observed. These possible interactions represent an interesting area for further investigations.

5.1.2 Domains of Brn-3a and Brn-3b involved in modulation of promoter activity

Different domains of the Brn-3 proteins appear to have specific effects on the activity of the protein. For instance, the amino terminal domain was shown to be critical for the transformation of fibroblasts by Brn-3a(l) since Brn-3a(s) or Brn-3b(s) which lacked sequences in this domain were incapable of achieving this effect (Theil and Moroy, 1994). In addition, the POU domain appeared to determine the effect of transcription on the synthetic Brn-3 binding site, PAP 3/4 derived from the papilloma virus (discussed below) (Morris *et al*, 1994). We were interested in identifying the domain/s of Brn-3a and Brn-3b involved in the activation or repression of transcriptional activity observed on the α -internexin promoter. Studies were carried out using chimeric molecules which were made by interchanging the domains derived from either Brn-3a or Brn-3b and cloned into the modified eukaryotic expression vector, pLTRpoly (figure 5.1.4) (Theil and Moroy 1994).

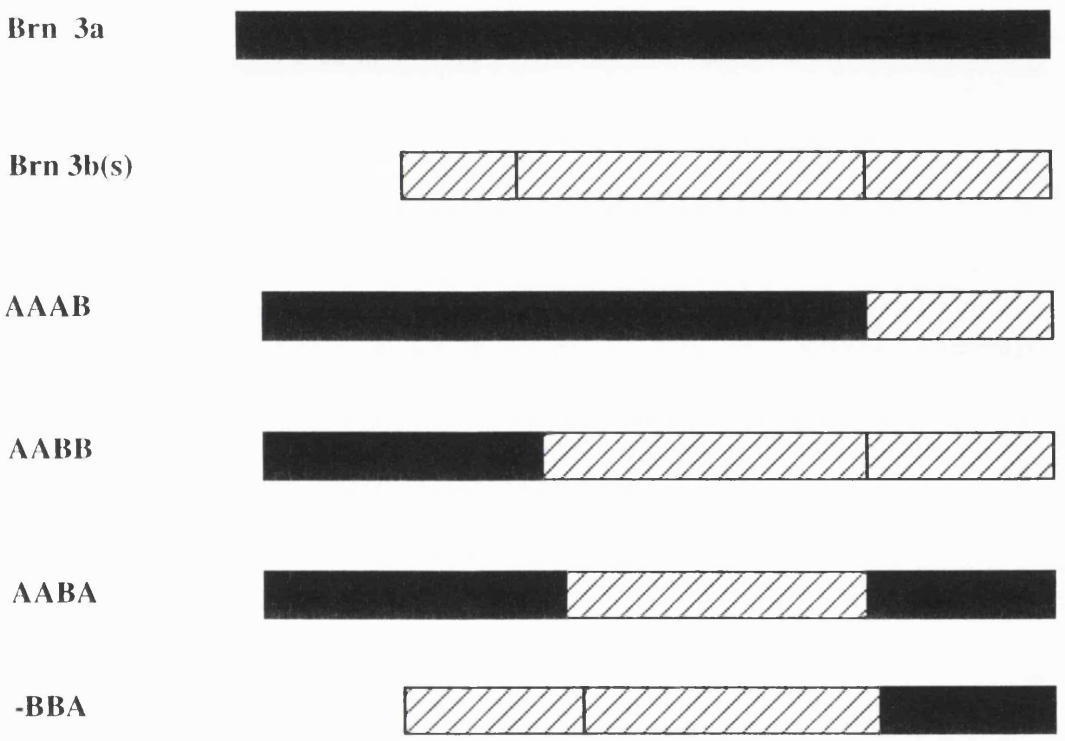
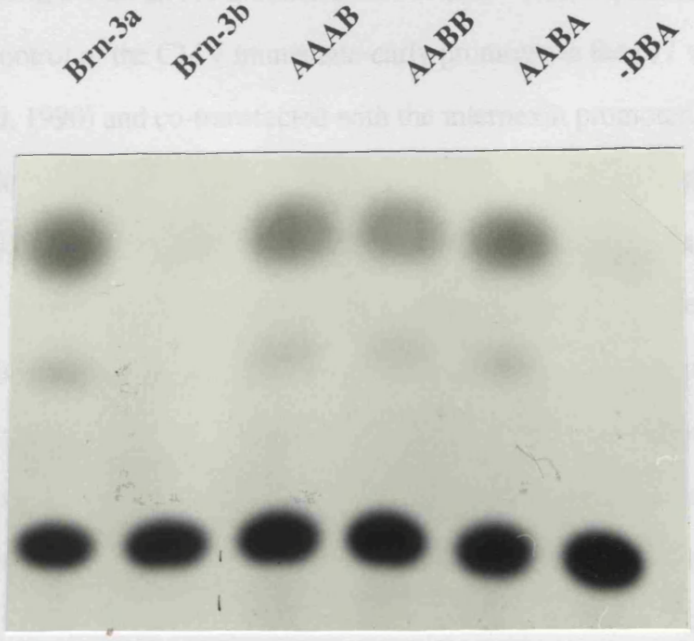


Figure 5.1.4: Schematic representation of the chimeras used to study the function of the domains of Brn-3a and Brn-3b on transcriptional activity. The domains of Brn-3a or Brn-3b indicated by (A) or (B) respectively, were ligated as shown. (-) indicates that no domain was present in -BBA at the amino terminus.

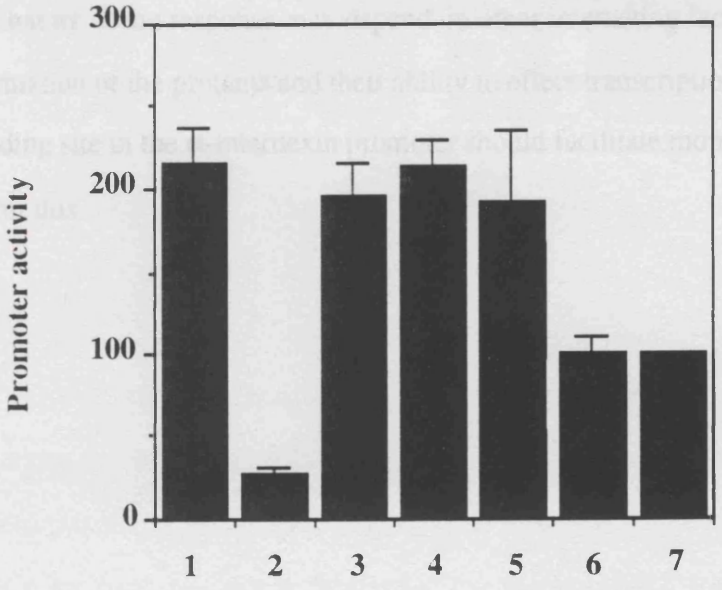
These constructs produced functional proteins which were shown to bind to the octamer related motif known to bind the Brn-3 proteins (Theil *et al.*,1993 and Morris *et al.*,1994). These chimeras were therefore co-transfected with the full length internexin promoter and assayed for their ability to modulate the activity of the promoter.

The results of these co-transfection experiments suggested that the amino terminal domain of the Brn-3a protein was critical for activation of this promoter since there was significant reduction of the activity when the construct lacking this domain (-BBA) was co-transfected compared with the activity induced by the full length Brn-3a (figure 5.1.5). The other chimeras AAAB, AABB and AABA containing the amino terminus of Brn-3a were all able to transactivate the promoter to a level similar to Brn-3a indicating that regions in the amino terminal domain rather than the POU domain was required for transactivation of this promoter. This effect was different to that observed previously with the synthetic promoter motif, PAP3/4 (Morris *et al.*,1994). Whereas activation via the this octamer motif was greater when the chimeras contained the Brn-3a POU domain, (-BBA and AABA) there was little stimulation by the constructs containing the POU domain from Brn-3b (AAAB and AABB). In contrast, the POU domain of Brn-3a in the -BBA construct was insufficient to support activation of the α-internexin promoter.

(A)


Brn-3a
Brn-3b
AAAB
AABB
AABA
-BBA

(B)


Promoter activity
300
200
100
0
1
2
3
4
5
6
7

To test if the POU domain of either factor alone could affect promoter activity, the POU domains of Brn-3a and Brn3b with an ATG codon added to allow transcriptional initiation were cloned under the control of the CMV immediate-early promoter in the pJ7 vector (Morgensterm and Land, 1990) and co-transfected with the internexin promoter. In these experiments, the POU domain of Brn-3a had little significant effect on transcriptional activity while Brn-3b POU domain repressed the promoter (figure 5.1.6). These effects may be achieved by the binding of the POU domain to the promoter and thus preventing access by endogenous Brn-3 proteins. However, this contrasts with the results obtained using the promoter containing the octamer motif, PAP3/4, which was trans-activated by the isolated Brn-3a POU domain and inhibited by Brn-3b POU domain (Morris *et al.*,1994, Budhram-Mahadeo *et al.*,1995). These results therefore suggest that the Brn-3a proteins contain two distinct activation sites with one located at the amino terminus and the other in the POU domain, which may be involved in mediating the response of this protein on gene activity. The different effects of the Brn-3 proteins on transcriptional activity when the binding site is in isolation from the cellular promoter compared with its presence in the context of such a promoter which generally contains binding sites for other factors may suggest that the nature of the response may depend on other interacting factors which may affect the conformation of the proteins and their ability to effect transcription. Identification of the Brn-3 binding site in the α-internexin promoter should facilitate more in-depth studies to confirm this.

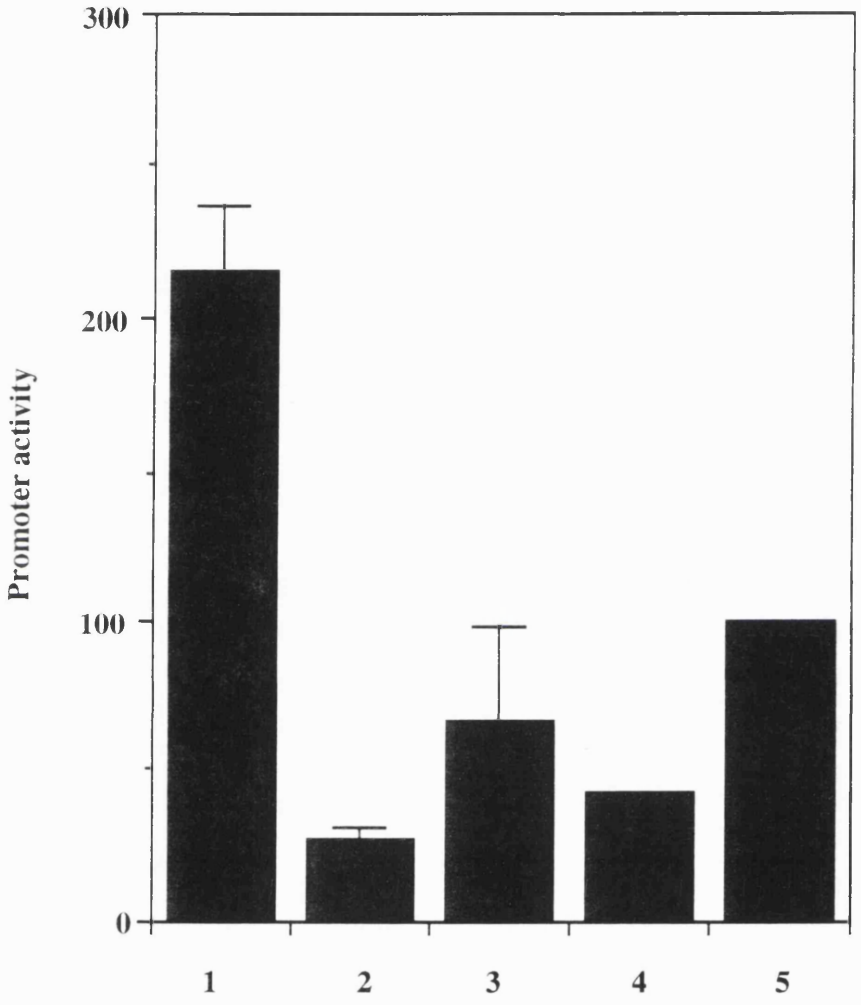
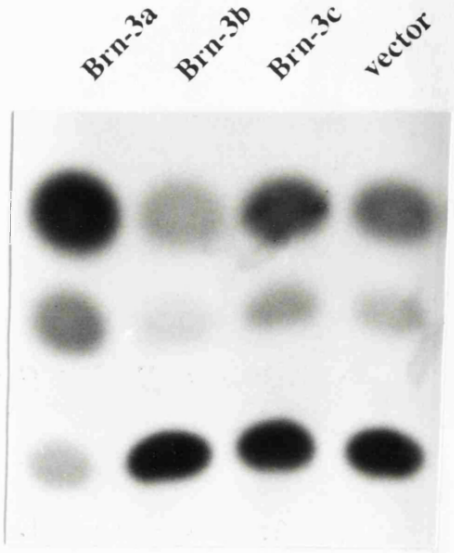


Figure 5.1.6: Levels of activity of the full length internexin promoter upon co-transfection with expression plasmids containing full length cDNA for Brn-3a(s) (track 1), Brn-3b(s) (track 2), or the POU domain of Brn-3a (track 3) and Brn-3b (track 4). Values were expressed as a percentage of the vector control (track 5). These results represent the values obtained from three independent experiments .

5.2.0 Identification of the Brn-3 binding site in the α-internexin promoter: Activity if the intermediate and minimal α-internexin promoter

In an attempt to identify the position of the DNA response element in the α-internexin promoter via which the Brn-3 proteins modulated transcriptional activity, constructs which contained shorter regions of the promoter sequences were used in transfection experiments. The intermediate length construct consisted of the sequence from -254 to +74 of the α-internexin promoter (Internexin I) and a minimal promoter sequence, -77 to +73 (Internexin S) cloned into the pBLCAT3 vector. Neither of these constructs contained any of the octamer sites. These promoter constructs were co-transfected with the expression vectors containing the Brn-3 cDNA and the CAT assay done to measure the promoter activity. The results of these experiments are shown in figure 5.2.1 and 5.2.2. Both constructs containing the shorter promoter sequences were activated by Brn-3a and Brn-3c proteins and repressed by Brn-3b, in a manner similar to that seen with the full length promoter. Moreover, there was stronger activation of the shorter promoter construct, Internexin S (-77 to +74) when co-transfected with plasmids expressing Brn-3a and Brn-3c proteins while Brn-3b still acted as a repressor (figure 5.2.2). This apparent higher activation may be the consequence of a lower basal levels by this minimal promoter but with the same activation in the presence of Brn-3a activity expressed. Hence expressing the activation by Brn-3a as a percentage of the control increases the fold activation compared with the longer promoter sequences. These observation also suggested that the sequence via which Brn-3a, Brn-3b and Brn-3c proteins mediate their responses in the α-internexin promoter was distinct from the classical octamer sequences found in the full length promoter and this site was located within the 77 bases upstream and / or 73 bases downstream of the transcriptional start site.

(A)



(B)

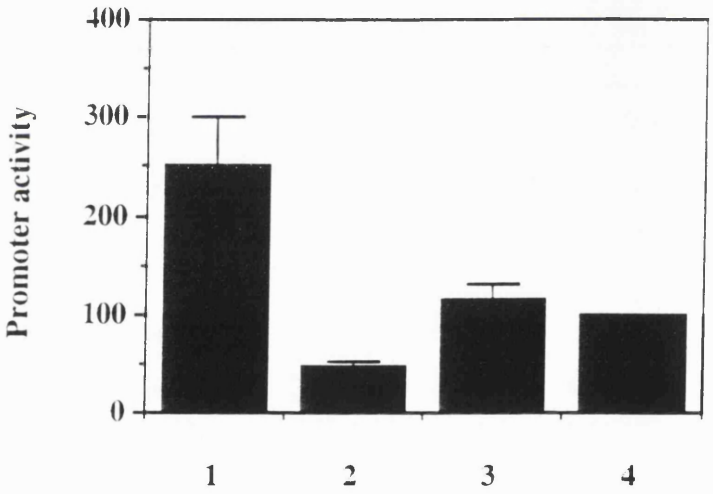
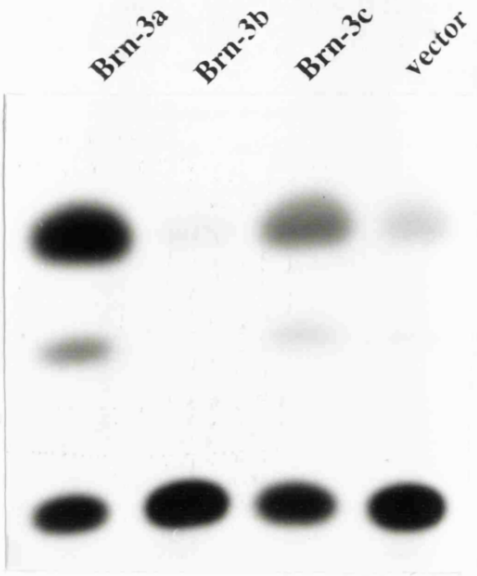


Figure 5.2.1: (A) Typical CAT assay showing the activity of a reporter construct containing the internexin promoter sequence from -254 to +73 bases (internexin I), co-transfected with the expression vectors encoding Brn-3a, Brn-3b, Brn-3c and vector control into ND7 cells. (B) The result of a number of independent experiments showing the activity of the intermediate length promoter when co-transfected with Brn-3a (track 1), Brn-3b (track 2) Brn-3c (track 3) and vector control (track 4). Values are expressed as a percentage of the vector control and the standard errors are indicated by the bars.

(A)



(B)

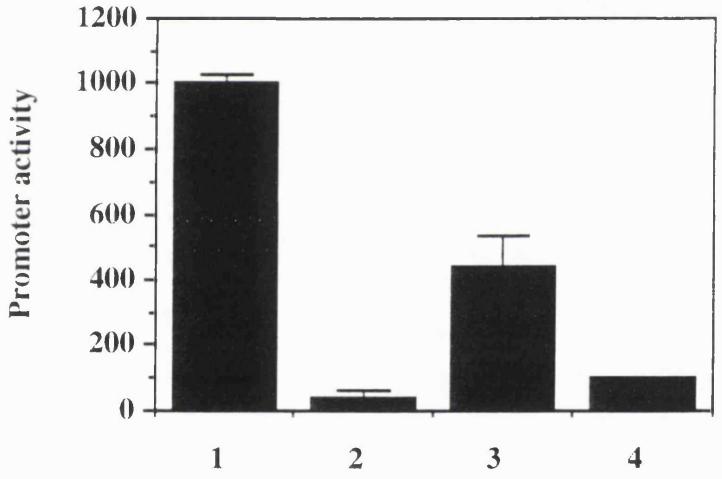


Figure 5.2.2: (A) Typical CAT assay showing the activity of the reporter construct containing the short internexin promoter sequence (-77 to +73 bases) co-transfected with the expression vectors encoding Brn-3a, Brn-3b, Brn-3c and vector control. (B) The result of a number of independent experiments showing the activity of the intermediate length promoter when co-transfected with Brn-3a (track 1), Brn-3b (track 2) Brn-3c (track 3) and vector control (track 4). Values are expressed as a percentage of the vector control and the standard errors are indicated by the bars.

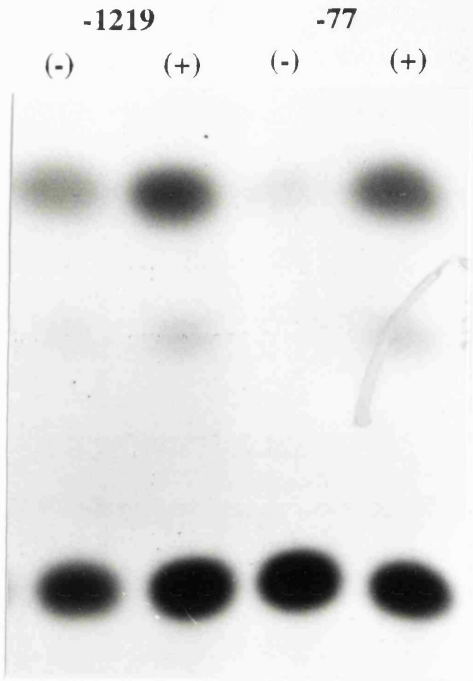
5.2.1 Cyclic AMP activation of the α-internexin gene promoter

The α-internexin mRNA was shown to increase following cyclic AMP treatment (Ching *et al*, 1991) although the gene promoter does not contain any cyclic AMP response elements (CRE) which normally mediate the transcriptional response to this substance (Lalli *et al*, 1994). Since the α-internexin promoter is activated by Brn-3a and Brn-3a mRNA was shown to be regulated by cyclic AMP, it is possible that the cyclic AMP mediated changes in the α-internexin expression is achieved by increasing Brn-3a expression and interactions.

To investigate this, the constructs containing the different lengths of the α-internexin promoter [long (L), intermediate (I) and short (S) constructs] were transfected into ND7 cells which constituitively express Brn-3a and Brn-3b and into BHK fibroblast cells which express no endogenous Brn-3 proteins.

The increase in the Brn-3a mRNA in cyclic AMP treated ND7 cells was accompanied by increased activity of all three α-internexin promoter constructs. The effect on the longest and shortest promoter sequences are shown in figure 5.2.3 since the effect on the intermediate length promoter was very similar to that observed with the full length promoter (Internexin L). While the effect on Internexin L and Internexin I promoter sequences were similar, showing approximately two fold increase in the promoter activity, a much greater activation (approximately nine fold increase) was observed with the shortest construct, Internexin S. This response was similar to that observed when the short promoter construct was co-transfected with the Brn-3a expression vector showing lower basal activity in untreated cells but with similar increase in the activity of the promoter in the presence of increased Brn-3a accomplished either by co-transfection of Brn-3a or by cyclic AMP treatment.

(A)



(B)

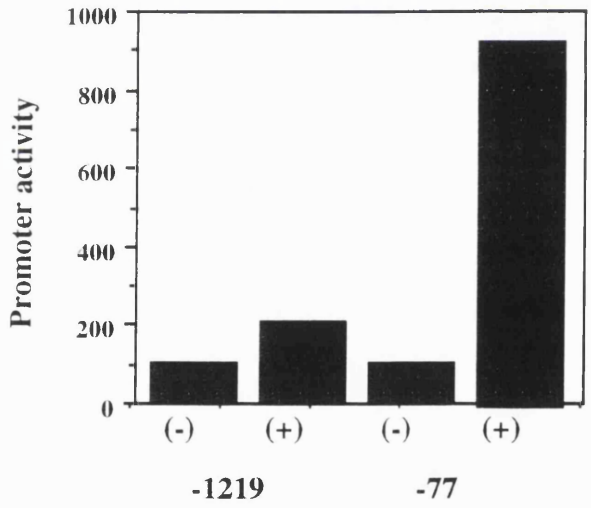


Figure 5.2.3: (A) A typical assay of the CAT activity mediated via the the full length (-1219) and the short (-77) internexin promoters in ND7 cells transfected in the absence (-) or presence (+) of cyclic AMP. The effect on the intermediate length promoter was similar to that observed for the full length promoter. (B) Levels of activity of the long and short internexin promoter constructs expressed as fold increase over the activity over untreated (-) cells.

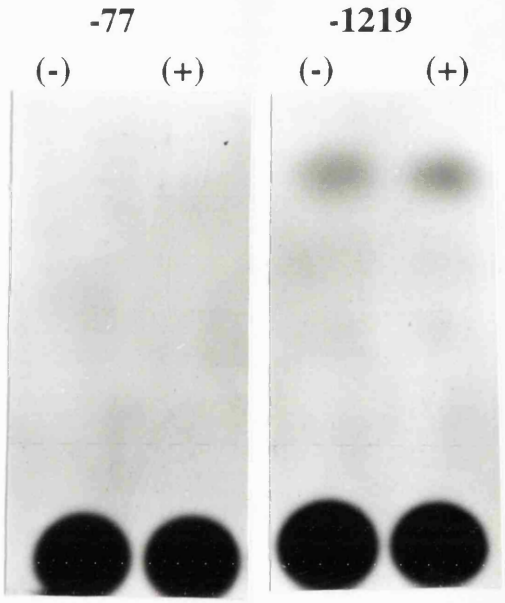


Figure 5.2.4: A representative CAT assay representing promoter activity in BHK cells transfected with the full length (-1219) and short (-79) internexin promoters, in the absence (-) or presence (+) of cyclic AMP.

This observation therefore suggests that neither the cyclic AMP effect or Brn-3a are dependent upon sites lost from the full length promoter and also that the binding and activity of the Brn-3 proteins which may accomodate other factors does not interfere with .

In contrast to the increased activity in cyclic AMP treated ND7 cells, no change in promoter activity was observed in the presence of cyclic AMP in similar experiments carried out in BHK cells which lack endogenous Brn-3 expression (figure 5.2.4) thus strongly supporting the idea that Brn-3a may play a significant role in the modulation of the α-internexin gene promoter in the presence of cyclic AMP.

5.2.2 Sequences in α-internexin gene promoter mediating Brn-3 effect

The results of the studies using the different lengths of α-internexin promoter suggested that the Brn-3a and Brn-3b proteins bind to and modulate gene expression via a non-classical octamer sequence which is present in the minimal internexin promoter. To try and locate the Brn-3 protein binding sites in this promoter, the sequence of the minimal promoter (Internexin S) was further analysed. Initial studies which were carried out aimed at deleting sequences from the 5' end of the Internexin S promoter construct, that is, from the -77 bases upstream of the transcription initiation site. This was achieved by BAL-31 nuclease digestion of the linearized plasmid followed by re-ligation, transformation, selection and sequencing of clones with deletions (N.Lakin). One of the clones obtained by this method had a deletion of 28 bases from 5' end of the original clone thus resulting in a sequence which started at -48 to + 73 bases (Int'xΔ2) (figure 5.2.5).

(A) [deletion clone starts]

↓

-77

5' GAT CTG AAG ATG AAG CTC CAC CCC TAG C GC GTC GCC CCA GCC CCG

CTA GAC TTC TAC TTC GAG GTG GGG ATC G CG CAG CGG GGT CGG GGC

TATA Box [+1]

CGC CTT AAA AGC CCC GCA CAC CGC CCC GCC GCA CCC AGC CTT GCC

GCG GAA TTT TCG GGG CGT GTG GCG GGG CGG CGT GGG TCG GAA CGG

GCA CCT CGC GTC CTC GCC AGG TCC GCC GCA GCC GCG CAC CCG GCC

CGT GGA GCG CAG GAG CGG TCC AGG CGG CGT CGGCGC GTG GGC CGG

CCG ACC CCG GCA CC

GGC TGG GGC CGT GG 3' +73

(B) 5' GAT CTG AAG ATG AAG CTC CAC 3'

3' CAT GAC TTC TAC TTC GAG GTG 5'

Figure 5.2.5: (A) Sequence of the short internexin promoter, (Internexin S) showing the position of the TATA box (underlined) and the transcription initiation site (+1) and indicated by bold underlined letter. The position of the start of the deletion clone obtained by Bal 31 nuclease digestion (-47) is also indicated.

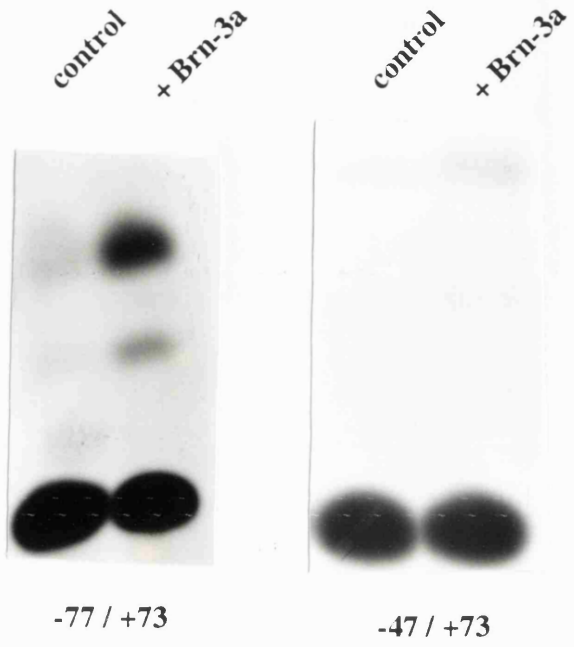
(B) Sequence of the oligonucleotide, IXB3, derived from -77 to -57 that was used in binding assays with the Brn-3 proteins and for transfection studies following cloning into pBLCAT2 vector. The Bam H1 site at the end allowed cloning of this sequence into a reporter construct for later studies.

To analysed for basal activity and to check if the Brn-3a binding site was abolished this clone was co-transfected either with the empty expression vector or with the Brn-3a expression vector into ND7 cells and the promoter activity analysed. The undigested clone was also co-transfected in parallel experiments as a control. The results showed a dramatic decrease in the activition of the deletion clone by Brn-3a compared with the control undigested construct (figure 5.2.6), thus implying that the sequences to which Brn-3a bind to activate the promoter lies within this region.

5.2.3 DNA sequence binding Brn-3 proteins in the α-internexin gene promoter

Investigation of the deleted sequence which contain the Brn-3a binding site revealed a short sequence, CTA, near the start of the minimal promoter which was related to the CAT of the octamer motif of Brn-2 binding site in the CRH promoter (Li *et al.*, 1993) which was also shown to bind Brn-3a (Gerrero *et al.*, 1993) and an A/T rich region (AAGAT), separated by the three nucleotides shown to be critical for Brn-3 activity (figure 5.2.5) (Li *et al.*, 1993; also see introduction). To test whether the Brn-3a binding site was localized to this region, oligonucleotides corresponding to the first 21 bases of the deleted sequence (referred to as IXB3) (Figure 5.2.5B) were synthesized and used in electrophoretic mobility shift assays.

(A)



(B)

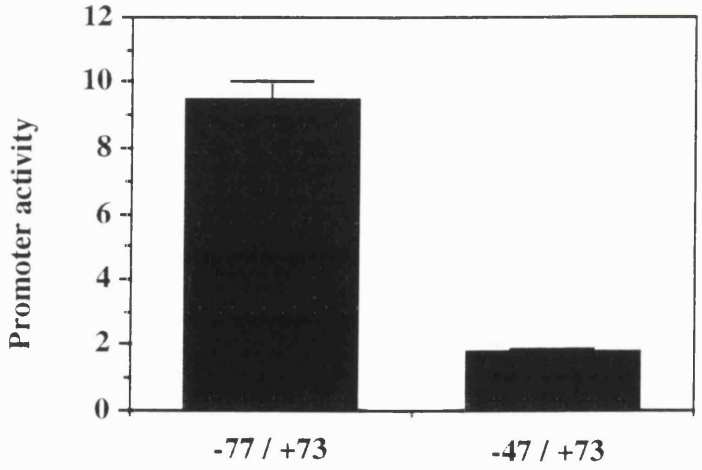


Figure 5.2.6 : (A) CAT activity of the short internexin promoter (-77 to +73) and the deletion construct (-47 to +73) showing the basal promoter activity (control) and the activity upon transfection with the Brn-3a expression vector. (B) Levels of activity combined from three independent experiments showing the activation associated with the presence of Brn-3a protein expressed as fold increase over the basal promoter activity in the minimal promoter and the deletion construct.

The labelled double stranded or single stranded oligonucleotide were incubated with *in-vitro* translated Brn-3a(l) and Brn-3b(s) protein either in the presence of no competitor, 100 fold molar excess of the unlabelled oligonucleotide used as the probe (specific competitor), 100 fold molar excess of an unrelated oligonucleotide sequence which acted as a non-specific competitor. The reactions were carried out as described in methods and following incubation, the protein / oligonucleotide complexes were resolved on a non-denaturing polyacrylamide gel.

The complexes formed upon incubation of the *in-vitro* translated Brn-3a and Brn-3b protein with the double stranded labelled oligonucleotide are shown in figure 5.2.7. Two of these weak complexes were present only in the tracks containing the Brn-3 *in-vitro* translated products and not in the control tracks were competed away upon the addition of the specific competitor but not with the non-specific competitor. These complexes were therefore assumed to be the Brn-3 / oligonucleotide complexes since there are no antibodies available to confirm these results at this time. The difference in the sizes of these complexes was suggestive of binding as monomers (complex 2) or dimers (complex 1) but could also represent truncated proteins which may result from the in-vitro translation reaction. This will therefore need to be further investigated perhaps by titration assays or cross-linking studies.

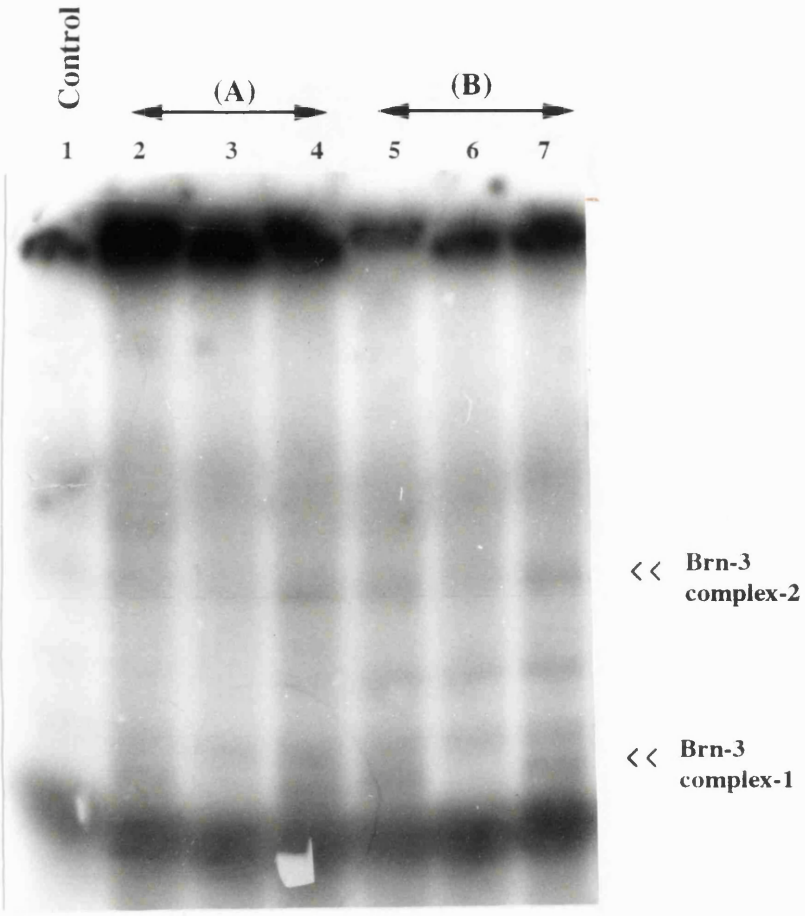


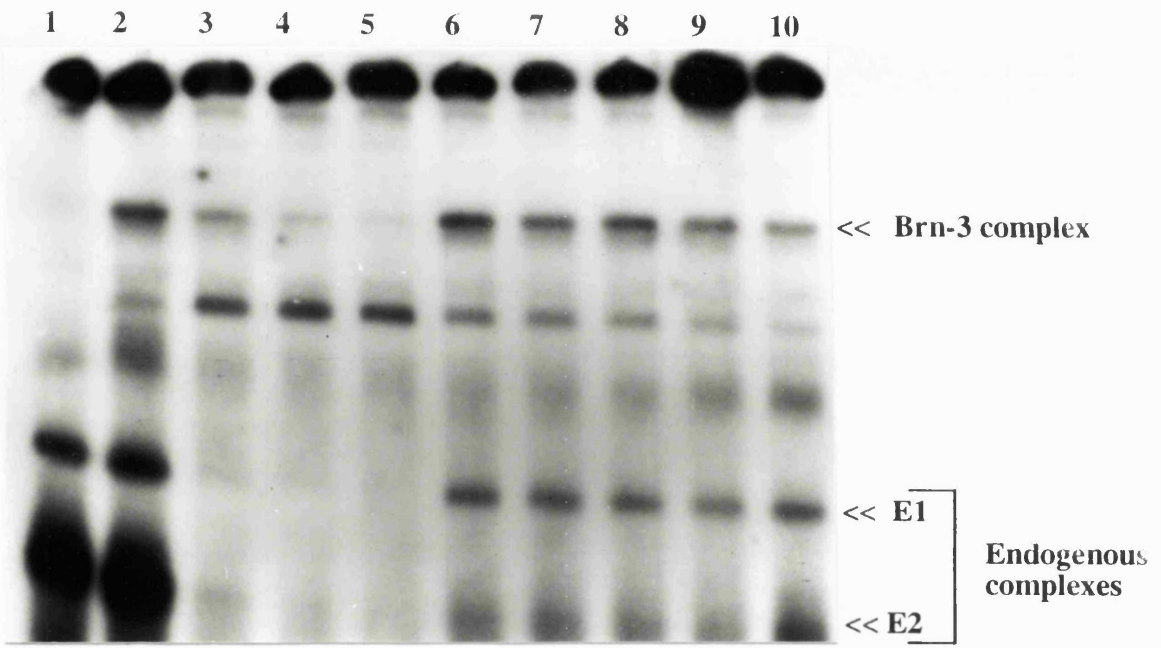
Figure 5.2.7: DNA binding activity of Brn-3a (A) and Brn-3b (B) in-vitro translated proteins incubated with double stranded, labelled sequence derived from the internexin promoter (IXB3) with no competitor (tracks 2 and 5), in the presence of 100 fold molar excess of the unlabelled oligonucleotide (specific competitor), (tracks 3 and 6) and with unlabelled, unrelated oligonucleotide (non-specific competitor), (tracks 4 and 7). The control track shows the probe incubated with the wheat germ lysate control used for the in-vitro translation so providing the control for binding to endogenous proteins in the lysate.

Whereas there was low affinity binding of the Brn-3 proteins on the DNA in the double stranded conformation, the single stranded 'coding' sequence of this oligo-nucleotide was found to bind Brn-3a and Brn-3b proteins with much higher affinity. The result of the EMSA following the incubation of the labelled coding strand with *in-vitro* translated Brn-3a proteins shows a number of complexes (figure 5.2.8) two of which were competed away with increasing amounts of the unlabelled 'specific' competitor but not by addition of equal amounts of the non-specific competitor or the unlabelled double stranded oligonucleotides previously shown to bind Brn-3a . However, one of these bands (E-2) appeared to be associated with endogenous proteins of the wheat germ lysate since it was also found in the control tracks containing the lysate used for the *in-vitro* transcription / translation reaction.

The larger complex which was specifically competed away with increasing concentration of the unlabelled oligonucleotide was present only in the tracks with Brn-3a protein and thus represent the complex formed with the Brn-3a protein. This complex was not competed by unlabelled double stranded oligonucleotides which were previously shown to bind Brn-3a suggesting that the binding specificities may be different. This observation together with the differences in the complexes formed with the double stranded oligonucleotides suggest that the binding affinities and pattern may be different.

Figure 5.2.8 (i): EMSA showing binding of the Brn-3a in-vitro translated protein to the labelled single stranded 'coding' sequence of the (IXB3) sequence derived from the putative binding site in the internexin promoter. The protein was incubated with no competitor (track 2), or with increasing amounts of unlabelled single 'coding' strand (specific competitor) with tracks 3 to 5 showing the addition of 25, 50 and 75 fold excess respectively of the specific competitor. Track 6 - 9 shows the effect of adding 50 fold molar excess of unlabelled oligonucleotides derived from the CRH promoter, and octamer related oligonucleotides, A and C respectively which were previously shown to bind Brn-3 proteins (see text). Track 9 represent the binding upon addition of 50 fold excess of an unlabelled, unrelated oligonucleotide (non-specific competitor) while track 10 shows the binding to the Brn-3b in-vitro translated proteins. Track 1 shows the probe incubated with the in-vitro translation control sample so providing the control for binding to proteins in the wheat germ lysate used for the translation of Brn-3 proteins.

The largest complex which is only present in the tracks with Brn-3a or Brn-3b proteins appears to be specifically competed away upon addition of the single stranded specific competitor but not by the non-specific competitor or the double stranded sequences. The endogenous band, E1, which is also present in the control track is also competed away specifically by the unlabelled specific competitor but not by the other oligonucleotides while E2 appears to be competed away by all the other competitors including the non-specific competitor.


1
2
3
4
5
6
7
8
9
10
<< Brn-3 complex
<< E1
<< E2
Endogenous complexes

A similar pattern was observed when this single stranded DNA probe was incubated with the *in-vitro* translated Brn-3b protein [figure 5.2.8(ii)] with the largest complex being specifically competed away upon addition of the specific competitor but not the non-specific competitor which may therefore represent binding of the complex with Brn-3b.

The specific recognition and binding of the single stranded oligonucleotide by the Brn-3 proteins was supported by the observation that incubation of the labelled coding strand of the DNA with another POU domain protein Oct-2.1, resulted in negligible binding compared with the complexes formed with the Brn-3 proteins. While the *in-vitro* translated Oct-2.1 protein was capable of binding strongly to the octamer motif (figure 5.2.9), incubation of the Oct-2.1 protein with the IXB3 sequence resulted in only a very weak band which was competed away upon addition of specific competitor and was not present in the control track.

These results therefore suggest that the sequence, IXB3, contain the binding site for Brn-3a and Brn-3b proteins and the single stranded 'coding' sequence binds both protein with higher affinity compared with the double stranded conformation. In addition, these complexes were not efficiently competed away with unlabelled double stranded oligonucleotides. The implications of these observations will have to be investigated in future experiments (see discussion).

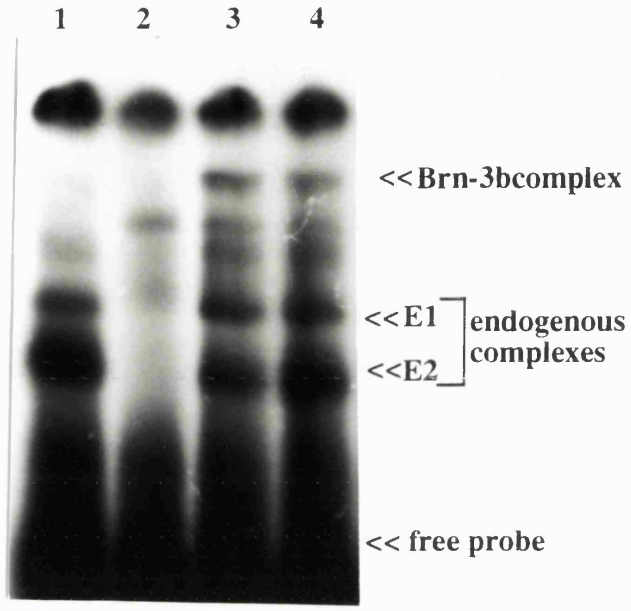


Figure 5.2.8 (ii): EMSA showing the binding of the Brn-3b in-vitro translated proteins with the single stranded DNA sequence derived from the sequence from the internexin promoter, IXB3, shown to bind Brn-3a in-vitro translated protein. The Brn-3b protein was incubated with the labelled 'coding' strand either in the presence of 100 fold molar excess of 'cold' unlabelled competitor (track 2), with no competitor (track 3) or with the non-specific competitor (track 4). The control track (1) shows the complexes resulting from incubation of the lysate control with the labelled probe. There was specific competition of the largest complex (Brn-3b) which was not present in the control tracks. Two other bands (endogenous complexes E-1 and E-2) were competed away with the specific competitor but these were also present in the control, indicating that the complexes resulted from association with the endogenous proteins in the wheat germ lysate used for the in-vitro transcription/ translation of Brn-3b.

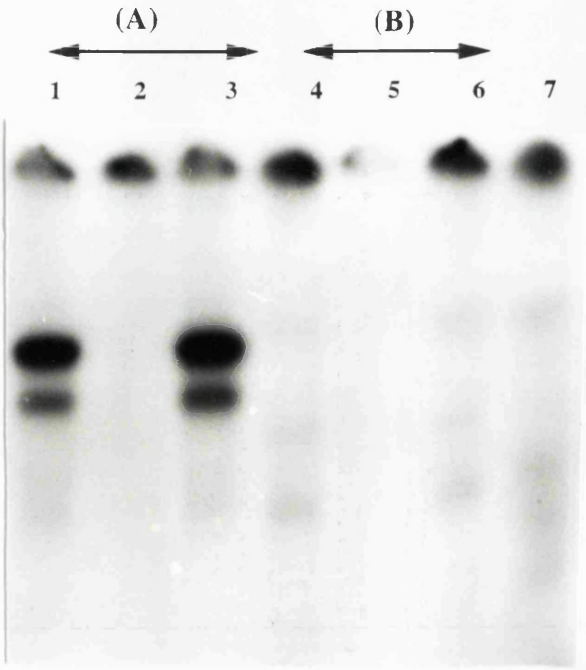


Figure 5.2.9: Comparison of the DNA binding by the Oct-2.1 in-vitro translated proteins with the coding strand of the sequence recognised by Brn-3, IXB3, and the octamer related oligonucleotide, oligo A. Oct-2.1 protein was incubated with either the labelled oligo A, (A), or the labelled coding stranded of the IXB3 sequence, (B) with either no competitor (tracks 1 and 4), in the presence of 100 fold molar excess of the unlabelled oligonucleotide (specific competitor), (tracks 2 and 5) or with unlabelled, unrelated oligonucleotide (non-specific competitor), (tracks 3 and 6). Track 7 represent the reaction carried out with the in vitro translation control showing bands resulting from interaction of the labelled oligonucleotides with proteins present in the wheat germ lysate.

5.2.4 Binding of the oligonucleotide to cellular extract

The double stranded DNA sequence also formed complexes with proteins in total cellular extracts obtained from proliferating ND7 cells. Two bands appeared to be competed away specifically in the presence of 100 fold molar excess of the specific competitor (figure 4.2.10). The largest, most intense band (represented as complex 1) and a smaller, much weaker complex (complex 2) may represent the complexes formed with the Brn-3 proteins but will have to be confirmed in later studies since there is no method at present of confirming which band corresponds to the complexes formed in the presence of Brn-3a or Brn-3b proteins.

There was also binding of the labelled 'coding' strand of this oligonucleotide to proteins in this total cellular extracts (figure 5.2.11). Two strong bands and one weaker band represented thecomplexeswhich were specifically competed away on addition of unlabelled oligonucleotide but not by the unrelated non-specific competitor sequence. Since it is not presently possible to identify the band resulting from complexes with the Brn-3 proteins no firm conclusions can be made at this time. However in view of the high affinity binding of the Brn-3 proteins to this sequence and the poor binding of Oct 2, the other abundant POU protein found in ND7 cells it is probable that these bands represent complexes with the different isoforms of the Brn-3a and Brn-3b proteins. The different binding patterns observed in these studies using cellular extracts also confirmed that proteins bind the single or double stranded DNA sequences with different affinities.

To confirm that this sequence contain the recognition site for the Brn-3 proteins, the double stranded oligonucleotide sequence has been cloned into the reporter construct pBLCAT 2, upstream of the thymidine kinase promoter which drives the expression of the CAT gene.

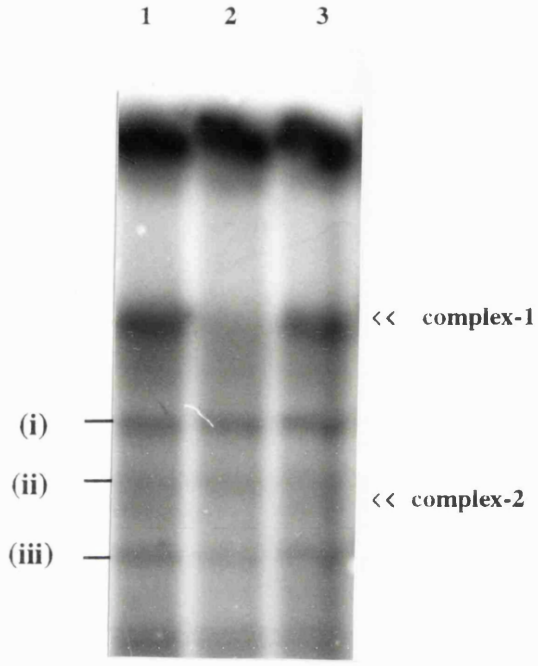


Figure 5.2.10: Binding of the double stranded sequence derived from the putative Brn-3 recognition site in the internexin promoter by proteins in total cellular extracts obtained from untreated proliferating ND7 cells . Cellular extracts were incubated with no competitor the labelled double stranded DNA sequence either with no competitor (tracks 1), in the presence of 100 fold molar excess of the unlabelled oligonucleotide (specific competitor), (tracks 2) or with an unlabelled, unrelated oligonucleotide (non-specific competitor), (tracks 3). The complexes 1 and 2 were specifically competed on addition of the 'cold' unlabelled oligonucleotide while bands (i), (ii) and (iii) remain unchanged.

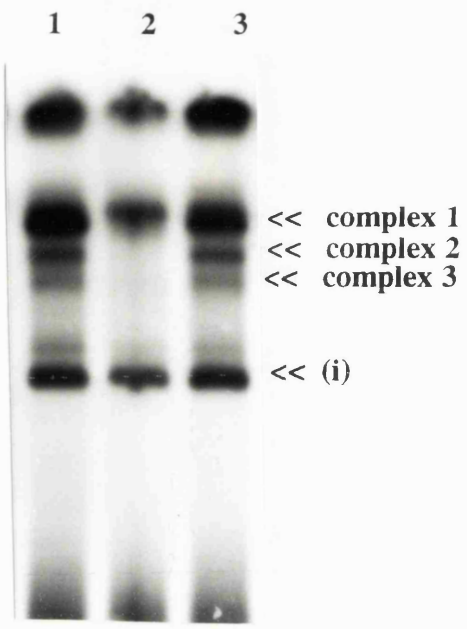


Figure 5.2.11: EMSA showing binding of the proteins in total cellular extracts obtained from untreated proliferating ND7 cells to the single 'coding' strand of the putative Brn-3 DNA binding site derived from the internexin promoter (IXB3). Cellular extracts were incubated with the labelled single stranded DNA and either with no competitor (tracks 1), in the presence of 100 fold molar excess of the unlabelled oligonucleotide (specific competitor) (tracks 2) or with unlabelled, unrelated oligonucleotide (non-specific competitor), (tracks 3). The complexes 1, 2 and 3 were competed away specifically upon addition of the unlabelled oligonucleotides but the invariant band (i) showed little changes in the presence of the specific or non-specific competitor.

Co-transfection of this construct with the expression vectors containing the cDNA sequence for the Brn-3 proteins into BHK cells and ND7 cells will assist in analysing the response of this sequence outside the context of the promoter.This work is currently being carried out by P.Morris.

5.3.0. Domains of Brn-3 proteins involved in regulation of the POMC promoter

Brn-3a was reported to bind to and activate the Pituitary Pre-opiomelanocortin (POMC) gene promoters via the sequence, GCATCCTTAAT (Gerrero *et al.*,1993). In addition, the CE2 sites in this promoter were shown to bind Brn-3a and mediate transcription activity. The POMC promoter was therefore used as a control for studying the effect of the Brn-3a and Brn-3b proteins on promoter activity. Two reporter constructs containing either the full length promoter of the POMC gene or multiple CE2 sites upstream of a minimal POMC promoter fused to the firefly luciferase reporter gene in the vector, pXP1, were co-transfected with the Brn-3a and Brn-3b expression vectors into ND7 cells and BHK cells.

In both ND7 cells and BHK cells, Brn-3a(s) strongly activated the transcription of the full length POMC promoter and Brn-3b repressed the basal activity in a manner which was similar to that observed previously with the heterologous promoter containing the octamer motif and the internexin promoter. However, the long isoform Brn-3a(l) repressed the activity of the full length POMC promoter in these cells (figure 5.3.1) but not the minimal promoter containing multiple CE-2 sites (figure 5.3.2). Similarly, Brn-3c could also activate the minimal promoter but not the full length promoter.Furthermore, while Brn-3b repressed the activity of the full length promoter quite significantly compared with the vector, a smaller decrease was observed on the basal activity of the minimal promoter containing the CE-2 sites.

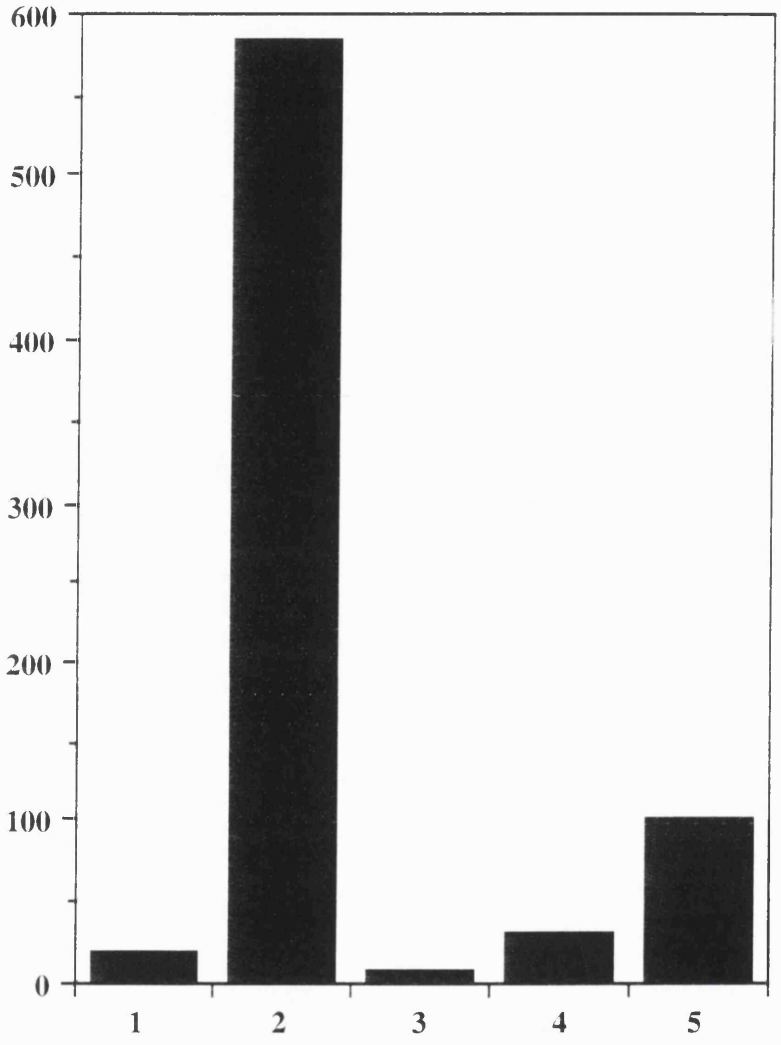


Figure 5.3.1: Luciferase activity of the full length POMC promoter co-transfected into ND7 cells with the expression vectors containing full length cDNA for Brn-3a(l) (track 1), Brn-3a(s) (track 2), Brn-3b (track 3) or Brn-3c (track 4). Levels are expressed as a percentage of the control levels (track 5).

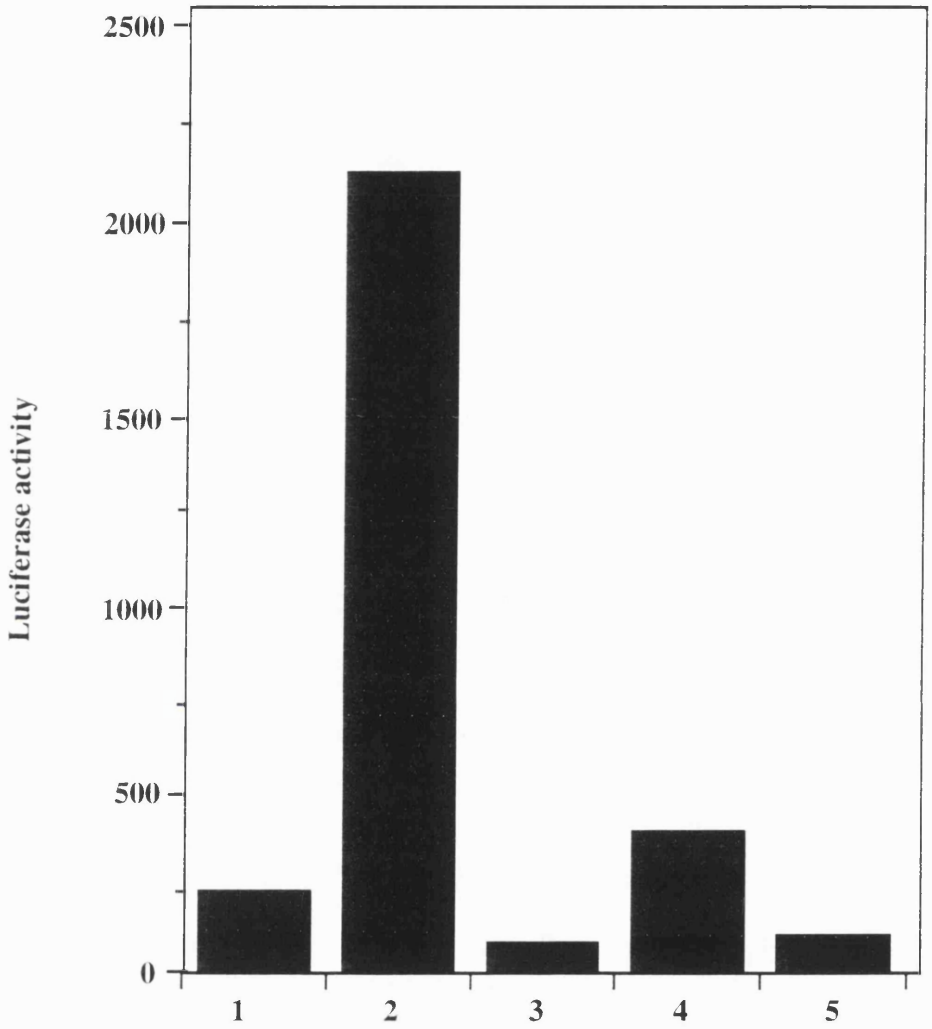


Figure 5.3.2: Luciferase activity of a heterologous promoter containing a minimal POMC promoter and multiple CE-2 sites which was co-transfected with expression vectors containing full length cDNA for the long Brn-3a(l) (track 1) or short Brn-3a(s) track 2, Brn-3b (track 3) or Brn-3c (track 4) and the empty vector control (track 5) into ND7 cells . Levels are expressed as a percentage of the control .

Brn-3a(l) was previously shown to be present in cellular extract derived from the corticotroph cell line, AtT20 could also transactivate the POMC promoter, or a heterologous promoter containing multiple CE-2 sites (Gerrero *et al.*,1993). However in ND7 cells where Brn-3a(l) acted as a repressor of the full length POMC promoter but not the minimal promoter while Brn-3a(s) was still a strong activator of both promoters. The differences between these two proteins lie in the aminio acids at the amino terminal of the proteins. These results therefore suggest that interactions of cell-specific factors which bind to the full length POMC promoter and the amino terminus of Brn-3a(l) may modulate its ability to activate transcription. Thus in the sensory neuronal cell line, ND7, which does not usually express POMC, the factors which interact with elements in the amino terminus of Brn-3a(l) and the promoter sequences inhibit its activation potential. In the minimal promoter which lacks the sites for binding of other factors the repression is lifted. Lack of the amino terminus in the Brn-3a(s) isoform prevents this interaction with the tissue specific repressor and therefore allow strong activation upon binding to the sites in the promoter. In the AtT20 cells, the presence of different cell specific factors which are co-expressed with the POMC promoter may interact directly with Brn-3a(l) or remove the factors which repress Brn-3a(l) activation in ND7 cells.

These results therefore suggest that the promoter activity may be modulated by interactions of the Brn-3 proteins and factors which may bind other promoter elements such as cell specific factors which may be differentially expresed in various cells. These interactions may thus determine the effect of these proteins on transcription which is observed in different cells. This combinatorial effect of different factors on transcriptional activity has been demonstrated to be important for cell-specific expression of proteins during development and in response to specific signals (see introduction). Isolation and characterization of cell specific factors which may modulate Brn-3a(l) and Brn-3c proteins activity on this promoter may provide an interesting area for future research.

5.4.0 Discussion

The Brn-3 proteins were all capable of regulating the α-internexin promoter with both Brn-3a isoforms, Brn-3a(l), Brn-3a(s) as well as Brn-3c trans-activating a reporter plasmid containing the full length promoter sequence while Brn-3b represses the activity. Although Brn-3a has been demonstrated to bind and transactivate an octamer related motif in a heterologous promoter (see chapter 4), the site to which the Brn-3 proteins bind in the α-internexin promoter was found to be distinct from the octamer related sequences since a minimal internexin promoter which lacks any of the classic octamer related sequences was still strongly activated by Brn-3a and Brn-3c and repressed by Brn-3b.

There was also significant interaction between Brn-3a and Brn-3b proteins on this promoter as observed by the ability of Brn-3b(s) to modulate transcriptional activity of both the Brn-3a(l) and Brn-3a(s) proteins. Upon co-transfection, both the Brn-3a(l) and Brn-3a(s) lose their ability to transactivate in the presence of Brn-3b proteins. Evidence for this interaction was also shown by Theil *et al.*, (1994) who demonstrated that increasing the concentration of the Brn-3b proteins in the presence of a fixed concentration of Brn-3a(l) resulted in loss of DNA binding by Brn-3a(l) to a target sequence. Moreover, this group demonstrated that in transformation experiments, both Brn-3a(s) and Brn-3b(s) could affect the ability of Brn-3a(l) to transform primary fibroblasts.

Study of the domains of the Brn-3a and Brn-3b proteins which were involved in mediating transcriptional effect suggested that the amino terminus of Brn-3a was necessary for the activation of the α-internexin promoter. This was in contrast to the activation of the octamer related motif isolated from the human papilloma virus in which the the POU domain was shown to be critical for the ability of Brn-3a to activate and Brn-3b to repress the promoter (Morris *et al.*, 1994). These results therefore suggests that there may be two activation domains in Brn-3a protein and its ability to regulate transcription may depend on the

position of the binding site within the promoter sequence as well as interaction with other factors which bind the promoter.

The binding site of the Brn-3 proteins in the α-internexin promoter was lost upon deletion of 28 bases from -77 bp upstream of the transcriptional initiation site. Study of this region revealed a sequence which was related to the Brn-3a binding site located in the corticotropin releasing hormone II (CRH II) promoter which was previously demonstrated to be related to, but distinct from, the octamer motif and was similar to the Brn-2 binding site (Gerrero *et al.*, 1993, Turner *et al.*, 1994 and Li *et al.*, 1994).Comparison of the sequence from the Brn-2 site showed that the site thought to bind the POU specific domain was inverted to CAT (CATnCAAAT) when compared with the ATG sequence found in the classic octamer sequence such as the (ATGCAAAT). Also of interest was the observation that I-POU which was shown to inhibit the transcriptional ability of an activator, Cf1a, by protein-protein interactions did not bind classical octamer sequences (Treacy *et al.*,1991) but was reported to bind to the CRH promoter sequence (Gerrero *et al.*,1993).

The sequence within twenty one bases from -77 to -57 upstream of the α-internexin promoter containing this site with similarity to the CRH site was used to study the binding affinity of the Brn-3 proteins. In the double stranded conformation, this sequence bound with low affinity to *in-vitro* translated Brn-3a and Brn-3b proteins but much higher affinity binding was observed upon incubation of these proteins with the labelled single 'coding' strand of this sequence. Moreover, in similar bandshift assays using total cellular extract obtained from ND7 cells, complexes were formed with both the double and single stranded DNA sequences. The complexes which represent Brn-3 protein binding will have to be confirmed in later studies when specific antibodies become available. In addition, to further confirm that this sequence contained the recognition site for the Brn-3 proteins, the double stranded oligonucleotide sequence cloned into a reporter construct is being used for co-

transfection studies with the Brn-3 proteins in BHK cells and ND7 cells to analyse the response of this sequence outside the context of of the promoter.

This sequence may therefore contain a novel binding site for Brn-3 proteins which binds both proteins with equal high affinity and which is distinct from but related to other POU factor binding sites. In addition, the observation that these proteins preferentially bind to the single stranded 'coding'sequence is also a novel observation for this group of proteins. However, other proteins which recognize and preferentially bind single stranded DNA have been reported in a number of cases. Many of these proteins showed affinity for the single stranded DNA which was comparable to or much greater than the binding observed with the corresponding double stranded DNA (Lannigan and Notides, 1989; Wilkinson *et al.*,1990. Pan et al.,1990, Kolluri *et al.*, 1992, Stark *et al.*,1992, Tanuma *et al.*, 1995). Furthermore, it was found that the single stranded DNA element may consist of either the coding or non-coding sequences and the proteins binding these sequences could be the same as or distinct from factors which bind double stranded DNA. For instance, proteins recognising a silencer element isolated in the Lipoprotein Lipase gene promoter bound with equal affinity to double stranded and 'non-coding' single stranded DNA. The same proteins were thought to form complexes with the single and double stranded DNA elements (Tanuma *et al.*, 1995). In contrast, distinct proteins were thought to bind a repressor element (proximal repressor element, PRE) in the growth hormone gene promoter. PRE binding proteins (PREB) bind this motif in the double stranded configuration while the single 'non-coding' strand was bound with higher affinity by single stranded PRE binding proteins, ssPREB (Pan *et al.*,1990). Similarly when oestrogen is complexed with its receptor there is low affinity binding to the oestrogen response element (ERE) in a double stranded configuration, but this complex has a 60 fold higher affinity for the single stranded 'coding' strand of this sequence but not the 'noncoding strand'(Lannigan *et al*, 1989).

This preferential recognition and binding of some proteins to specific single-stranded sites on DNA may provide an additional level of complexity for controlling gene expression. The activation or repression by such factors would require the assembly of other factors to facilitate the unwinding of the DNA thus exposing single stranded DNA to which these proteins may bind preferentially to mediate full transcriptional regulation. A possible mechanism by which this may be achieved has been proposed for the oestrogen receptor complex. The process may be initiated by weak binding of the proteins to double stranded DNA which facilitates low levels of initial activity but upon transistory strand separation conformational changes may occur in the single stranded DNA to form a structure which is recognised and bound strongly by the target protein with resultant accentuated transcriptional effect. Since *in-vitro* studies have suggested that the Brn-3 proteins bind their DNA site in the α-internexin promoter in both the double and single- stranded conformation, this could be a possible mechanism by which these proteins achieve transcriptional regulation. The implications of this finding remains to be determined but this may facilitate the search for other target genes regulated by these factors. The exact sequences which constitute the Brn-3 recognition sequence will need to be established perhaps by DNAse1 footprinting and tested by mutation studies in future studies.

The α-internexin gene promoter was reported to be cyclic AMP responsive but has no cyclic AMP responsive element (CRE) in the promoter sequence. We have previously shown that Brn-3a levels increased upon treatment of ND7 cells with cyclic AMP and the increase in promoter activity associated with increased Brn-3a was reversed in the presence of the antisense Brn-3a POU domain sequence (chapter 4). Since this promoter was shown to be regulated by Brn-3 proteins the effect of cyclic AMP treatment on the α-internexin promoter activity in ND7 cells which express constituitive levels of Brn-3 proteins and in BHK cells which have CRE response but which lacked endogenous Brn-3 protein

expression. The increase in promoter activity was observed on promoter of different lengths which contained the Brn-3 binding site, only in ND7 cells and not in BHK cells. These results therefore strongly suggested that the increase in the expression of the α-internexin promoter in response to cyclic AMP may be mediated via Brn-3a and that a CRE sequence may be present in the promoter sequence of Brn-3a and Brn-3b. However, the sequence of these promoters are still to be established.

The cellular POMC promoter which was previously reported to be activated by Brn-3a(l) in the pituitary cell line, AtT20 was shown to be strongly activated by Brn-3a(s) in ND7 cells but its activity was repressed by Brn-3a(l), Brn-3b(s) and Brn-3c. However in the minimal promoter containing CE-2 sites, Brn-3a(s) was still a strong activator but Brn-3a(l) and Brn-3c also activated the promoter. The observation that Brn-3a(l) could activate a minimal promoter but not the full length POMC promoter in the sensory neuronal cell line, ND7 but was shown to be an activator of the full length promoter in the AtT20 cell line may indicate that the activation potential of this protein is further regulated by interactions with other cell specific factors which bind to recognition sites in the promoter itself or interact with the amino terminus of the Brn-3a(l) but not Brn-3a(s) proteins.

Thus, it appears that while Brn-3a, Brn-3b and Brn-3c proteins can recognize and bind an octamer related motif, both the α-internexin and the POMC promoters contain sites for these proteins which are distinct from this sequence. Also, whereas Brn-3a and Brn-3c generally activated gene transcription, Brn-3b(s) repressed basal promoter activity. In addition, these proteins appear to interact with each other and possibly with factors which as still unidentified to modulate promoter activity. It is possible therefore, that the Brn-3a and Brn-3b protein which have overlapping patterns of expression may act together to regulate promoter activity. Ongoing work which aims to characterize the expression pattern of the isoforms of Brn-3a and Brn-3b in sensory neurons during development and in adult

tissues may facilitate a greater understanding of their functions and interactions in regulating promoter activity.

Chapter 6

Results

Cell cycle arrest and differentiation of the neuronal ND7 cell line in the absence of apoptosis

6.0.0 Background

During normal development of the nervous system there is a balance between proliferation of neuronal precursors, differentiation to non-dividing phenotypes which are characteristic of the mature neurons and cell death by programmed cell death or apoptosis (Wylie *et al.*,1980 reviewed in Stellar, 1995). This process of programmed cell death plays an important part in establishing and maintaining the morphology and functional properties of specific tissues and organs. In the nervous system, this process allows the appropriate number of mature neurons to develop and innervate specific regions (Oppenheim, 1991). Extensive cell death has been observed in areas of the brain and spinal ganglia during early development when proliferating neuroblasts exit the cell cycle and differentiate into non-dividing neurons (Hamburger *et al.*, 1981). The cells induced to undergo apoptosis were shown to be neuronal precursors that have recently replicated their DNA but have not undergone full morphological differentiation (Carr and Simpson, 1982). This elimination serves to match the number of innervating neurons to the size of the target. Later, more mature non-dividing neurons were also found to undergo apoptosis and this process was associated with the limited supply of trophic factors derived from the target field (Barde, 1989; Oppenheim, 1989).

The commitment of the cells to undergo apoptosis depends on the signals which may be derived either from the target field such as trophic factors (the nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin -3 (NT-3), ciliary neurotrophic factor (CNTF) (Hamburger *et al*, 1981; Cattano and McKay,1990; Ruit *et al,* 1992, Deckworth and Johnson, 1993; Raff *et al.*,1993), or from neighbouring cells. For instance extensive cell death of oligodendrocytes occurs during development and experimental evidence suggested that in isolation these cells do not survive but require the signals from neighbouring cells such as the astrocytes. Furthermore, *in-vitro* studies suggested that these cells can be rescued from apoptosis by the addition of growth factors such as PDGF,

FGFs and IGFs (McKinnon *et al.*,1990; Collarini *et al.*,1991; Bondy, 1991; Raff *et al.*, 1993).

Growth factors have been demonstrated to play a role in the proliferation and/or survival of cells, such as the fibroblast growth factors (FGF), (Walicke, 1986; Walickie *et al,* 1989; Ferrari *et al,* 1989; Zurn, 1992) insulin derived growth factors (IGFI and IGFII) (Bondy 1991; D'Mello *et al,* 1993) and platelet derived growth factor (PDGF) (McKinnon *et al,* 1990; Collarini *et al,* 1991) as well as cyclic nucleotides such as cyclic AMP (Deckworth and Johnson, 1993; D'Mello *et al.,* 1993; Buckmaster and Tolkovsky, 1994). In addition factors which increase the intracellular calcium concentration have also been shown to be protective in sympathetic neurons (Johnson *et al.*, 1993). Many of these factors have been shown to enhance neuronal survival in *in-vitro* studies often by acting synergistically (McKinnon *et al,* 1990; Rukenstein *et al,* 1991).

A number of molecules have also been described which appeared to enhance the cell death process. For instance in the nematode *C.elegans* the products the genes, *ced 3* and *ced 4* are required for cell death to occur while *ced 9* gene product appears to be protective against cell death. The product of *ced 9* gene is similar to the members of the human Bcl-2 family. In addition, treatment of sympathetic neurons with factors such as the leukemia inhibitory factor (LIF) induces apoptosis in a dose and time dependent manner (Kessler *et al.*, 1993) and this effect was rescued by addition of NGF. This therefore suggests that the number of neuronal in a target field may depend on a balance of factors which promote neuronal survival against those which cause cell death. Although previous studies had proposed that RNA and protein synthesis are required for apoptosis to occur, this is not true for all systems. Recent evidence suggest that many cells are constituitively programmed to apoptose and require signals to prevent this process (Raff *et al.*, 1992, 1993, Steller, 1995).

While it is clear that the balance between apoptosis and cellular differentiation plays a vital role in the development and function of the nervous system, the precise signals and factors which mediate these responses are still under intensive investigations. However the limited amounts of homogeneous materials which can be obtained from regions of the nervous system which contain proliferating neuronal precursors or differentiating cells restricts the detailed molecular analysis of the processes which regulate neuronal cell proliferation, differentiation and apoptosis.

Cell lines provide a useful model system for analyzing processesin sensory neurons which could then be confirmed in primary cultures. The sensory neuronal ND7 cell line which could be maintained in a proliferative state indefinitely when grown in full growth medium containing 10% FCS could be induced to undergo cell cycle arrest and morphological differentiation upon transfer to serum free medium (see introduction) when they display many characteristics and responses of sensory neuronal cells. This behaviour mimicked that of primary isolates of proliferating neuronal precursor cells which also undergo morphological differentiation upon exposure to serum free medium (Catteneo and McKay, 1990).

Previous studies have shown that upon transfer of ND7 cells into serum free medium, a number of cells differentiated while a proportion which did not undergo differentiation died by apoptosis (Howard *et al,* 1993). This was detected on the basis of DNA fragmentation and morphological changes observed by light and electron microscopy. Thus in the neuronal derived ND7 cells, cell cycle arrest may result in both morphological differentiation and programmed cell death in a manner which parallels the events observed during neuronal cell development and survival *in-vivo*. This system therefore provides a

suitable model for studying factors which regulate the balance between proliferation, differentiation and programmed cell death in neuronal cells *in-vitro*.

The experimental evidence of this study suggests that in ND7 cells the serum requirement for the processes of proliferation, differentiation or prevention of apoptosis are different. In addition it appears that cell survival can be dissociated from proliferation and inhibition of morphological differentiation. These results are discussed in this chapter.

Results

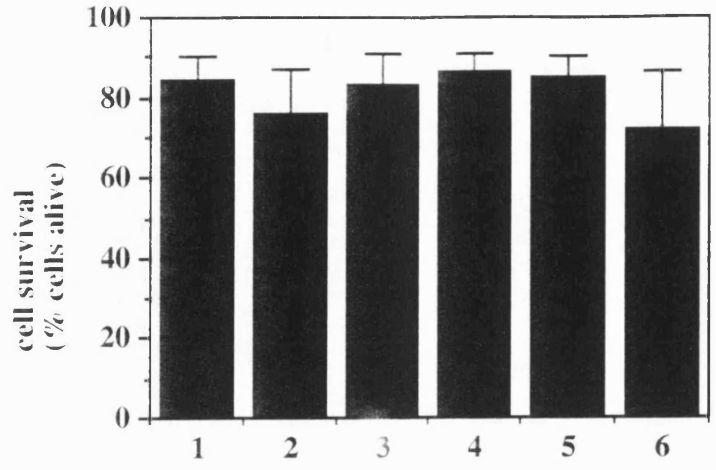
6.1.0 Effect of serum concentration on cell survival

It was previously demonstrated that ND7 cells which were grown in 10% FCS, proliferated and did not show significant cell death but transfer of these cells to serum free medium resulted in either morphological differentiation or apoptosis (Howard *et al*, 1993). Therefore, ND7 cells were transferred from media containing 10% FCS to medium containing either 0.5% or 1% FCS. In addition, the serum from new born calf (NCB) serum or adult bovine serum (ABS) were also studied to check if factors required for survival, differentiation or apoptosis were specific to FCS. Control cells were maintained in medium containing10% FCS. Analysis of the cell viability was measured by the ability of the cells to exclude Trypan Blue while the total cell counts were representitive of the cellular proliferation. Morphological changes were observed by light microscopy and were expressed as changes in the percentage of cells extending processes while the uptake of tritiated thymidine (^{3}H thymidine) from culture medium was taken as a measure of the DNA synthesis occuring in the different cultures.

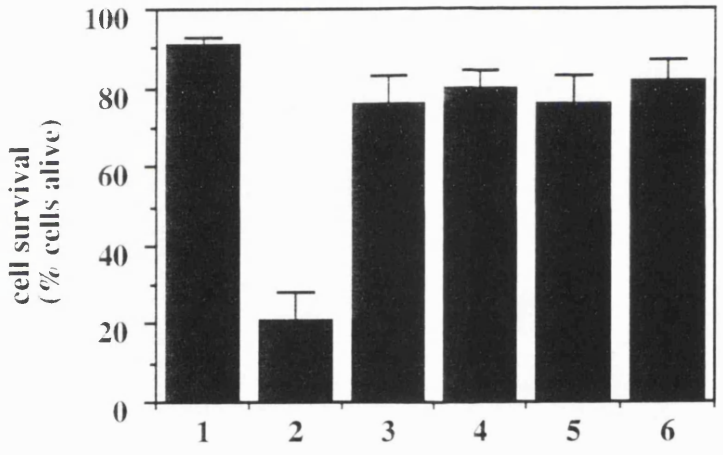
Analysis of the cell viability was carried out at 24 and 48 hours and the results are shown in figure 6.1.1. Very little cell death was observed in any of the cultures by 24 hours following transfer to the different media but by 48 hours, only 20% of the cells in the culture without serum were able to exclude Trypan Blue compared with the the cells which were maintained in media which contained either 0.5% FCS, 1% FCS or 10% NBC serum or ABS all of which showed cell survival approaching that in the control cells grown in 10% FCS. This therefore indicates that at this time point even 0.5% FCS can inhibit the programmed cell death observed in the cells maintained in the complete absence of serum. By 72 hours, however, the cells in media containing lower serum concentrations were showing lower survival rates, indicating that a serum factor which prevents cell death must be depleted by this time in these cultures.

Figure 6.1.1: Percentage of live cells (as determined by their ability to exclude trypan blue) at 24 hours (A), 48 hours ((B) and 72 hours (C) after the transfer of ND7 cells from medium containing 10% FCS (track 1) to medium containing either no serum (track 2), 0.5% FCS (track 3), 10% of newborn calf serum (4) or 10% adult calf serum (5). The values show the average of cell survival determined in three experiments.

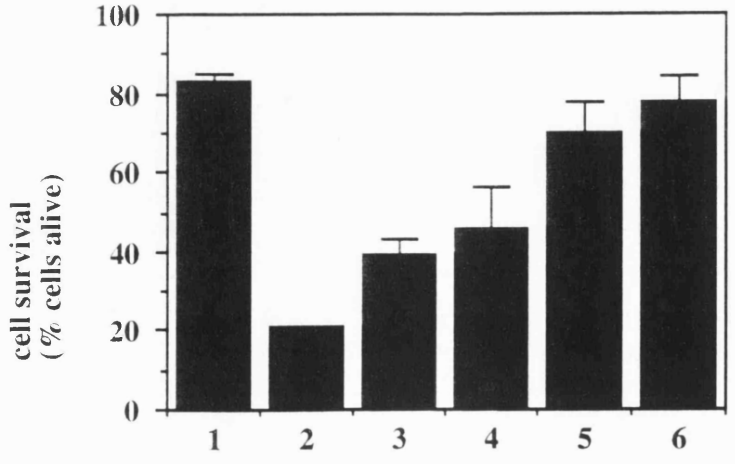
(A)


cell survival
(% cells alive)
100
80
60
40
20
0
1
2
3
4
5
6

(B)


cell survival
(% cells alive)
100
80
60
40
20
0
1
2
3
4
5
6

(C)

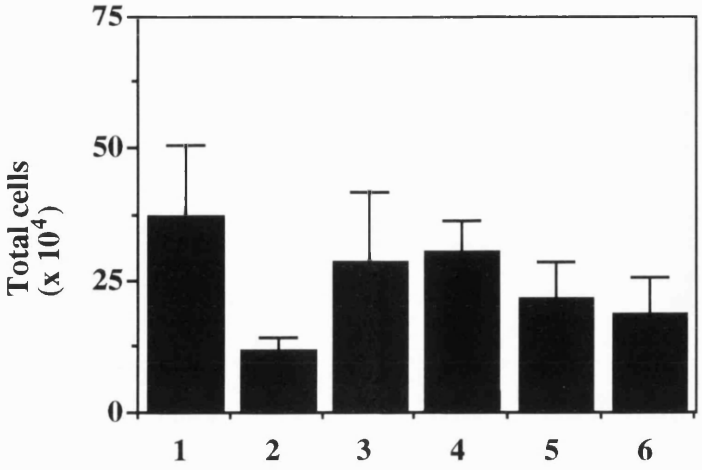

cell survival
(% cells alive)
100
80
60
40
20
0
1
2
3
4
5
6

In addition to the cell survival, the serum concentration also affected the rate of cellular proliferation as shown by measurement of the percentage of viable cells in each culture. After 24 hours of transfer to serum free medium, there was a significant decrease in the total cell number (figure 6.1.2A), but not in the cultures containing some serum. This effect on cells in the serum-free medium was more pronounced after 48 hours of transfer. However at this time point there was also a decrease in the total number of cells which were maintained in medium containing 0.5% FCS compared with the other cultures with the cell numbers being similar to that observed in serum free medium (figure 6.1.2B). These results therefore suggest that the cells in 0.5% serum had cease proliferation.

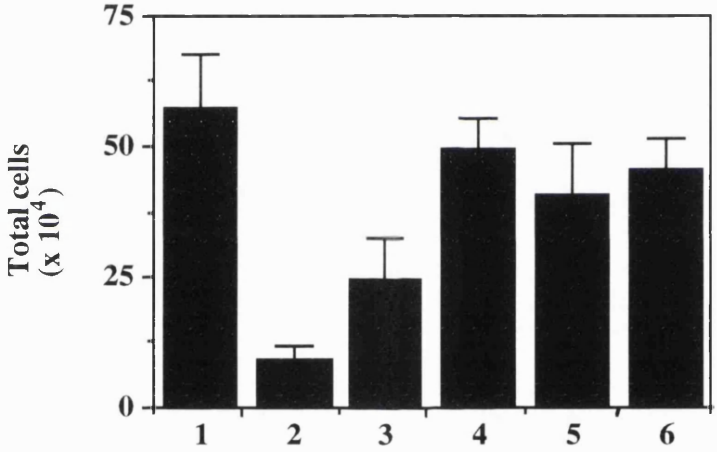
Thus it appears that although the cells had exited the cell cycle and ceased proliferation at 48 hours in medium containing 0.5% FCS there was no significant increase in the cell death hence there was little change in percentage of cells that remain viable in the culture at this time point. However, by 72 hours, the lower serum concentration also affected the ability of the cells to proliferate by such that even in 1% FCS there were markedly less cells than in 10% serum.

Figure 6.1.2: Total cell number in ND7 cell cultures after 24 hours in growth medium containing either 10% FCS (track 1), no serum (track 2), 0.5% serum (track 3), 1% serum (track 4), 10% new born calf serum (track 6) or 10% adult bovine serum. Values show the average of three experiments with the standard deviation shown by the bars.

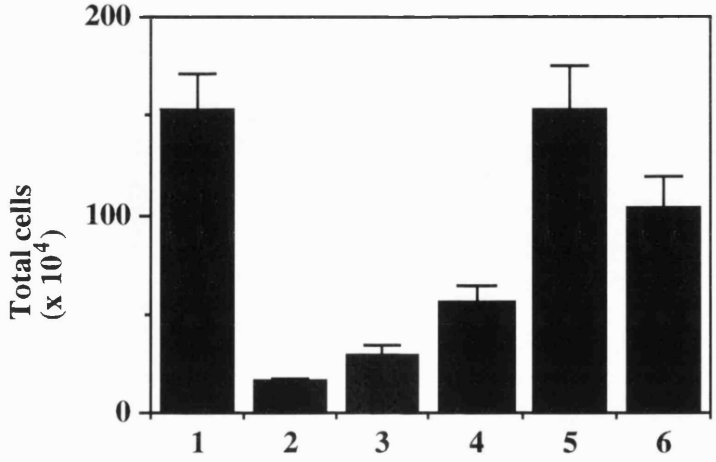
(A)


75
50
25
0
Total cells
(x 10^4)
1
2
3
4
5
6

(B)


75
50
25
0
Total cells
(x 10^4)
1
2
3
4
5
6

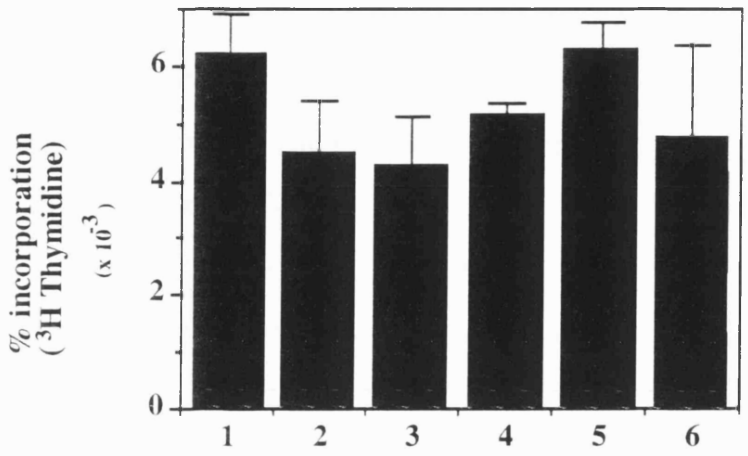
(C)


200
100
0
Total cells
(x 10^4)
1
2
3
4
5
6

Since 48 hours of treatment in 0.5% serum appear to define the state in which cell cycle arrest correlates with lack of significant cell death, the relative proportion of cells undergoing DNA synthesis and division in different culture media was estimated by measuring the level of [^{3}H] thymidine incorporation in cells at 24 and 48 hours. While there was a small difference at 24 hours in the levels of incorporation in cells grown in medium which contained no serum or 0.5% FCS compared with 10% FCS the levels were not markedly different to the cells grown in NBC serum and ABS indicating that some DNA synthesis was ongoing. By 48 hours however, the [^{3}H]-Thymidine incorporation by cells in 0.5% serum was similar to that observed in cells transferred to serum free media and these levels were significantly lower than the incorporation by cells maintained in 1% or 10% FCS as well as in 10% NBC serum or ABS (figure 6.1.3). These results suggest that whereas the ND7 cells continued to proliferate in medium containing 1% FCS they ceased dividing in the presence of 0.5% FCS in the medium. Moreover, the newborn calf or adult bovine serum were as effective as the FCS in maintaining the proliferation and viability of the cells.

Previous studies reported that ND7 cells transferred to serum free medium, ceased to proliferation and either undergo morphological differentiation or apoptosis. The results of cell survival and proliferation have indicated that in the presence of 0.5% serum there is arrest of proliferation without significant apoptosis. To test if the differentiated state of the ND7 cells changed in response to the different levels of FCS, the number cells extending neurite processes were taken as a measure of cells undergoing morphological differentiation (Suburo *et al.*, 1992) in ND7 cells grown in media containing either 10% FCS, no serum , 0.5% or 1% FCS. The number of cells with significant neurite outgrowth was expressed as a percentage of the total cells.

(A)



(B)

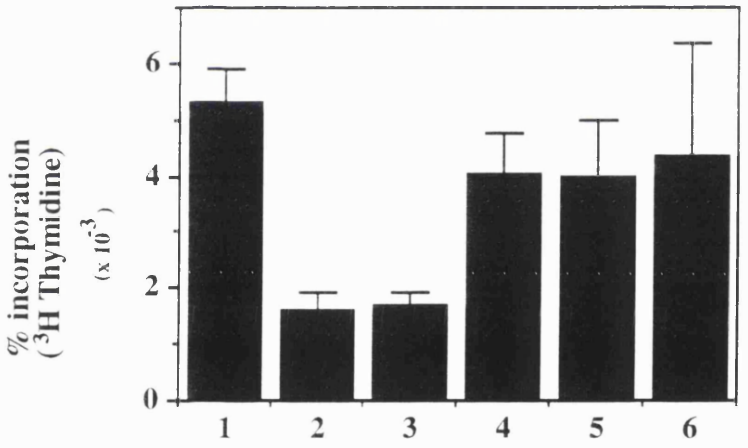
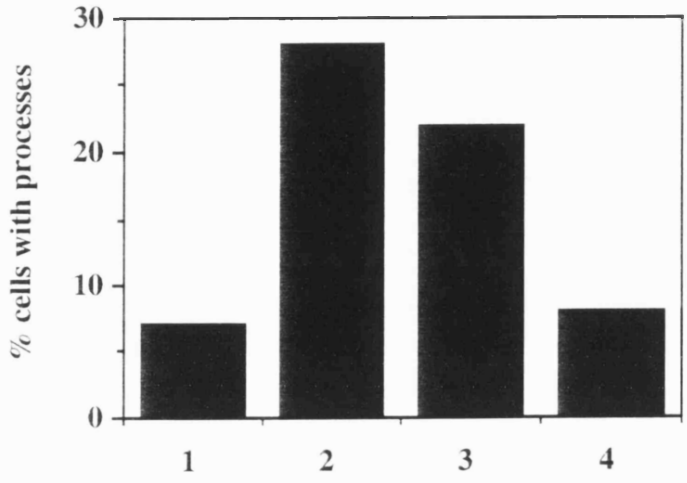


Figure 6.1.3: Incorporation of tritiated thymidine by ND7 cells at 24 hours (A) and after 48 hours (B) following transfer to medium containing 10% FCS (track 1), no serum (track 2), 0.5% FCS (track 3), 1% FCS (track 4), 10% newborn calf serum (track 5) or adult bovine serum (track 6). The values represent the incorporation per 10^{-4}cells and represent the average of three experiments.

There was little change in the number of cells with processes in the different media at 24 hours. However at 48 and 72 hours, the cells maintained in medium containing 0.5% FCS had extensive neurite outgrowth with numbers which were similar to the proportion observed in serum free medium (figure 6.1.4). In contrast, cells maintained in 1% FCS showed no enhancement of process formation when compared with cultures maintained in 10% FCS.By 72 hours there was a small increase in the number of cells with processes in 1% serum. This correlates with the finding that up to 48 hours, 1% FCS was able to mimic all the effects of full serum (10%) medium in cell replication, survival and differentiation while 0.5% serum was unable to maintain cellular proliferation or stop cells undergoing morphological differentiation but did prevent apoptosis. Thus at 48 hours the medium containing 0.5% FCS may define a state in which cell cycle arrest and differentiation can occur without significant programmed cell death. However by 72 hours there appears to be a limiting factor which may be depleted in 1% but not 10% serum which result in some changes in the proliferation, survival of these these cells. These results therefore suggests that factors present in serum may play a part in prevention of apoptosis in these cells and these may be different from factors required for cellular proliferation or if they are the same then much lower concentrations which have no effect on cell division are required to prevent apoptosis.

In an attempt to identify such factor(s) in serum which are required to inhibit apoptosis in ND7 cells, growth factors were added individually or in combination to serum free medium and cell survival was compared with cells maintained in 10% FCS or in serum free medium. None of these factors alone or in combination was sufficient to rescue these cells from apoptosis or to increasing proliferation (figure 6.1.5). However this combination of growth factors was shown to reduce the number of cells with neurite processes (see section 4.1.4, chapter 4) thus indicating that in these cells the signals regulating these processes may be independent.

(A)



(B)

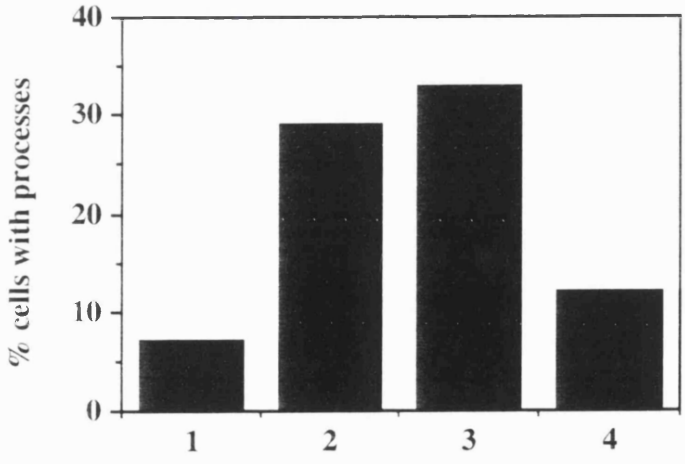


Figure 6.1.4: Percentage of ND7 cells bearing neurite processes after 48 hours (A) and 72 hours (B) of transfer from medium containing 10% FCS (track 1), no serum (track 2), 0.5% serum (track 3) or 1% serum (track 4).

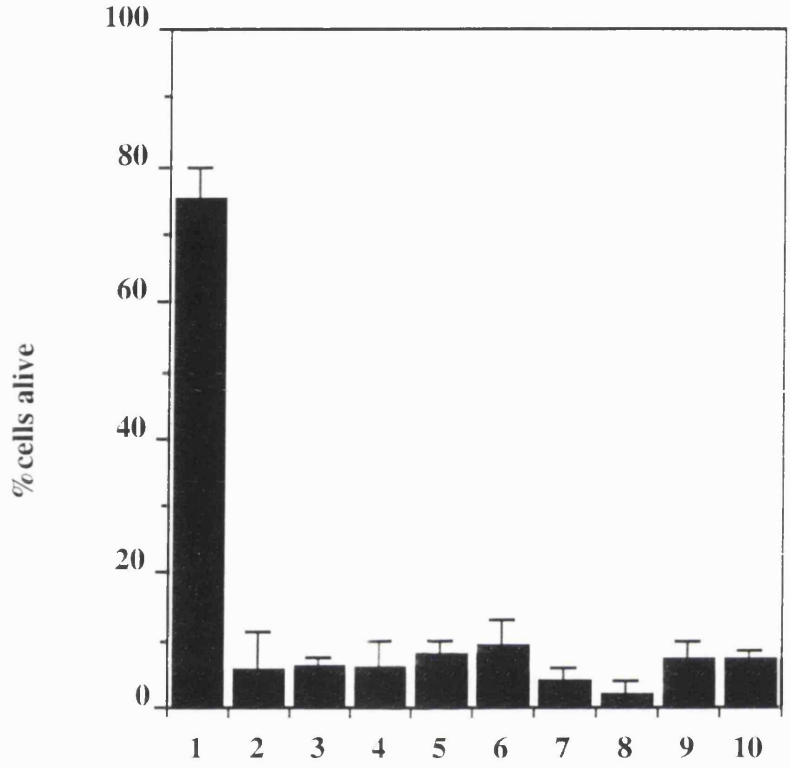


Figure 6.1.5: Percentage of cells which were alive (measured by the ability to exclude trypan blue) seventy two hours after transfer of ND7 cells to media containing either 10% FCS (track 1), no serum (track 2) or no serum supplemented with either 20 ng/ml EGF (track 3), 5 ng/ml acidic FGF (track 4), 5 ng/ml basic FGF (track 5), 20 ng/ml of IGF I (track 6), 20 ng/ml of IGF II (track 7), 50 ng/ml of NGF (track 8) or 10 ng/ml of PDGF (track 9) or a combination of different growth factors (track 10).

6.2.0 Discussion

While the phenomenon of apoptosis is well defined the mechanisms which mediate this process are still not clear and is an area of intensive research. During development, the limited number of homogeneous population of cells which can be acquired while the precursor cells are proliferating and before cell commitment have taken place restricted the studies to elucidate the mechanisms involved in cell cycle arrest, growth and commitment. Howard *et al.*, had shown that in the sensory neuronal cell line ND7, serum removal which induced the cells to exit the cell cycle resulted in either differentiation or apoptosis. The results of these studies therefore indicate that under certain circumstances cell cycle arrest can occur without significant cell death. While it appears that factors in serum may be involved in maintaining cellular proliferation and in inhibiting apoptosis, the precise nature of the factors responsible are still to be established. However, it appears that under certain conditions apoptosis can be dissociated from cell cycle arrest and morphological differentiation. These conditions therefore define an environment in which the distinct processes of differentiation and apoptosis can be studied the sensory neuronal derived ND7 cell line and may thus allow the elucidation of signals which induce processes such as neurite outgrowth independently of cell death.

Chapter 7

General Discussion

The growing numbers of POU domain proteins which are being identified and characterized provide the evidence for the importance of this class of transcriptional regulators in normal development and function of the nervous system. The mechanisms by which this increasingly complex group of proteins achieve precise transcriptional control is still not clear and much research is ongoing in this field.The results of this study in combination with the reports of other research groups during this period have lead to a number of important observations about the Brn-3 family of POU domain transcription factors. The initial isolation of Brn-3a POU domain sequence from brain cDNA and the subsequent identification of its expression in sensory neurons has shown that it is highly homologous to the unc-86 gene in the POU domain and it has been suggested that Brn-3a may be the mammalian homologue for the nematode factor (He *et al.*, 1989). Furthermore, the unc-86 gene product has been shown to be critical for the normal development and differentiation of specific sensory neurons thus implying that Brn-3a may be involved in the development and function of sensory neurons.

The subsequent isolation of the Brn-3b POU domain from cDNA obtained from a sensory neuronal cell line, ND7, in our laboratory has demonstrated that these two highly related factors are co-expressed in sensory neurons (Lillycrop *et al.*, 1992). Isolation of another related factor, Brn-3c, from the DRG (Ninkina *et al.*, 1993) have supported the evidence for a family of related transcription factors which are highly homologous in the POU domain. The isolation of the full length cDNA clones by other groups have revealed that there is limited homology outside the POU domain except for the highly conserved POU IV box in the amino terminus of these proteins and other members of the POU IV subgroup (Theil *et al.*, 1993, Gerrero *et al.*, 1993). The complexity of the Brn-3 family of proteins is further increased by the identification of different isoforms of the Brn-3a and Brn-3b proteins (Theil *et al.*, 1993, Gerrero *et al.*, 1993, Turner *et al.*, 1994) with the differences occuring primarily in the amino termini of these proteins which contain the POU IV box.

A study of the mRNA expression of Brn-3a and Brn-3b have revealed that both factors are expressed in adult rat brain and DRG but not in other tissues such as heart, liver, lung, kidney or spleen. This is in agreement with reports from other groups who have demonstrated the high expression of transcripts of both Brn-3a and Brn-3b in regions of the brain, retina, DRG and trigeminal ganglia both during development and in the adult but not in other tissues. However, we have also identified the mRNA of Brn-3a and Brn-3b in rat ovary and testis by semi-quantitative RT-PCR and in preliminary in-situ hybridization studies. While Brn-3a mRNA has been found at low levels, Brn-3b transcripts appeared to be more abundant. In addition, Brn-3b but not Brn-3a has also been identified in uterus and cervix. Furthermore, while Brn-3b mRNA appears to be localized to cells which may be undergoing proliferation, the identity of cells expressing Brn-3a is less clear and this will have to be established in additional experiments. A number of other POU proteins have also been identified in the testis and ovary, suggesting a role for these proteins in events which are in many cases still to be established. The ovarian and spermatogenic cycles share a number of common events such as cellular proliferation and differentiation under the influence of hormones such as the gonadotropins, FSH and LH, as well as the androgens, oestrogen, progesterone and testosterone. Furthermore, there are also changes in the expression of a number of factors such as cyclic AMP and growth factor which have been shown to regulate the Brn-3 mRNAs. Experiments to identify the precise cells in these tissues which express the Brn-3 mRNAs and the factors which affect expression will form the basis for future studies since identification of cell or stage specific expression may facilitate the identification of target genes regulated by these factors.

In neuronal cells there is overlapping expression of Brn-3a and Brn-3b, with Brn-3a being expressed at higher levels in cells with mature, differentiated, neuronal phenotypes while Brn-3b is more highly expressed in proliferating cells. Brn-3a expression appears to correlate with neurite outgrowth in these cells, an observation which has been supported by studies with stable cell lines transfected with the antisense

Brn-3a POU domain. Shorter neurites and lower levels of some neuronal proteins have been observed in the cells expressing the antisense mRNA (Lakin *et al.*, 1995).

The transcription of Brn-3a and Brn-3b mRNA appear to be regulated in opposite directions by common signalling pathways mediated by factors such as cyclic AMP and growth factors. This opposite regulation of the mRNA expression in the ND7 cells is accompanied by antagonistic effect on promoter activity. Brn-3a is a strong activator of transcription of either a heterologous promoter containing an octamer-related DNA binding site which was previously shown to bind the POU domain of both factors (Theil, *et al.*, 1993; Budhram-Mahadeo *et al.*, 1994; Morris *et al.*, 1994) or cellular promoters such as the α-internexin (Budhram-Mahadeo *et al.,* 1995), SNAP-25 (Lakin *et al.*, 1995); CRH and POMC promoters (Gerrero *et al.*, 1993). Studies with Brn-3b(s) has shown that this protein act as a repressor of promoter activity of the heterologous promoter containing an octamer-related DNA binding site (chapter 4 and Budhram-Mahadeo *et al.*, 1994) and the α-internexin (Budhram-Mahadeo *et al.,* 1995). Furthermore, Brn-3b appears to interact with Brn-3a to repress its activating potential. This observation has been reinforced by reports of experiments by Theil *et al.*, (1994) who reported that Brn-3a(l) isoform is capable of transforming fibroblast by cooperative interactions with Ha-Ras but in the presence of Brn-3b this transforming potential is lost. Further evidence for interactions between these proteins has also been provided by the binding studies carried out by this group. Thus it appears that these proteins are capable of interactions which may provide a mechanism by which they modulate gene activity in cells which co-express both factors.

While we have observed repression of gene activity by Brn-3b(s), Turner *et al.*, (1994) have reported that Brn-3b(l) activates the CRH gene promoter. It is possible therefore, that the differences in the amino terminus of these proteins allow conformational changes which modulate their effect on promoter activity. In experiments to analyse the domains of the Brn-3a and Brn-3b proteins which contribute to the observed

transcriptional effect, it has been shown that the POU domain of Brn-3b(s) acts as a repressor of promoter activity either when present with other domains derived from Brn-3a in chimeric constructs or in isolation from other domains. However, in the Brn-3a proteins there appears to be two activation domains, one in the POU domain which is required for the activation of a heterologous promoter containing the octamer-related binding site while the other, present in the amino terminus, has been shown to be critical for activation of the α-internexin promoter (Morris, *et al.*, 1994; Budhram-Mahadeo *et al.*, 1995). It may be speculated that Brn-3b(l) which contains the amino terminal domain may contain an activation domain in this region which is not present in the Brn-3b(s) isoform and that this domain in Brn-3b(l) overrides the repressive nature of the POU domain depending on the promoter. This remains to be established.

The amino terminal domains of Brn-3a(l) and Brn-3b(l) contain the highly conserved POU IV box which has some homology to a region of the c-myc protein thought to be involved in protein-protein interactions (Gerrero *et al.,* 1993). The precise function of this domain in the POU-IV class of proteins remains to be established. However, it appears that the activation domain present in this N' terminus of the Brn-3a proteins is distinct from this POU IV box since Brn-3a(s) which lacks the POU-IV box is still a strong activator. However, the ability of Brn-3a(l), but not Brn-3a(s) to transform fibroblasts by cooperative interactions with Ha-Ras may suggest that this POU-IV box facilitates interactions with other proteins which cannot be achieved by Brn-3a(s). Furthermore, we have shown that Brn-3a(s), but not Brn-3a(l) can activate the full length POMC promoter in ND7 cells and BHK cells which do not normally express this protein while Gerrero *et al.*, (1993) have shown that Brn-3a(l) strongly activated this promoter in AtT20 pituitary cell line which express endogenous POMC. These results therefore also suggest that interactions of cell-specific proteins with the amino terminal domain of Brn-3a(l) may modulate the activation potential of the protein. Thus, Brn-3a(s) may have stronger transcriptional activation potential but is unable to transform

cells while Brn-3a(l) can activate some promoters depending on the cell type but also has the ability to transform cells.

The highly conserved nature of this region suggests an important function. Hence, this provides an area for future investigations which will aim to characterize the activation domain in the amino terminal domain of Brn-3a proteins and to study the function of the POU IV box. Ongoing work to determine the expression of these isoforms during development and in tissues and cell lines are being carried out by others in our laboratory. Such information may provide a clearer picture of the mechanisms of action and the functions of these proteins.

A final interesting observation arising from this study is that the site in the α-internexin promoter which binds the Brn-3 proteins is distinct from the octamer binding sites and from any of the established POU protein binding sites. Furthermore, it appears that the 'coding strand' of this sequence binds both Brn-3a and Brn-3b with higher affinity in the single stranded conformation. This represents a novel site with distinct binding property by the POU proteins. It is possible that while these proteins can bind with weak affinity to the octamer site, this novel site may represent the true Brn-3 binding site and provide another mechanism for diversity of interactions by these factors. The DNA sequences which are contacted by the POU domain of these proteins will be investigated in future studies by DNAse 1 footprinting and mutational analysis studies.

Thus, Brn-3a and Brn-3b which are two members of the Brn-3 family of proteins have been shown to be related but distinct proteins which have overlapping expression but different functions. Much work remains to be done to establish the precise targets and roles within the cells but the evidence so far suggests that this may prove to be exciting and interesting in this rapidly changing field of research.

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The levels of the antagonistic POU family transcription factors Brn-3a and Brn-3b in neuronal cells are regulated in opposite directions by serum growth factors

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Abstract

The Brn-3a and Brn-3b proteins are closely related POU family transcription factors with generally antagonistic effects on gene expression. We show that transfer of ND7 neuronal cells to medium containing either no foetal calf serum or low concentrations of serum results in a rise in Brn-3a mRNA levels and a fall in Brn-3b mRNA levels, although the precise serum dependence of these two effects differ. These effects can be reversed by addition of specific growth factors to the medium lacking serum, although not all growth factor treatments which suppress the rise in Brn-3a can reverse the fall in Brn-3b levels. These effects do not correlate with the effects of each treatment on cellular proliferation indicating that they are not simply a consequence of changes in proliferation. Interestingly however, treatments which produce a rise in Brn-3a levels also induce the outgrowth of neuritic processes. Hence the expression of a functionally antagonistic pair of POU factors is regulated in opposite directions by treatments with serum growth factors and this is likely to represent one means by which such growth factors modulate the gene expression patterns and ultimately the behaviour of neuronal cells.

Keywords: POU family; Transcription factors; Brn-3a, -3b; Neuronal cells; Growth factors

The development of the nervous system involves a balance between proliferation of neuronal precursors, the differentiation of these cells into mature non-dividing neurons and the death of a significant proportion of the neuronal cells which are initially present by apoptosis (for review, see Ref. [13]). The balance between these processes is regulated by specific neurotrophic factors such as nerve growth factor [3,12] as well as by growth factors such as the fibroblast growth factors [5,17].

Ultimately such factors act by regulating the transcription of specific genes whose protein products are required for the appropriate response to the factor [8]. In turn such genes are activated by the binding of specific cellular transcription factors to their regulatory regions. To do this the transcription factors must themselves be appropriately regulated so that they produce gene activation only in response to the appropriate treatment. This is achieved either by regulating the synthesis of the transcription factor so that it is synthesized only in response to the appropriate treatment or by post-translational regulation of the activity of a pre-existing factor [9].

In order to analyze the processes involved in neuronal differentiation we have used the ND7 neuronal cell line which was obtained by fusing non-dividing rat dorsal root ganglion cells with the N18 mouse neuroblastoma cell line [19]. Although these cells proliferate indefinitely in culture, following transfer to serum free medium, they undergo cell cycle arrest and morphological differentiation into a mature neuronal phenotype exhibiting numerous dendritic processes [14,18] whilst a proportion of the cells undergo cell death by apoptosis [1,7].

We have previously shown [10] that during this differentiation process, the levels of the POU family transcription factor Brn-3a rise whilst that of the closely related factor Brn-3b fall. Such a finding is of particular interest since Brn-3a acts as an activator of gene transcription whereas Brn-3b both inhibits basal promoter activity and prevents activation by Brn-3a [2,11]. Moreover, a gene promoter containing the binding site for these factors is activated during ND7 cell differentiation [2] indicating

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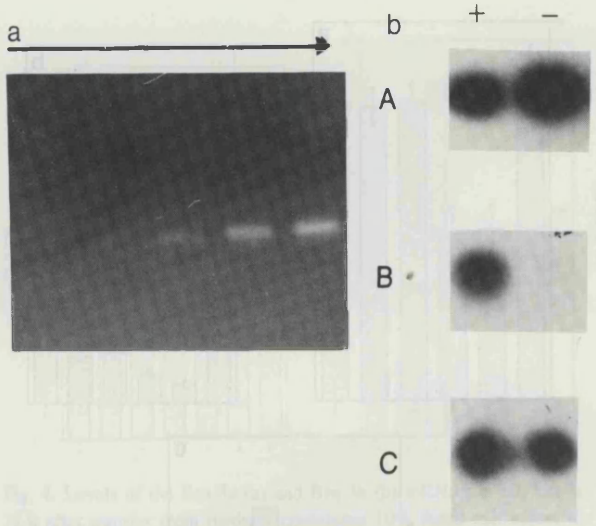

Fig. 1. (a) Amplification of the Brn-3a mRNA with increasing number of PCR cycles (indicated by the arrow). Cycle numbers were 15, 20, 25, 30, 35. Note the increase in the product between 20 and 30 cycles indicating the linear range of the assay. (b) PCR amplification of the Brn-3a (A) , Brn-3b (B) and L6 ribosomal protein (C) mRNAs in samples prepared from cells grown in the presence or absence of foetal calf serum.

that the changes in transcription factor levels occurring during this process can have functional effects on gene activity.

To investigate further the processes regulating the expression of these factors in ND7 cells, we wished initially to study their response to different levels of serum in the growth medium. Thus we have previously shown that the effects of serum removal on growth arrest and process formation can be dissociated from its effect on apoptosis since low concentrations of serum (0.5%) which allow growth arrest and process formation can effectively prevent apoptosis [1].

ND7 cells were therefore grown in RPMI medium containing 10% foetal calf serum and either maintained in this medium or transferred to medium lacking serum or containing 0.5% or 1% foetal calf serum. Seventy-two hours after transfer, RNA was isolated by the guanidinium isothyocyanate procedure [4]. One microgram of total RNA was used as a template for the preparation of cDNA using random hexanucleotide primers (Pharmacia LKB Biotechnology Ltd.). The levels of the Brn-3a and Brn-3b mRNAs were then measured by polymerase chain reaction (PCR) amplification using primers specific for each of these mRNAs [2,10].

Amplifications were routinely carried out using 23, 28 and 33 cycles of amplification for Brn-3a and 20, 25 and 28 cycles for Brn-3b which we had previously determined to be in the exponential phase of the reaction allowing reliable quantitation of the product (Fig. 1a). Any differences in the total amount of mRNA in each sample or in the efficiency of amplification were controlled by equalizing the values obtained on the basis of control amplification using primers specific for the constitutively expressed mRNA encoding the L6 ribosomal protein. All samples were run on a 2% agarose gel and southern blotted with random primer labelled probe for Brn-3a or Brn-3b. The resulting filters were exposed to X-ray film and the signal quantitated by densitometric scanning. After equalization for the value obtained in the control L6 amplification values were expressed as a percentage of the value in untreated ND7 cells.

In these experiments (Figs. 1b and 2) the level of the Brn-3a mRNA rose in the cells transferred to serum free medium as expected from our previous results [10]. A similar rise was also observed in the cells transferred to medium containing 0.5% serum, a condition under which growth arrest and process outgrowth still occur but the cells do not undergo apoptosis [1]. In contrast, no increase in the level of the Brn-3a mRNA occurred in the cells transferred to medium containing 1% foetal calf serum in which cellular proliferation continues and no processes are extended [1]. Indeed under these conditions the level of the Brn-3a mRNA actually fell below that in cells maintained in medium containing 10% foetal calf serum.

In contrast, and as expected from our previous results [10], the level of the Brn-3b mRNA fell upon transfer to serum free medium (Fig. 2b). Interestingly however, similar although less extensive decreases were also consistently observed in the cells transferred to either 0.5% or 1% serum indicating that this effect occurs at different low concentrations of serum regardless of their effect on cellular proliferation and process outgrowth. Similar changes in the Brn-3a and Brn-3b mRNA levels were observed in three similar experiments using different

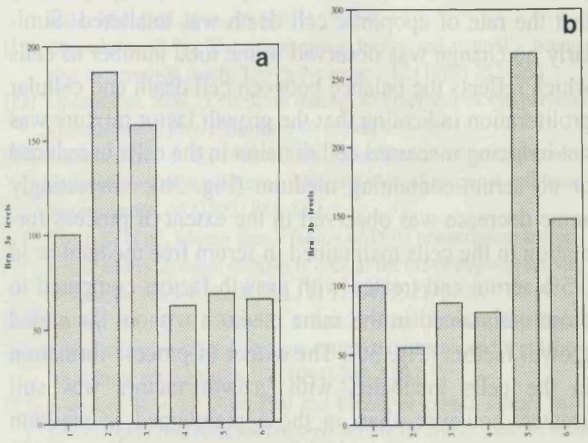

Fig. 2. Levels of the Brn-3a (a) and Brn-3b (b) mRNAs 72 h after transfer from medium containing 10% foetal calf serum to either the same medium (track 1), or medium containing no serum (track 2), 0.5% serum (track 3), 1% serum (track 4), no serum plus 5 ng/ml acidic FGF, 5 ng/ml basic FGF, 20 ng/ml insulin-like growth factor I and 20 ng/ml insulin-like growth factor II (track 5) or 0.5% serum plus same growth factor mixture (track 6). All values are normalized relative to the level observed in cells grown in medium plus 10% foetal calf serum and are the average of at least three determinations with independent RNA preparations.

:NA preparations and in experiments using an internal ontrol template capable of being amplified with Brn-3 rimers but yielding a product of different size to the en- ogenous mRNA (date not shown).

Having established that Brn-3b mRNA levels fell in ıedium containing various low concentrations of serum vhereas the rise in Brn-3a mRNA levels appeared to cor- elate with the cessation of cellular proliferation and ex- ension of neurite processes, we endeavoured to test this orrelation further. To do this, ND7 cells were transferred o serum free medium containing a mixture of several erum growth factors, namely acidic and basis fibroblast growth factors and insulin-like growth factors types I and I. The levels of the Brn-3a and Brn-3b mRNAs were neasured as before. In these experiments (Fig. 2a), the nixture of growth factors was able to abolish the increase n the Brn-3a mRNA which is normally observed upon ransfer to serum free medium without growth factors and ven resulted in a decrease below the basal level observed n full serum-containing medium. Conversely, the growth actors abolished the fall in the Brn-3b mRNA normally bserved in ND7 cells transferred to serum free medium nd produced an increase in the level of this mRNA bove that observed in full serum-containing medium Fig. 2b). Similar results were also observed in cells trans- erred to medium containing 0.5% serum with the added growth factors (Fig. 2).

To correlate these changes with other changes occur- ing in the ND7 cells, we investigated the effects of growth factor addition on proliferation, survival and process formation of ND7 cells. In these experiments Fig. 3a) growth factor addition had no effect on the per- entage of cells able to exclude trypan blue which was aken as a measure of cellular survival [2,7] indicating hat the rate of apoptotic cell death was unaltered. Simi- arly no change was observed in the total number of cells which reflects the balance between cell death and cellular proliferation indicating that the growth factor mixture was ıot inducing increased cell division in the cells in reduced or no serum-containing medium (Fig. 3b). Interestingly some decrease was observed in the extent of process for- nation in the cells maintained in serum free medium or in 0.5% serum and treated with growth factors compared to hose maintained in the same medium without the added growth factors (Fig. 3c). The extent of process formation n the cells incubated with growth factors was still greater, however, than in those maintained in medium ontaining 1% serum or in full serum-containing medium.

These findings suggest that an increase in Brn-3b lev- els and a decrease in Brn-3a levels occurs under condi- ions where either serum or a mixture of growth factors are added to the cells and are independent of any effects of these treatments on cellular proliferation. The precise requirements of this modulation in the levels of these factors are distinct, however, since Brn-3b levels decline in 1% serum whereas no corresponding rise in Brn-3a

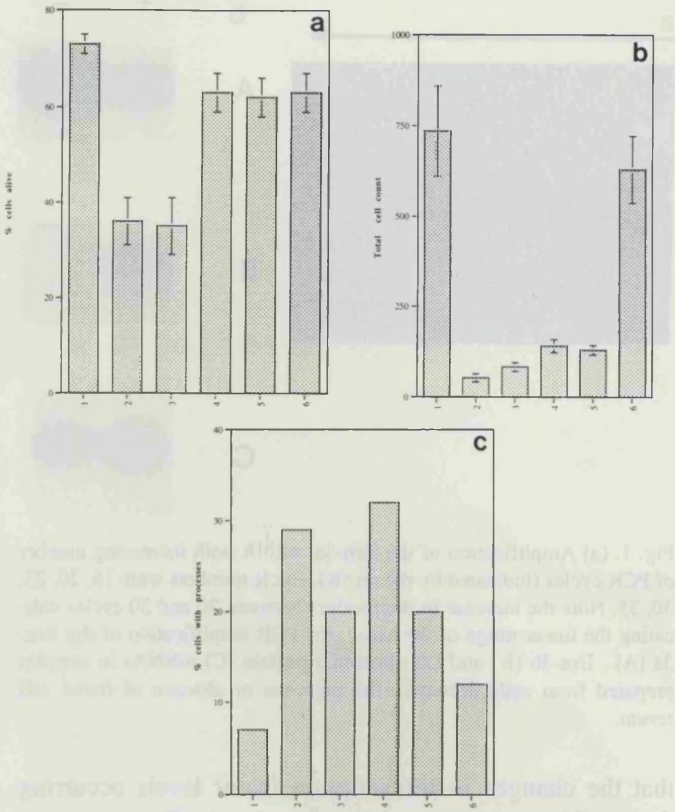

Fig. 3. Effect on the percentage of cells able to exclude trypan blue (a), the total number of cells (b) and on the percentage of cells bearing neuritic processes (c) of transferring ND7 cells from medium containing 10% foetal calf serum to the same medium (track 1), medium containing no serum (track 2) medium containing no serum plus added acidic and basic FGF, IGF-I and II (track 3) 0.5% serum-containing medium (track 4) 0.5% serum-containing medium plus added acidic and basic FGF, IGF-I and II (track 5) and 1% serum-containing medium (track 6).

levels occurs. Thus the modulation in Brn-3b levels does not correlate with any specific change in the cellular behaviour of the ND7 cells whereas the rise in Brn-3a levels is associated with a high degree of neurite outgrowth and the levels of this mRNA fall under conditions where reduced process formation occurs either following growth factor addition or in high serum concentrations.

To investigate the effects of the individual growth factors on Brn-3 levels, ND7 cells were transferred to serum free medium in the presence of each individual growth factor. In these experiments each of the factors was able to abolish the rise in Brn-3a levels which normally occurs upon transfer to serum-free medium indicating that each factor independently can mimic the effect of the mixture (Fig. 4a). In contrast, none of the factors was able to reproduce the rise in Brn-3b levels produced by the mixture of the four factors and in fact the individual factors acted to decrease the level of the Brn-3b mRNA (Fig. 4b). A similar absence of any increase in Brn-3b mRNA levels was seen with pairwise combinations of the factors (data not shown) indicating that increased expression requires all the factors acting together.

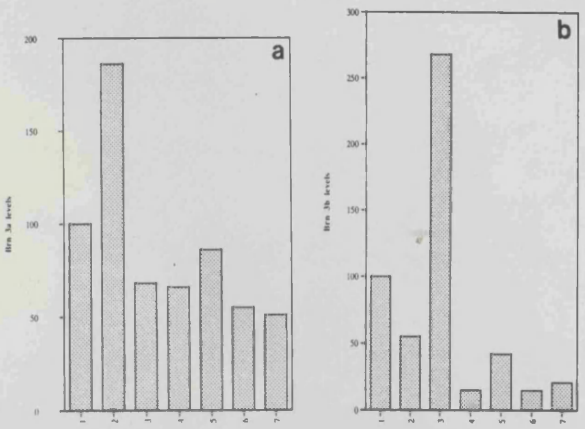

Fig. 4. Levels of the Brn-3a (a) and Brn-3b (b) mRNAs in ND7 cells 72 h after transfer from medium containing 10% foetal calf serum to the same medium (track 1) or to serum free medium either alone (track 2) or containing a mixture of acidic and basic FGF and IGFI and II (track 3), acidic FGF alone (track 4), basic FGF alone (track 5), IGFI alone (track 6) or IGFII alone (track 7). All values are normalised relative to the level in cells grown in medium plus 10% foetal calf serum and are the average of at least three determinations with independent RNA preparations.

These findings suggest therefore that the levels of the Brn-3a and Brn-3b mRNAs are oppositely regulated in ND7 neuronal cells with serum growth factors producing a rise in the level of the Brn-3b mRNA and a fall in the level of the Brn-3a mRNA. Interestingly, however, the precise requirements of these two effects are distinct both in terms of their serum dependence and the effects of individual growth factors. Moreover, since they can be dissected away from the effect of these factors on cellular proliferation, the changes in Brn-3a or Brn-3b mRNA levels are unlikely to be simply a consequence of changes in cellular growth. Rather they are likely to reflect direct effects of cellular signalling pathways on the activity of the genes encoding Brn-3a and Brn-3b.

As Brn-3a and Brn-3b have generally antagonistic effects on the activity of specific genes, such changes in their relative levels of expression are likely to affect the expression of these genes. Since both Brn-3a and Brn-3b are expressed in the developing and adult nervous system [6,15,16,20] such changes may have effects on cellular events occurring in neuronal cells. In particular, the data presented here suggest a correlation between a rise in Brn-3a mRNA levels and process outgrowth by ND7 cells. In agreement with this we have recently shown (Lakin et al., submitted) that the anti-sense RNA-mediated inhibition of the rise in Brn-3a levels greatly reduces neurite outgrowth by ND7 cells transferred to serum free medium whilst cellular proliferation is arrested as in control cells. Although these results require further confirmation in primary neuronal cells in vivo they do suggest that changes in Brn-3 expression can exert specific functional effects on neuronal phenotype. The data presented here establish that such expression changes can occur in response to treatment with specific serum-containing growth factors and that these treatments frequently but not always produce opposite changes in the expression of Brn-3a and Brn-3b.

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Cell cycle arrest and morphological differentiation can occur in the absence of apoptosis in a neuronal cell line

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Key words: Apoptotic cell death; Cell cycle arrest; Morphological differentiation

Apoptotic cell death plays a critical role in the development of the nervous system. Although the apoptotic death of mature non-dividing neurons has been extensively studied, the mechanisms mediating the extensive cell death in areas of the developing brain where proliferating neuroblasts are differentiating into mature neurons have not been analyzed. We have previously shown that the cell cycle arrest of a proliferating neuronal cell line by transfer from medium containing 10% foetal calf serum (FCS) to serum-free medium results in the morphological differentiation of some cells and the death of others by apoptosis. Here we show that the effect of 10% FCS can be mimicked by medium containing either similar concentrations of newborn or adult bovine serum or 1% FCS all of which maintain cellular proliferation and inhibit differentiation and apoptosis. In contrast, the presence of 0.5% FCS in the medium effectively prevents apoptosis but does not allow cellular proliferaton or inhibit morphological differentiation. Hence cell cycle arrest and differentiation can occur in the absence of apoptosis in cells of neuronal origin and the factors in serum responsible for modulating these processes are likely to be distinct.

The normal development of the nervous system involves the correct balance between the proliferation of neuronal cell precursors, their differentiation to the non-dividing phenotype characteristic of mature neurons and the death of a significant proportion of the neuronal cells which are initially present by the process of programmed cell death or apoptosis (for reviews see refs. 9, 14). Thus early in development extensive cell death is observed in areas of the nervous system where proliferating neuroblasts are ceasing division and differentiating into mature neurons [5, 7] and this has been shown to involve cells which have recently undergone DNA replication and are not yet fully differentiated [2]. Similarly more mature non-dividing neurons also undergo programmed cell death which is regulated by the limited supply of neurotrophic factors derived from the target field [1, 5].

A number of factors influencing neuron survival and/or proliferation have been defined including nerve growth factor and other neurotrophic factors [8] growth factors such as the fibroblast growth factors [4, 11] and cyclic nucleotides such as cyclic GMP [12]. However, the limited amounts of material from regions containing proliferating or differentiating neuronal precursors has thus far prevented a detailed molecular analysis of the processes regulating neuronal cell proliferation, differentiation and apoptosis.

In an attempt to analyze the mechanisms involved in these processes, we have developed a model system using the ND7 neuronal cell line, one of a series of cell lines obtained by fusing non-dividing rat dorsal root ganglion cells with the N18 mouse neuroblastoma cell line and selecting for the HAT resistance of the ganglion cells [13]. The ND7 cell line proliferates indefinitely in serum-containing medium but retains many ganglion cell characteristics which were absent in the parental neuroblastoma [10, 13]. Following exposure to serum free medium however, the cells undergo cell cycle arrest and morphological differentiation into a mature neuronal phenotype exhibiting numerous dendritic processes [10]. This behaviour mimics that of primary isolates of proliferating neuronal precursor cells which also undergo morphological differentiation upon exposure to serum free medium [3]. Interestingly however, following transfer to serum free medium, a number of the ND7 cells do not undergo differentiation but instead die by apoptosis which can be detected on the basis of DNA fragmentation and morphologically by light and electron microscopy [6].

Hence, in this system, cell cycle arrest appears to result in both morphological differentiation and programmed cell death providing a system in which the balance be-

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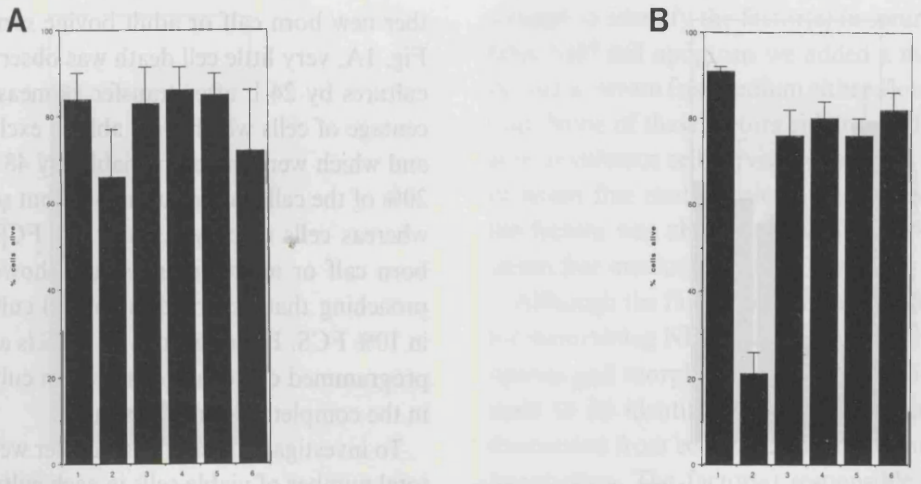

Fig. 1. Percentage of live cells (as determined by the ability to exclude Trypan Blue) 24 h (A) and 48 h (B) after transfer of ND7 cells from medium containing 10% FCS to medium containing either 10% FCS (1), no serum (2), 0.5% FCS (3) 1% FCS (4), 10% new born calf serum (5) or 10% adult bovine serum (6). Values are the average of three determinations whose standard deviation is shown by the bars.

tween proliferation, differentiation and death can be studied in neuronal cells in vitro. In this report, we have studied the serum requirements of each of these processes and have shown that cell survival can be dissociated from proliferation and inhibition of morphological differentiation on the basis of its requirement for a lower concentration of serum.

Cell culture. Routinely, ND7 cells were grown in RPMI medium supplemented with 10% FCS. To induce the cells to cease dividing and undergo morphological differentiation they were transferred to serum free medium consisting of a 1:1 mix of Dulbecco's MEM (Gibco) and Nutrient mix Ham's F12 (Gibco) supplemented with human transferrin (5 μg/ml), bovine insulin (250 ng/ml) and sodium selenite (30 nM). In other experiments cells were incubated in the same medium with the appropriate concentration of serum as indicated.

Trypan blue staining. Cells were aspirated and spun for 10 min at 4,000 rpm at 4°C. The cell pellet was resuspended in 50 μl of medium and an equal volume of 0.4% Trypan Blue in PBS was added. The mixture was incubated at room temperature for 5 min and the proportion of cells able to exclude Trypan Blue counted in a counting chamber.

Cell counts. (A) Total cells: Total cell number was determined as the total number of both live and dead cells in the Trypan Blue staining test. A minimum of three counts was done per sample with triplicate samples for each treatment.

(b) Cells with processes: Cells were photographed at the specified periods after treatment. The number of cells with processes was then determined and expressed as a percentage of the total.

(c) [^{3}H]Thymidine incorporation: Cell cultures were

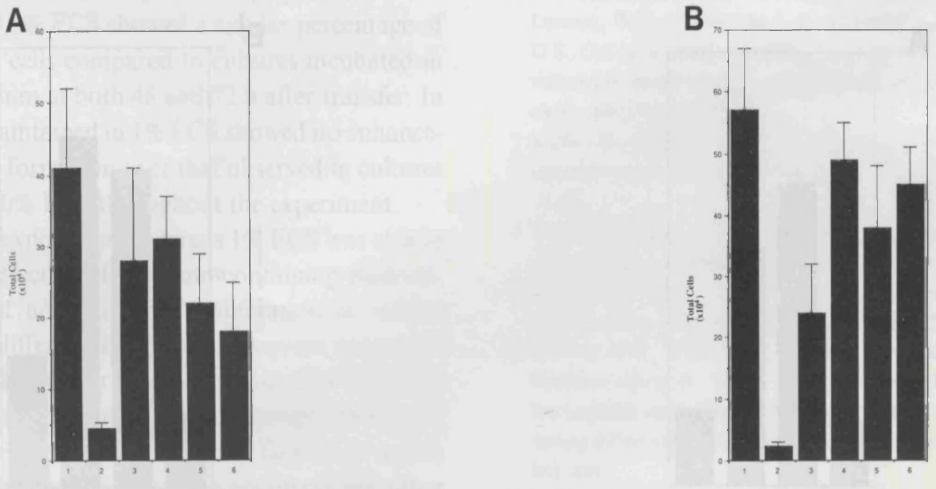

Fig. 2. Total cell number in ND7 cell cultures 24 h (A) and 48 h (B) after transfer to medium containing 10% FCS (1), no serum (2), 0.5% FCS (3), 1% FCS (4), 10% new born calf serum (5) or 10% adult bovine serum (6). Values are the average of three determinations whose standard deviation is shown by the bars.

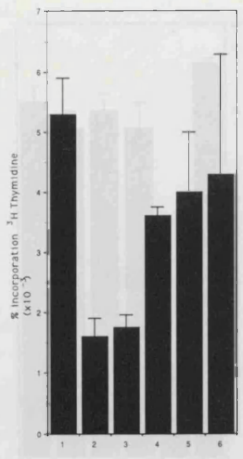

Fig. 3. Incorporation of tritiated thymidine by ND7 cell cultures after transfer to medium containing 10% FCS (1), no serum (2), 0.5% FCS (3), 1% FCS (4), 10% new born calf serum (5) or 10% adult bovine serum (6). Values represent the incorporation per 10^4 cells and are the average of three determinations whose standard deviation is shown by the bars.

incubated in the presence of [^{3}H]thymidine for 4 h. Cells were harvested and the incorporation of ^{3}H]thymidine was determined by precipitating cellular DNA with 10% trichloroacetic acid (TCA). Values shown represent the average relative amounts of thymidine incorporation in triplicate cultures compared to triplicate control cultures (proliferating ND7 cells).

In previous experiments [6], we showed that whilst ND7 cells grown in 10% FCS proliferated and did not show significant cell death, cells transferred to serum free medium ceased proliferation and whilst some cells underwent morphological differentiation, others underwent programmed cell death. ND7 cells were therefore transferred from medium containing 10% FCS to medium containing different concentrations of FCS or either new born calf or adult bovine serum. As shown in Fig. 1A, very little cell death was observed in any of the cultures by 24 h after transfer as measured by the percentage of cells which were able to exclude Trypan Blue and which were therefore viable. By 48 h, however, only 20% of the cells in the culture without serum were viable whereas cells in only 0.5% or 1% FCS or in 10% new born calf or adult bovine serum showed viabilities approaching that observed in control cultures maintained in 10% FCS. Hence even 0.5% FCS is able to inhibit the programmed cell death observed in cultures maintained in the complete absence of serum.

To investigate these effects further we also counted the total number of viable cells in each culture. At 24 h after transfer (Fig. 2A), the cells maintained in the absence of serum already showed a significant fall in total cell number. This effect was exacerbated by 48 h after transfer (Fig. 2B). Interestingly however, a decrease in total viable cell number was also observed at this time point in the cultures maintained in 0.5% FCS compared to other cultures. The lower total number of viable cells in this culture even though it did not show enhanced cell death suggested that the cells in this culture had ceased to proliferate paralleling the absence of proliferation we previously observed in serum free medium [6]. Hence this culture would show reduced cell numbers compared to the cultures in which the cells were proliferating even in the absence of significant cell death.

To test this possibility we measured the level of tritiated thymidine incorporation in each culture. As illustrated in Fig. 3, the cells maintained in 0.5% FCS showed no incorporation of thymidine above the background level observed in cells maintained in the absence of serum. In contrast significant incorporation was observed in cells maintained in 1% or 10% FCS as well as in

Fig. 4. Percentage of ND7 cells bearing neuritic processes 48 h (A) and 72 h (B) after transfer to medium containing 10% FCS (1), no serum (2), 0.5% FCS (3) or 1% FCS (4).

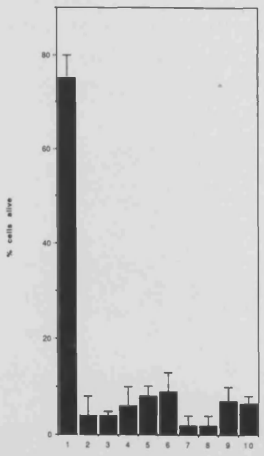

Fig. 5. Percentage of live cells able to exclude trypan blue seventy two h after transfer of ND7 cells to medium containing 10% FCS (1), no serum (2) or no serum supplemented with either 20 ng/ml epidermal growth factor (3), 5 ng/ml acidic FGF (4), 5 ng/ml basic FGF (5), 20 ng/ml Insulin-like growth factor I (6), 20 ng/ml Insulin-like growth factor II (7), 50 ng/ml nerve growth factor (8), 10 ng/ml platelet derived growth factor (9) or with all the indicated growth factors [10]. Values are the average of three determinations whose standard deviation is shown by the bars.

the cells cultured in new born calf or adult bovine serum. Hence as indicated by a comparison of the total numbers and percentage of viable cells in each culture, ND7 cells continue to proliferate in 1% FCS but do not do so to a significant extent in 0.5% FCS.

In our previous experiments [6] the absence of serum, induced arrest of ND7 cell proliferation, induction of apoptosis and morphological differentiation. In the experiments described so far we have shown that two of these effects can be dissociated with 0.5% FCS resulting in the arrest of cell proliferation without significant apoptosis. We therefore tested the effect of transfer to medium containing different concentrations of FCS on the morphological differentiation of ND7 cells as measured by the extension of neuritic processes [10]. As shown in Fig. 4, cells in 0.5% FCS showed a similar percentage of process bearing cells compared to cultures incubated in serum free medium at both 48 and 72 h after transfer. In contrast cells maintained in 1% FCS showed no enhancement of process formation over that observed in cultures maintained in 10% FCS throughout the experiment.

Hence in our experiments whereas 1% FCS was able to mimic all the effects of full serum-containing medium, 0.5% serum did not maintain proliferation or inhibit morphological differentiation but did prevent apoptosis. This indicates that under some circumstances cell cycle arrest can occur without significant programmed cell death. Moreover, it suggests that the factors in serum which are required to prevent ND7 apoptosis are either different from those which are required for cellular proliferation or are required at a lower concentration. In an attempt to identify the factor(s) in serum required to inhibit ND7 cell apoptosis we added a number of growth factors to serum free medium either alone or in combination. None of these factors either together or singly was able to enhance cell survival compared to that observed in serum free medium alone (Fig. 5). Similarly none of the factors was able to allow ND7 cell proliferation in serum free medium (data not shown).

Although the factor(s) in serum which are responsible for maintaining ND7 cell proliferation and inhibiting apoptosis and morphological differentiation, therefore remain to be identified it is clear that apoptosis can be dissociated from cell cycle arrest and morphological differentiation. The factor(s) responsible for these events are therefore likely to be distinct. Similarly, our observations indicate for the first time conditions under which ND7 cells can undergo cell cycle arrest and morphological differentiation in the absence of apoptosis, allowing easier study of the process of differentiation.

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Activation of the α-Internexin Promoter by the Brn-3a Transcription Factor Is Dependent on the N-terminal Region of the Protein*

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The Brn-3a, Brn-3b, and Brn-3c proteins are closely related POU (Pit-Oct-Unc) family transcription factors which are expressed predominantly in neuronal cells. We have identified the α-internexin gene as the first reported, neuronally expressed, target gene whose promoter activity is modulated by these factors. Both the Brn-3a and Brn-3c factors can activate the α-internexin promoter while Brn-3b represses it and can prevent activation by Brn-3a. Using chimeric constructs containing different regions of Brn-3a or Brn-3b, we show that activation of the α-internexin promoter requires the N-terminal region of Brn-3a. In contrast the activation by Brn-3a but not Brn-3b of an artificial promoter containing a synthetic Brn-3 binding site can be shown using the same constructs to be dependent on the POU domain of Brn-3a. Moreover, the isolated POU domain of Brn-3a can activate this artificial promoter but not the α-internexin promoter. Hence Brn-3a contains two distinct transactivation domains, at the N terminus and within the POU domain, whose effect is dependent upon the target promoter. The relationship of gene transactivation by Brn-3a to its ability to transform primary cells which is also dependent on the N-terminal region of the protein is discussed.

The POU (Pit-Oct-Unc) family of transcription factors are defined on the basis of a common POU domain which constitutes the DNA binding domain of these proteins and which can be subdivided into a POU-specific domain and a POU-homeodomain separated by a short linker region (for reviews, see Refs. 1 and 2). In contrast to the classical homeodomain proteins, the POU proteins bind to extended DNA sequences related to the consensus octamer motif ATGCAAAT and thereby influence transcription (1–3). Such modulation of transcription by POU factors plays a critical role in the development of specific cell types. Thus, for example, the Pit-1 factor has been shown to be essential for pituitary gland development in both mice and humans (4, 5), while the *unc*-86 mutation in the nematode results in the failure to develop specific neuronal cell types particularly sensory neurons (6, 7).

Among the mammalian POU proteins, the Brn-3 family of factors are the most closely related to *unc*-86 within the POU domain and these proteins together with the *Drosophila* factors I-POU and tI-POU (8, 9) constitute the POU-IV class of POU factors (1, 2). Following the identification of the founder member of the Brn-3 family (10), now known as Brn-3a (11–13) or Brn-3.0 (10, 14), two other members of this family have been identified. Like Brn-3a, each of these factors, Brn-3b (12, 15) (also known as Brn-3.2) (16) and Brn-3c (17) (also known as Brn-3.1) (14) are expressed at high levels in sensory neurons paralleling the important role of Unc-86 in this cell type and are also present in other neuronal cells but not in most non-neuronal cell types (12, 14, 17). The three different Brn-3 factors are encoded by three distinct genes (13) and show only restricted homology to one another outside the POU domain (10–18).

These sequence differences between the different Brn-3 factors are paralleled by differences in their activity. Thus both Brn-3a and Brn-3c can co-operate with the Ha-*ras* oncogene to transform primary rat embryo fibroblasts (11). In contrast Brn-3b does not possess this ability and can inhibit the transforming effect of Brn-3a in co-transfection experiments.[1] The transforming activity of Brn-3a is dependent on the N-terminal region of the protein (11)[1] which contains a short region known as the POU-IV box that is common to several different POU-IV family members (11, 14, 15).

In addition to differences in their transforming ability, the Brn-3 proteins can also have radically different effects on promoter activity. Thus, while the POU domains of all three Brn-3 factors can bind to the octamer motif, a thymidine kinase promoter containing an added synthetic octamer motif is transactivated by co-transfection with Brn-3a and Brn-3c expression vectors and inhibited by Brn-3b (20). Although, as in the transformation experiments, Brn-3b can inhibit trans-activation by Brn-3a (20), the ability of Brn-3a to trans-activate the target promoter is not dependent on the N-terminal region of the protein. Rather chimeric molecules containing only the POU domain of Brn-3a linked to the rest of the Brn-3b molecule are able to activate this promoter, whereas a molecule containing the Brn-3b POU domain linked to the remainder of Brn-3a represses it (21). Hence, the differences in the activity of the Brn-3 factors on this test promoter are determined by the POU domain.

These differences in the region of the Brn-3a protein required for transactivation of an artificial test promoter and

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[1] T. Theil and T. Möröy, submitted for publication.

transformation suggest the possibility that transformation by Brn-3a might not involve transactivation of target genes. Alternatively, the region of Brn-3a required for transactivation may be different depending on the target promoter with some naturally occurring promoters whose protein products are necessary for transformation requiring the N-terminal domain for transactivation rather than the POU domain.

To investigate this question, we have identified the gene encoding the neuronal intermediate filament protein α-internexin (22) as the first neuronally expressed target genes transactivated by Brn-3a and have shown that such transactivation requires the N-terminal region of the protein.

MATERIALS AND METHODS

Plasmid DNAs—The longest α-internexin promoter constructs contained the region of the promoter from 1219 bases upstream of the start site of transcription to 73 bases downstream linked to the chloramphenicol acetyl transferase (CAT)[2] gene (22). The other constructs contained either 254 or 77 bases of upstream sequence linked to the identical downstream sequence (22). The artificial reporter plasmid contains the Brn-3 octamer-related binding site ATGCTAATGAGAT cloned into the *Bam*HI site in the vector pBL CAT 2 which contains the herpes simplex virus thymidine kinase promoter from −105 to +51 driving expression of the CAT gene (23). The Brn-3a, -3b, and -3c expression vectors contain full-length cDNA or genomic clones for each of these proteins (11) cloned under the control of the Moloney murine leukemia virus promoter in the vector pLTR poly which has been modified by deletion of a cryptic splice site in the SV40 3′ untranslated region (24).

DNA Transfection—Transfection of DNA was carried out according to the method of Gorman (24). Routinely 1×10^6 BHK-21 cells (25) or ND7 cells (26) were transfected with 10 μg of the reporter plasmid and 10 μg of the Brn-3 expression vectors. In all cases cells were harvested 72 h later. The amount of DNA taken up by the cells in each case was measured by slot blotting the extract and hybridization with a probe derived from the ampicillin resistance gene in the plasmid vector (27). This value was then used to normalize the values obtained in the CAT assay as a control for differences in uptake of plasmid DNA in each sample.

CAT Assay—Assays of chloramphenicol acetyl transferase activity were carried out according to the method of Gorman (24) using samples which had been equalized for protein content as determined by the method of Bradford (28).

RESULTS

To identify a natural target gene for the Brn-3 factors we tested the promoters of several genes which like the Brn-3 factors (10, 12, 17) are expressed in sensory neurons and which contain octamer or octamer-related sequences in their promoters that could potentially bind Brn-3. In each case, the gene promoter driving the gene encoding the marker CAT protein was co-transfected with vectors directing the expression of each of the forms of Brn-3 (11). Transfections were carried out both into BHK-21 fibroblast cells (25) which lack endogenous Brn-3 as well as into the ND7 cell line derived by the immortalization of primary sensory neurons (26) which expresses all the different forms of Brn-3 (12).

In these experiments, no effect of any of the forms of Brn-3 was observed on the promoters of the genes encoding either calcitonin gene related peptide or tyrosine hydroxylase (data not shown) despite the presence of octamer-related motifs in these promoters (29, 30). In contrast the promoter of the gene encoding the neuronal intermediate filament protein α-internexin showed strong activation by both Brn-3a and Brn-3c expression vectors when co-transfected into the BHK cells, whereas the Brn-3b expression vector repressed the basal activity of the α-internexin promoter (Fig. 1). These effects were also observed when the promoter was co-transfected with the various Brn-3 expression vectors into the ND7 cell line which expresses endogenous Brn-3 (Fig. 2).

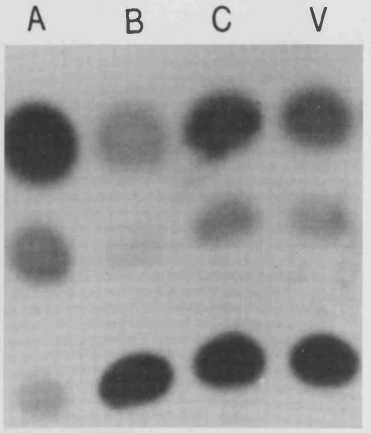

FIG. 1. **Assay of CAT activity in BHK-21 cells transfected with a reporter construct containing the α-internexin promoter (from −1219 to +73 relative to the transcriptional start site) linked to the CAT gene and expression vectors encoding Brn-3a (*A*), Brn-3b (*B*), Brn-3c (*C*) or the empty expression vector (v).**

These results with a natural promoter therefore parallel our previous results with a promoter containing synthetic octamer-related Brn-3 binding sites with activation being observed both with Brn-3a and Brn-3c while Brn-3b has an inhibitory effect on promoter activity (20, 21). As in our previous experiments (20, 21), Brn-3b was able to inhibit the stimulatory effect of Brn-3a when the two expression vectors were co-transfected together with the α-internexin promoter (Fig. 2). Hence the natural α-internexin promoter responds to the different forms of Brn-3 in a similar manner to the tK promoter containing added synthetic octamer-related motifs.

The α-internexin promoter contains three octamer-related motifs in the region upstream of the transcriptional start site which might act as targets for Brn-3 binding (22). Surprisingly, however, a construct containing 254 bases of upstream sequence which lacks all these motifs still responded to the various forms of Brn-3 in a manner identical to the full length construct (Fig. 3*A*) while a construct containing only 77 bases of upstream sequence was stimulated more strongly by Brn-3a and Brn-3c and was still repressed by Brn-3b (Fig. 3*B*). Hence the sequences which mediate the response of the α-internexin promoter to Brn-3 appear to be located within the 77 bases upstream of the transcriptional start site and/or the 73 bases downstream which are present within the shortest construct. In experiments involving more extensive deletions we have been unable to eliminate the responsiveness of the promoter without also eliminating its basal activity suggesting that this responsiveness is a property of the minimal α-internexin promoter. The recent observation that sequences very different from the classical octamer motif can bind Brn-3 very strongly (14, 31) suggests that the minimal α-internexin promoter contains such sequences allowing it to respond to Brn-3.

In previous experiments we have shown that treatment of ND7 cells with dibutyryl cyclic AMP results in an increase in the level of the Brn-3a mRNA and a decrease in the level of the Brn-3b mRNA (12). This results in a corresponding increase in the activity of a transfected tK promoter linked to a synthetic octamer motif which is not observed for the unmodified tK promoter lacking this added binding site for Brn-3 (20). Interestingly, the level of the α-internexin mRNA has been shown to be increased following cyclic AMP treatment (22), although the gene promoter does not contain any cyclic AMP response elements (CRE) which normally mediate the transcriptional response to this compound (32).

We therefore transfected ND7 cells with the constructs con-

[2] The abbreviations used are: CAT, chloramphenicol acetyltransferase; CRE, cyclic AMP response element.

taining different amounts of the internexin promoter 5′ upstream sequence and measured their response to treatment with 1 mM dibutyryl cyclic AMP. In these experiments (Fig. 4) all three constructs were stimulated by cyclic AMP with the strongest response being observed for the shortest construct paralleling its strong activation by Brn-3a. These findings together with the absence of a CRE in either the shortest construct or further upstream (22) suggest that the Brn-3 factors may play a role in the activation of the α-internexin promoter by cyclic AMP paralleling their role in the cyclic AMP inducibility of the tK promoter carrying a synthetic octamer motif (20). Consistent with this possibility, the α-internexin promoter constructs did not respond to cyclic AMP in BHK-21 fibroblast cells which do not express Brn-3a (data not shown).

As discussed above, the effects of cyclic AMP and the different forms of Brn-3 on the α-internexin promoter parallel our findings using an artificial promoter containing a Brn-3 binding site and extend them to a natural promoter. In our previous experiments using this artificial promoter (21), we used chimeric constructs containing different regions of Brn-3a or Brn-3b (Fig. 5) to show that the ability of Brn-3a to activate this promoter was dependent on its POU domain so that for example construct 1 (AAAB) was not able to activate the promoter, whereas construct 4 (-BBA) was able to do so (21) (Fig. 5). We therefore tested the effect of these chimeric constructs in co-transfections with the full internexin promoter (−1219 to +73).

As illustrated in Fig. 6, the effect of these chimeric constructs was completely different to that which we had previously observed. Thus for example construct 1 (AAAB) was now able to activate this promoter as was construct 3 (AABB). In contrast construct 4 (-BBA) did not activate the promoter, although unlike intact Brn-3b it did not repress it. These findings indicate, therefore, that in the case of the α-internexin promoter, the N-terminal region of Brn-3a rather than the POU domain is of critical importance for transactivation. Similar results were also obtained with the shorter α-internexin promoter con-

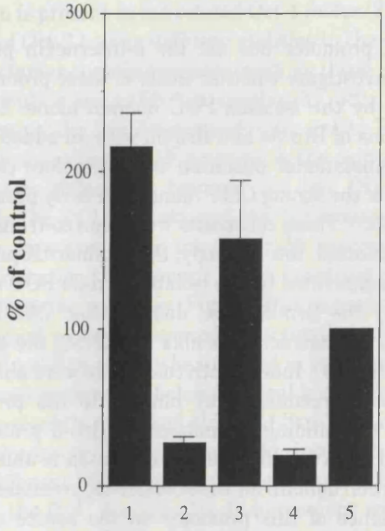

FIG. 2. **Assay of CAT activity in ND7 cells transected with the α-internexin promoter and expression vectors encoding Brn-3a (*track 1*), Brn-3b (*track 2*), Brn-3c (*track 3*), a mixture of the Brn-3a and Brn-3b vectors (*track 4*) or the empty expression vector alone (*track 5*).** Values are expressed relative to the level of CAT activity upon co-tranfection of α-internexin and the empty expression vector alone and are the average of four determinations whose standard error is shown by the *bars*.

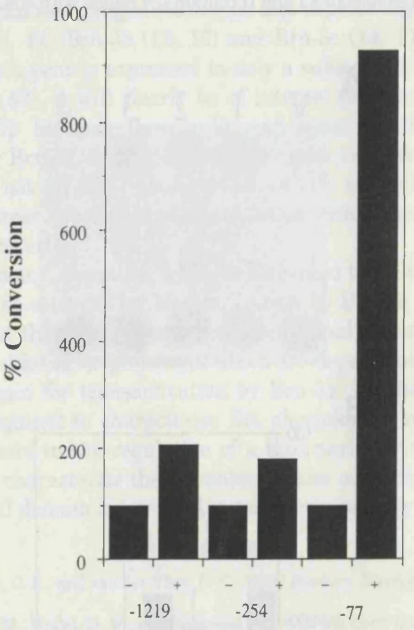

FIG. 4. **Assay of CAT activity in ND7 cells transfected with the indicated α-internexin constructs and either left untreated (−) or treated with 1 mM dibutyryl cyclic AMP (+).** Values are expressed relative to the level observed with each construct in the absence of cyclic AMP.

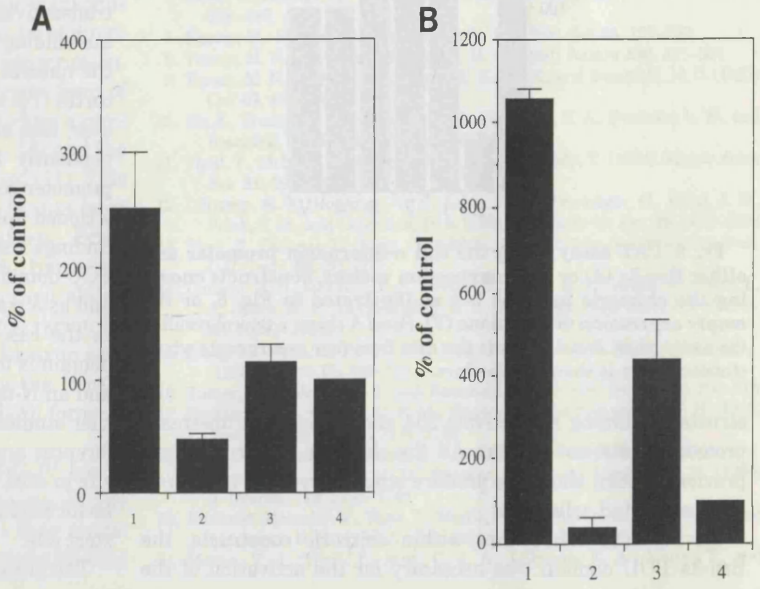

FIG. 3. **Assay of CAT activity in ND7 cells transfected with reporter constructs containing the α-internexin promoter from −254 to −73 (*Panel A*) or −77 to +73 (*Panel B*) linked to the CAT gene and expression vectors encoding Brn-3a (*track 1*), Brn-3b (*track 2*), Brn-3c (*track 3*) or the empty expression vector alone (*track 4*).** Values are expressed relative to the value obtained with expression vector alone and are the average of three determinations whose standard error is shown by the bars.

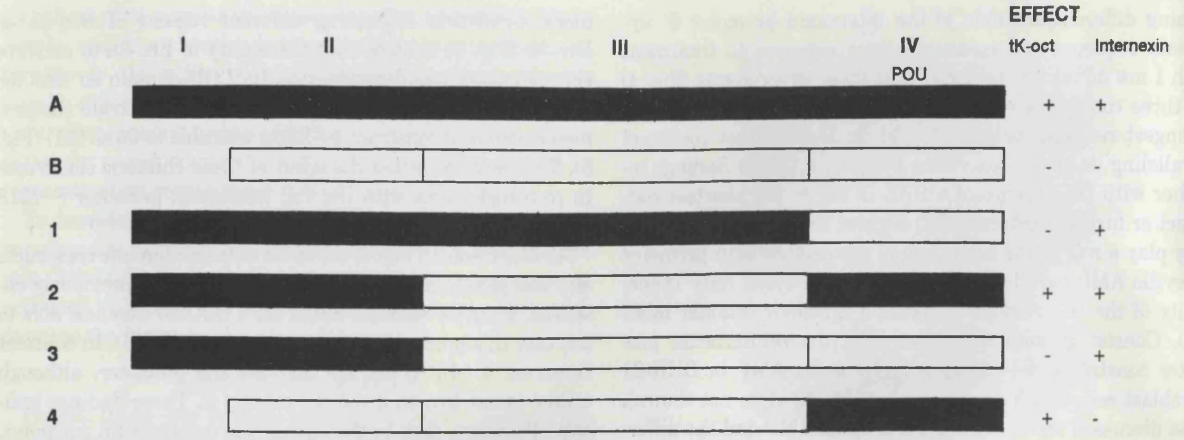

FIG. 5. **Summary of gene trans-activation data obtained using either the tK promoter carrying a synthetic octamer motif (tK-Oct) (data from Morris *et al.* (21)) or the α-internexin promoter (see Fig. 6) together with Brn-3a or -b or with constructs encoding chimeric proteins with different regions derived from Brn-3a or Brn-3b.** The division of Brn-3a and -3b subdomains I, II, III, and IV is as follows. For Brn-3a: *domain I*, amino acids 1–40; *domain II*, amino acids 41–108; *domain III*, amino acids 109–267; *domain IV* (POU domain), amino acids 268-end. For Brn-3b: *subdomain II*, amino acids 1–92; *subdomain III*, amino acids 93–169; subdomain IV (POU domain), amino acids 170-end. The domains I and II contain the region with similarity to the POU factors I-POU and UNC 86 (11, 14) while region IV contains the POU domain.

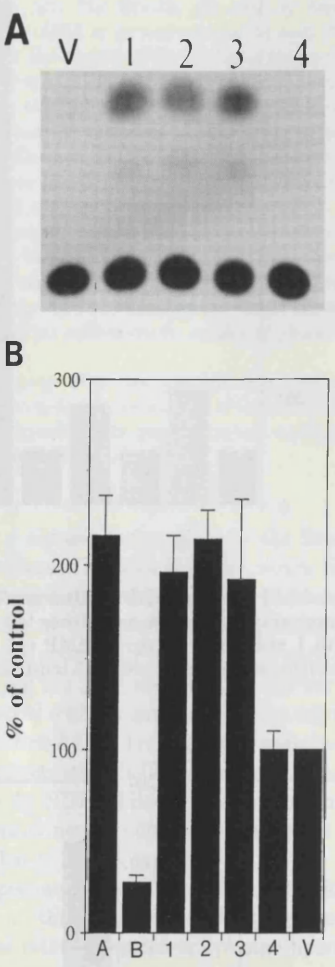

FIG. 6. **CAT assay using the full α-internexin promoter and either Brn-3a (*A*) or b (*B*) expression vectors, constructs encoding the chimeric proteins 1–4 as illustrated in Fig. 5, or the empty expression vector alone (*V*).** *Panel A* shows a typical result of the assay while *Panel B* shows the data from four experiments whose standard error is shown by the *bars*.

structs containing respectively 254 and 77 bases of upstream promoter (data not shown). All the chimeric constructs have previously been shown to produce similar levels of protein in the transfected cells (21).[1]

Having established that within chimeric constructs, the Brn-3a POU domain was necessary for the activation of the octamer-tK promoter but not the α-internexin promoter we wished to investigate whether either of these promoters would be affected by the isolated POU domain alone. The isolated POU domains of Brn-3a and Brn-3b with an added ATG codon to allow translational initiation were therefore cloned under the control of the strong CMV immediate-early promoter in the pJ7 vector (33). These constructs were then co-transfected with the two promoters. Interestingly, the octamer-tK promoter was clearly transactivated by the isolated Brn-3a POU domain and inhibited by the Brn-3b POU domain (Fig. 7*A*). In contrast, neither of the constructs was able to activate the α-internexin promoter (Fig. 7*B*). Indeed both constructs were able to repress this promoter presumably by binding to the promoter and preventing the binding of endogenous Brn-3 proteins. Hence the isolated DNA binding domain of Brn-3a is able to act as a transactivation domain for the octamer-tK promoter explaining the dependence of this promoter on the source of the POU domain when it is co-transfected with different natural and chimeric forms of Brn-3. In contrast this domain is inactive on the α-internexin promoter in agreement with the data indicating that the N-terminal domain of Brn-3a is required for transactivation of this promoter.

DISCUSSION

The Brn-3a transcription factor has been shown to be able to transactivate both artificial promoters containing an appropriate binding site cloned upstream of a test promoter (14, 21) or the natural promoters for the genes encoding proopiomelanocortin (14) and α-internexin (this report). Here we show, however, that at least two distinct regions of the protein can independently mediate such transactivation depending on the promoter concerned. Thus in the case of the tK promoter with a cloned upstream binding site we have extended our previous findings using chimeric constructs to show that the isolated POU domain of Brn-3a can act both as a DNA binding domain and as a transactivation domain for this promoter. In contrast in the case of the α-internexin promoter, the Brn-3a POU domain is inactive either in isolation or in chimeric constructs and an N-terminal region is required for transactivation. Further studies will be required to determine whether these differences are determined by the sequence of the Brn-3 binding site in each promoter, its context relative to other transcription factor binding sites or its position relative to the transcriptional start site.

The presence of two distinct activation domains in Brn-3a is

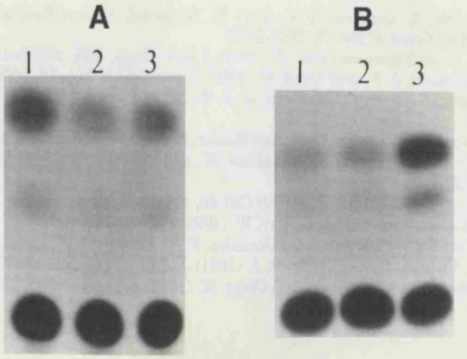

FIG. 7. **Assay of CAT activity in cells transfected with either the tK promoter carrying a synthetic octamer motif (*Panel A*) or the α-internexin promoter (*Panel B*) together with expression vectors encoding the isolated POU domain of Brn-3a (*1*) or Brn-3b (*2*) or the empty expression vector alone (*3*).**

paralleled in the Oct-2 POU transcription factor which contains two distinct activation domains at the N and C termini of the protein (35, 36). Interestingly only the N-terminal activation domain is present in the related Oct-1 factor (37), resulting in Oct-1 and Oct-2 having differing abilities in the activation of different octamer-containing genes such as those encoding the immunoglobulins and snRNA molecules (37, 38).

In the case of the Brn-3 factors, Brn-3b unlike Brn-3a is not able to activate either the tK-octamer or the α-internexin promoters due to differences between the two POU factors in respectively the POU domain and the N-terminal region. Interestingly, however, while intact Brn-3b represses the α-internexin promoter, the chimeric -BBA construct did not activate or repress the promoter (Fig. 6). This suggests that whilst the N-terminal region is required for activation by Brn-3a, the POU domain of Brn-3b may be involved in its ability to repress the α-internexin promoter below its basal level. This is likely to involve the recently described ability of Brn-3b to interact with Brn-3a and prevent its binding to DNA[1] since such interactions between different POU proteins normally involve heterodimerization via the POU domain (34). In agreement with this idea, unlike Brn-3b, the -BBA construct was not able to interfere with activation of the internexin promoter when co-transfected with Brn-3a (data not shown).

The N-terminal region of Brn-3a which activates transcription contains the POU-IV box, a region of 40 amino acids which is also present in Brn-3c, and the other members of the POU-IV family, I-POU, tI-POU, and Unc-86 (11, 14). Interestingly only a fragment of this POU-IV box is present in the form of Brn-3b we have used here which is derived from the major transcript in the spinal cord (11). Hence the POU-IV box may be of direct relevance to the ability of Brn-3a to activate transcription. Interestingly however, a transcript encoding a longer form of Brn-3b with additional N-terminal sequences including a complete POU-IV box has been detected in the retina (15) and the CNS (16), although it was absent in the spinal cord (11). This longer form of Brn-3b (also known as Brn-3.2:-) (16) has been shown to activate a promoter containing a synthetic Brn-3 binding site upstream of the prolactin promoter (16). Hence two distinct forms of Brn-3b may exist which differ in their ability to activate promoters which require the N-terminal region for transactivation. However, neither form would be likely to activate promoters which rely on transactivation by the Brn-3a POU domain since the POU domain common to both forms of Brn-3b is inactive in this assay.

Interestingly the N-terminal region of the POU-IV box is similar to a domain found in the N terminus of all c-*myc* family members (14) which has been shown to modulate their transforming ability (39). It has therefore been suggested (11) that the POU-IV box may be involved in the ability of Brn-3a to co-operate with the Ha-*ras* protein in transforming primary cells in the same manner as the c-*myc* proteins. Whatever the precise role of this box, it is clear that the N-terminal region of Brn-3a is involved both in transactivation of some promoters (this report) and transformation (11).[1] Hence the transforming ability of Brn-3a is likely to be dependent on the ability of its N-terminal region to activate specific target genes whose protein products are required for transformation.

Although the activation of the gene encoding the neuronal intermediate filament protein α-internexin is unlikely to be relevant to transformation of fibroblasts by Brn-3a, this gene may well represent a physiologically relevant target for Brn-3a within the nervous system. Thus the only previously described natural promoter to be activated by Brn-3a is derived from the gene encoding proopiomelanocortin which is expressed in the pituitary gland where levels of Brn-3a are so low as to be undetectable by *in situ* hybridization (14). In contrast α-internexin is widely expressed in the developing and adult nervous system (19, 40) with high levels being found in sensory neurons within dorsal root ganglia (40) which also express high levels of Brn-3a (10, 14) Brn-3b (12, 16) and Brn-3c (14, 17). As the α-internexin gene is expressed in only a subset of cells within the DRG (40), it will clearly be of interest to determine the relationship between these cells and those which express Brn-3a or Brn-3c, both of which have also been detected in some but not all DRG neurons (10, 14, 17) as well as those which express Brn-3b whose distrubution within the DRG has not been reported.

In summary, therefore, we have identified the α-internexin promoter as a target for transactivation by Brn-3a and have shown that this effect requires an N-terminal transactivation domain unlike other promoters which are dependent upon the POU domain for transactivation by Brn-3a. Further studies will be required to characterize the physiological role of the Brn-3 factors in the regulation of α-internexin expression as well as to characterize the promoters whose activation via the N-terminal domain is involved in transformation by Brn-3a.

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The DNA target site for the Brn-3 POU family transcription factors can confer responsiveness to cyclic AMP and removal of serum in neuronal cells

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ABSTRACT

The POU factors Brn-3a and Brn-3b are closely related transcription factors which are expressed in neuronal cells. The levels of the transcripts encoding these factors are regulated in opposite directions in neuronal cells by specific cellular signalling pathways with dibutyryl cyclic AMP treatment and serum removal enhancing the level of Brn-3a and reducing the level of Brn-3b expression. This opposite expression pattern is paralleled by the ability of Brn-3a to specifically trans-activate a target promoter bearing its DNA binding site whereas this promoter is repressed by Brn-3b. As predicted from these observations this target promoter is strongly activated by serum removal or addition of dibutyryl cyclic AMP. Therefore changes in Brn-3a and b expression can have a functional effect on promoter activity indicating that Brn-3a and Brn-3b can regulate gene expression via a specific binding site in response to the activation of specific cellular signalling pathways. The reasons for the differences in activity between these two related factors and their role in regulating gene activity in the nervous system are discussed.

INTRODUCTION

The POU (Pit-Oct-Unc) family of transcription factors was originally defined on the basis of a common 150–160 amino acid DNA binding domain which was present in the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and in the nematode regulatory protein encoded by the *unc*-86 locus (for reviews see 1–3). It was subsequently shown that these POU factors played a critical role in regulating growth and differentiation in a number of different cell types, particularly those of neuronal origin. Thus in the nematode the *unc*-86 mutation results in the failure to develop specific neuronal cell types particularly sensory neurons (4, 5). Similarly, the Pit-1 factor is essential for the correct development of the mammalian pituitary gland and inactivation of Pit-1 by mutation results in congenital dwarfism in both mic (6) and humans (7).

The important role of the founder members of the POU famil in neuronal cells led to a search for other members of this famil expressed in these cells. Polymerase chain reaction (PCR experiments using degenerate oligonucleotides derived from th conserved region of the POU domain led to the isolation of severa mRNAs encoding novel members of the POU family expresse in the mammalian brain (8).

A similar approach to isolate cDNA clones for the POU factor expressed in the immortalized ND7 cell line derived from sensor neurons (9) led to the isolation of several cDNA clones derive from Brn-3, one of the factors originally isolated by He *et al.*, (8). In addition however, we also isolated several clones whic were closely related to Brn-3 but differed at seven amino acid within the POU domain (10). The factor encoded by these clone therefore represents a novel member of the POU family whic was designated Brn-3b to distinguish it from the original Brn-3 factor (8) which we now refer to as Brn-3a.

The distinction between these two factors has been confirmed by the isolation of full length cDNA clones of murine Brn-3a and b (11) and of human Brn-3a (also called RDC-1: 12) and Brn-3b (13) which indicate that they are derived from distinct genes showing only limited homology outside the POU domain. The close homology between Brn-3a and b in the POU domain has led to their classification as a separate sub-family (Group IV) within the POU domain together with the product of the *unc*-86 gene and the *Drosophila* factors I-POU and twin of I-POU (3).

Despite the strong homology within their POU domains, Brn-3a and b show distinct expression patterns in the developing rodent (10) and human (12, 14) brain. Moreover, in our initial experiments (10) the two factors showed opposite changes in expression pattern during the *in vitro* differentiation of the ND7 neuronal cell line. Thus when ND7 cells were differentiated to a non-dividing process bearing phenotype by transfer to serum

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free medium (9, 15), the expression level of Brn-3a rose dramatically whilst the level of Brn-3b declined (10). Similarly Brn-3a has been shown to co-operate with Ha-ras in transforming rat primary cells whereas Brn-3b does not have this property and inhibits the transforming action of Brn-3a (11).

This finding and the opposite regulation of Brn-3a and Brn-3b expression during the *in vitro* differentiation of ND7 cells suggested that these two POU factors might have antagonistic effects on target gene expression similar to the closely related *Drosophila* POU factors I-POU and twin of I-POU (16, 17). We have therefore further investigated the regulation of Brn-3a and b expression in neuronal cells and their effect on the activity of a target promoter.

MATERIALS AND METHODS

Cell culture

ND7 cells (9) were grown in L15 medium (Gibco) with 10% foetal calf serum, 0.3% D-glucose, 0.37% sodium bicarbonate 0.2 mM L-glutamine and 1.0% penicillin/streptomycin (10,000 units per ml). To differentiate the cells to a non-dividing phenotype, they were transferred to DMEM/HAMS (1:1, Gibco) medium with 5 μg/ml transferrin, 250 ng/ml bovine insulin, 30 mM sodium selenite in the absence of serum. Treatment with 1 mM dibutyryl cyclic AMP was carried out for the time indicated.

RNA isolation

RNA was isolated as previously described (18) and used as a template for the preparation of cDNA using random hexanucleotide primers (Pharmacia LKB Biotechnology Ltd). The cDNA equivalent to 0.01 μg of total RNA was amplified by polymerase chain reaction (PCR) according to the method of Kawasaki (19). Amplification of Brn-3a and b cDNAs was carried out with the common primers 5'GTGGGCTCGGCGCTGGC-C3' and one or other of two specific primers, 5'CGGGGTTG-TACGGCAAAA3' (Brn-3a) or 5'CTTGGCTGGATGGCGA-AG3' (Brn-3b) (see reference 10). Amplifications were also carried out with control primers specific for the constitutively expressed mRNAs encoding the L6 ribosomal protein (5'ATC-GCTCCTCAAACTTGAAC3' and 5'AACTACAACCACCTC-ATGCC3') and the glucose-6-phosphate dehydrogenase protein (5'CACCTCAACAGCCACATGAA3' and 5'GTTCGACAGT-TGATTGGAGC3').

For all PCR reactions aliquots of the products were taken at different cycle times to ensure that the reaction was in the exponential phase in the samples quantitated. To confirm the identity of the PCR products in each case and to quantitate their level, southern blotting and hybridization was performed with appropriate random primed labelled probes as described (20).

Competitive PCR

Specific competitor species for the Brn-3a and b PCR products were made by the method of Celi *et al.*, (21) using the common 5' Brn-3a and b primer and new 3' primers containing the sequence of the Brn-3a or b specific primers linked to a sequence which is present 100 bases nearer to the 5' primer in the Brn-3a or b mRNAs. In the case of Brn-3a this 3' primer had the sequence 5'CGGGGTTGTACGGCAAAAGGCCTCCTCCAG-CCAGGC3' and in the case of Brn-3b it had the sequence 5'G-TTGGCTGGATGGCGAAGAGCTTCCTCCAGCCA-CGC3'. The use of these primers in an initial experiment therefore produces a product which has the two standard PCR primers 100 base pairs closer together than in the Brn-3 mRNA itself and which can therefore be used as an internal control in subsequent PCR amplifications (21, 22).

In initial experiments a series of dilutions of the competitors was used to determine the concentration which would produce an approximately equimolar level of the normal PCR productand the competitor product. This was then used in all subsequent experiments.

Transient infections

Transfections were carried out according to the calcium phosphate method of Gorman (23). Cells were exposed to the calcium phosphate precipitate for three hours, washed and transferred to fresh medium with or without 1 mM dibutyryl cyclic AMP. In all cases cells were harvested seventy two hours later. The amount of DNA taken up by the cells in each case was measured by slot blotting the extract and hybridization with a probe derived from the ampicillin resistance gene in the plasmid vector (24). This value was then used to normalize the values obtained in the CAT assay as a control for differences in uptake of plasmid DNA in each sample.

Cat assay

Assays of chloramphenicol acetyl transferase activity were carried out according to the method of Gorman (23) using samples which had been equalized for protein content as determined by the method of Bradford (25).

Plasmid DNA

The test plasmid contains the Brn-3 binding site ATGCTAAT-GAGAT cloned into the Bam HI site in the vector pBL CAT 2 which contains the herpes simplex virus thymidine kinase promoter from -105 to $+51$ driving expression of the chloram-phenicol acetyl transferase (CAT) gene (26). The Brn-3a and b expression vectors contain full length cDNA or genomic clones for each of these proteins (11) cloned under the control of the Moloney murine leukaemia virus promoter in the vector pLTR poly which has been modified by deletion of a cryptic splice site in the SV40 3' untranslated region (27). In the case of Brn-3a, two different expression vectors were used containing cDNAs or genomic clones derived from the longer spliced form of the Brn-3a mRNA and from a shorter intronless mRNA which encodes a shorter protein (11). The Brn-3b vector contains a full length cDNA derived from the intronless mRNA which encodes this protein (11).

RESULTS

In our previous experiments (10) a larger increase in the level of the Brn-3a mRNA was observed in ND7 cells differentiated by transfer to low serum-containing medium (0.5% foetal calf serum) together with 1 mM dibutyryl cyclic AMP than in cells that were differentiated by transfer to serum free medium in the absence of dibutyryl cyclic AMP. However, we had not previously investigated whether the level of the Brn-3a mRNA could be modulated by treatment with dibutyryl cyclic AMP alone. We therefore added 1 mM dibutyryl cyclic AMP to ND7 cells growing in serum-containing medium (10% foetal calf serum) and measured the effect on the expression levels of Brn-3a and Brn-3b (Figure 1a and b). The levels of the Brn-3a and b mRNAs were measured as before by reverse transcriptase-

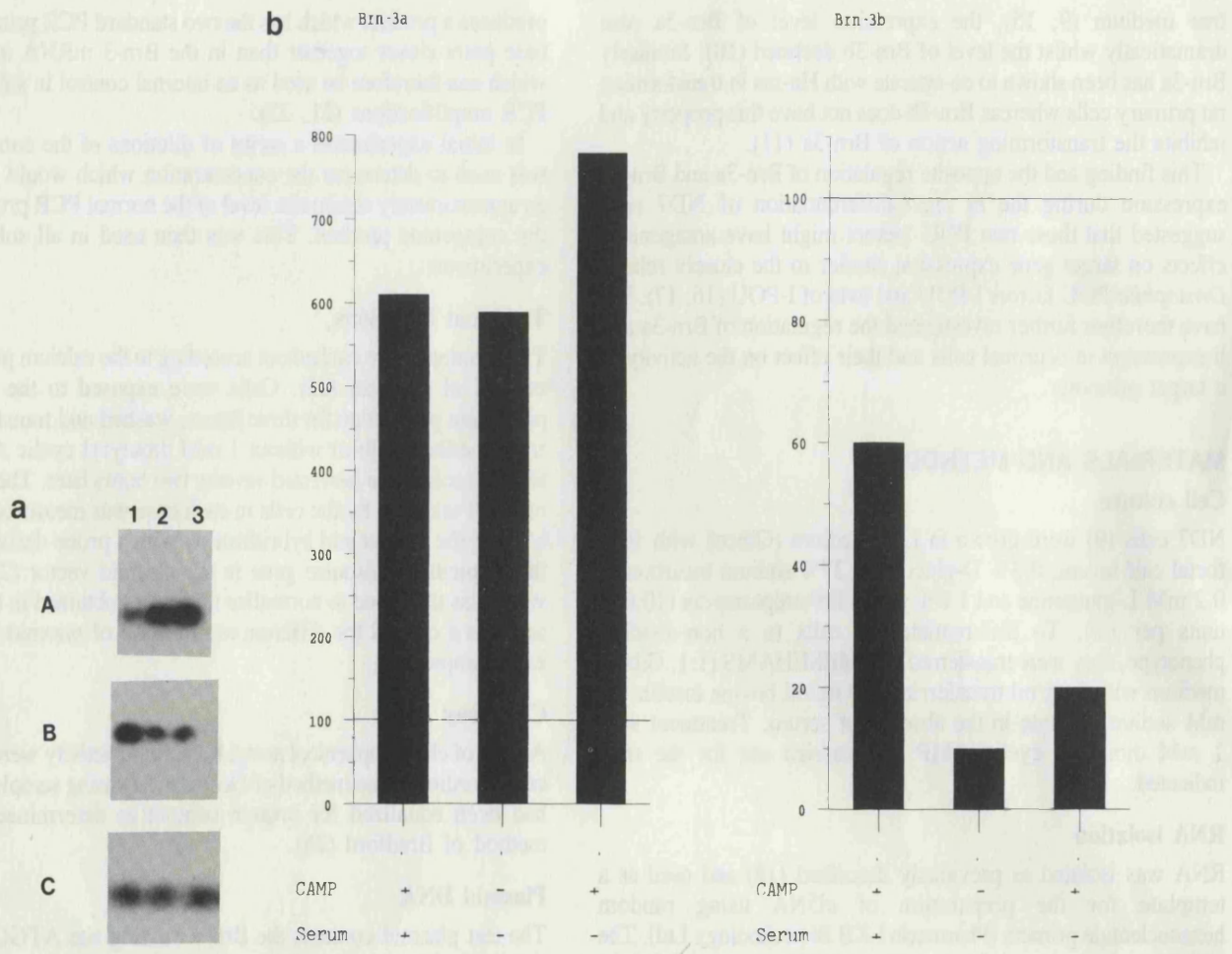


Figure 1. (**a**) Polymerase chain reaction/reverse transcriptase assay of the transcripts encoding Brn-3a (panel A), Brn-3b (panel B) and the ribosomal L6 protein (panel C) using cDNA prepared from the mRNA of cells in serum containing medium (track 1), in the absence of serum (track 2) or in the presence of serum with the addition of 1mM dibutyryl cyclic AMP (track 3). (**b**) Quantitation of the results of a typical experiment measuring the levels of the Brn-3a or Brn-3b mRNAs seventy two hours after the onset of the indicated treatment. All values are expressed relative to the level in untreated ND7 cells (100%) and have been equalized on the basis of the levels of the L6 ribosomal protein mRNA.

polymerase chain reaction amplification using an upstream primer whose sequence is common to the POU domain of each factor and a pair of downstream oligonucleotides corresponding to the region of the POU domain which is most diverged between the two factors (10). Both these primer pairs showed similar amplification efficiencies when tested with a given amount of template and a similar number of amplification cycles was used in all experiments. Hence the levels of the Brn-3a and Brn-3b mRNAs observed in our experiments can be directly compared with one another. Variations between different samples in the total amount of RNA or in the efficiency of reverse transcription were equalized by carrying out control amplifications using primers specific for the constitutively expressed transcripts for glucose-6-phosphate dehydrogenase or the ribosomal L6 protein.

In these experiments (Figure 1), cells grown in full serum-containing medium with added dibutyryl cyclic AMP showed a clear increase of Brn-3a expression over the low level normally present in cells growing in full serum-containing medium in the absence of cyclic AMP. In contrast the level of Brn-3b expression declined in cells treated with dibutyryl cyclic AMP whereas the level of the control mRNA encoding the ribosomal L6 protein remained unchanged. Similarly the removal of serum resulted in an increase in Brn-3a mRNA levels and a fall in that of Brn-3b (Figure 1) in accordance with our previous results (10). The combined treatment of removing serum and adding cyclic AMP also resulted in an increase in the level of the Brn-3a mRNA and a fall in that of Brn-3b (Figure 1b). The two treatments did not have a synergistic effect however, with the elevation observed in Brn-3a with both treatments being only marginally above that obtained with each treatment independently whilst the fall in the Brn-3b mRNA was similar to that observed upon serum removal in the absence of cyclic AMP.

Hence ND7 cells show an increase in the level of Brn-3a expression and a decline in the level of Brn-3b expression in response to dubutyryl cyclic AMP treatment or serum removal. This results in a significant change in the ratio of Brn-3a to Brn-3b transcripts as detected by PCR with a common oligonucleotide and a pair of discriminatory oligonucleotides.

The comparative PCR method described above, controls for any variations in the amount of total RNA in each sample or in the efficiency of the reverse transcriptase reaction by comparing the level of product produced from the Brn-3 RNAs with that for the mRNAs encoding the constitutively expressed G6PDH or ribosomal L6 proteins. This method does not control however, for tube to tube variations in the amplification efficiency of the PCR itself. In order to confirm the data obtained in this way we

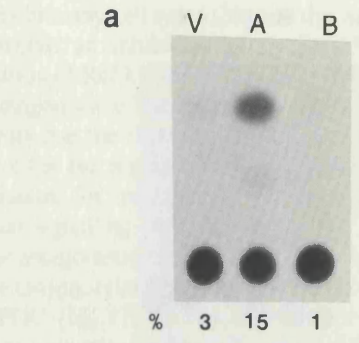
Figure 2. PCR assay of the Brn-3a transcript in cells incubated for twenty four hours in the absence (−) or presence (+) of 1 mM dibutyryl cyclic AMP. A control template which will produce a PCR product 100 base pairs smaller than that produced by the natural Brn-3a transcript has been added to all samples. The products produced by this control template (C) and that from the Brn-3a transcript in the samples under test (T) are indicated.

therefore used the method of Celi *et al.*, (21) to synthesize Brn-3a or Brn-3b templates which when amplified would produce a product approximately one hundred bases shorter than the cDNA produced from the natural Brn-3a or Brn-3b mRNAs. Co-amplification of an equal amount of these competitor templates with the experimental samples can therefore provide an internal control for the efficiency of the PCR reaction with the competitor template being distinguishable from the product of the natural mRNA on the basis of its smaller size (22).

These experiments confirmed the results of our earlier experiments using comparative PCR with a clear increase in the Brn-3a mRNA and fall in the Brn-3b mRNA being observed following dibutyryl cyclic AMP treatment or serum removal although the levels of the internal control template remained the same indicating a similar efficiency of PCR amplification in each case (see for example Figure 2). Overall, in four replicate experiments, an increase in the Brn-3a to Brn-3b ratio was observed at twenty four, forty eight and seventy two hours of exposure to cyclic AMP or at similar times following serum removal.

It is clear therefore that both the removal of serum and the addition of cyclic AMP can result in an increase in the level of the Brn-3a mRNA and a decline in the level of the Brn-3b mRNA. To understand the significance of these changes we wished to investigate the functional activity of Brn-3a and Brn-3b. To do this we inserted the octamer-related motif ATGCTAATGAGAT upstream of the herpes simplex virus thymidine kinase promoter in the vector pBL CAT 2 (26). This motif was chosen since it binds the POU domain of both Brn-3a and Brn-3b (11 and unpublished observations) but not the neuronal octamer binding protein Oct-2 (28).

The resulting CAT reporter construct was co-transfected into BHK-21 fibroblast cells (29) which lack significant endogenous levels of Brn-3a or Brn-3b, together with either the empty expression vector alone (11) or expression plasmids containing clones capable of encoding similar levels of functional Brn-3a or Brn-3b protein (11 and Theil and Moroy, submitted). In these experiments, (Figure 3a and b) a strong stimulation of CAT activity was observed upon co-transfection of the reporter construct with the Brn-3a expression plasmid. A similar stimulation of activity was observed using two distinct Brn-3a



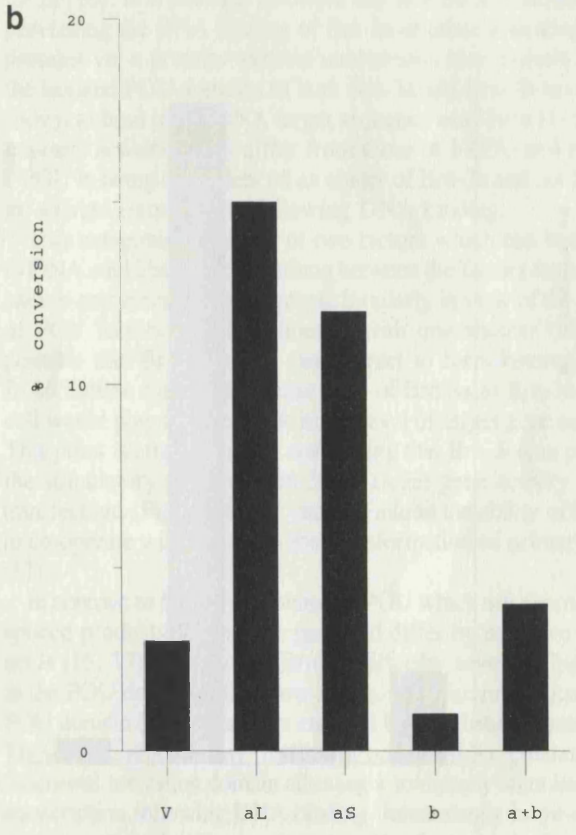


Figure 3. (**a**) Assay of chloramphenicol acetyl transferase (CAT) activity in BHK-21 cells transfected with a reporter construct containing the Brn-3 binding site ATGCTAATGAGAT together with either the empty expression vector (V) or the same vector containing the entire Brn-3a gene (A) or Brn-3b gene (B). The values indicate the percentage of chloramphenicol acetylated in each case. (**b**) Quantitation of a typical CAT assay in which the reporter construct used in panel a was transfected with 10 μg of the empty expression vector (V), 10 μg of the same vector expressing either the long (aL) or short (aS) forms of Brn-3a or the Brn-3b gene (b) or with 5 μg of Brn-3a and 5 μg of Brn-3b expression vectors (a + b).

expression plasmids encoding either a longer spliced form of the Brn-3a mRNA and a shorter intronless form which encodes a smaller protein that lacks the 84 amino terminal residues but is otherwise identical to the longer protein (11) (Figure 3b). In contrast the Brn-3b expression plasmid consistently repressed activity approximately three fold below that observed with plasmid vector alone (Figure 3a and b) and was also capable of blocking the stimulation of the target promoter by Brn-3a when

Figure 4. (**a**) Assay of CAT activity in ND7 cells grown in the presence (+) or the absence (−) of 10% foetal calf serum and transfected with either the plasmid vector pBL CAT 2 (V) or the reporter plasmid in which the Brn-3 binding site ATGCTAATGAGAT has been cloned upstream of the tK promoter in this vector (R). (**b**) Levels of CAT activity in two replicate experiments of this type using plasmid vector (V) or the reporter construct (R) transfected into cells grown in the presence (1) or absence (2) of 10% foetal calf serum. Values are the average of two experiments whose range is shown by the bars.

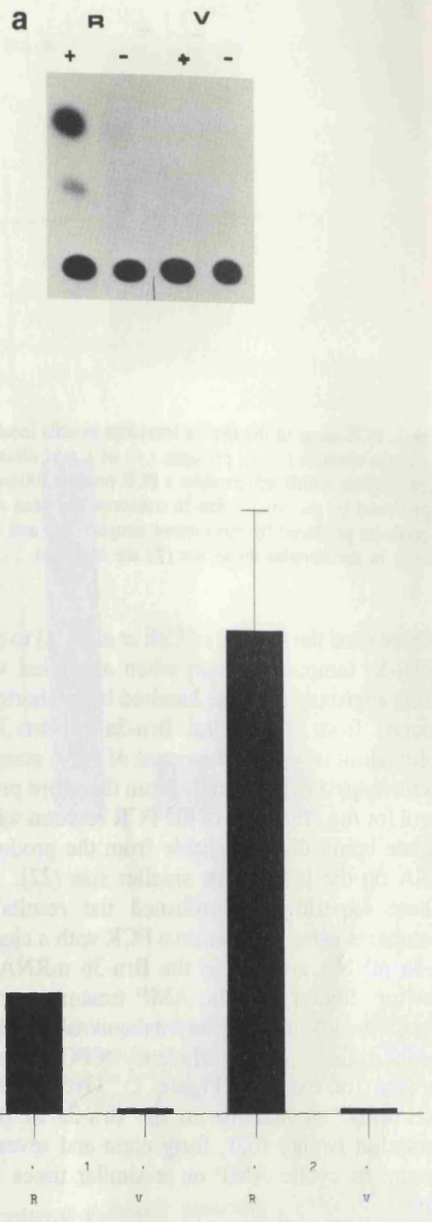


Figure 5. (**a**) Assay of CAT activity in ND7 cells grown in the presence (+) or absence (−) of 1mM dibutyryl cyclic AMP and transfected with either plasmid vector (V) or the reporter plasmid consisting of the vector with the addition of the Brn-3 binding site (R). (**b**) Levels of promoter activity in this experiment using the plasmid vector (V) or the reporter construct transfected into cells maintained in the absence (1) or presence (2) of 1 mM dibutyryl cyclic AMP. Values are the average of two experiments whose range is shown by the bars.

the two forms were transfected together confirming that it expressed functional Brn-3b protein (Figure 3b).

Thus the long and short Brn-3a proteins (11) are capable of stimulating the activity of a promoter carrying the appropriate binding site whereas Brn-3b represses such a promoter. This observation suggested that factors such as serum removal and dibutyryl cyclic AMP treatment which produce a rise in the Brn-3a to Brn-3b ratio, should lead to activation of this target promoter. To test this prediction we transfected ND7 cells with the same CAT reporter construct in the presence or absence of ten per cent foetal calf serum and in the presence or absence of 1 mM dibutyryl cyclic AMP.

In these experiments a clear stimulation of promoter activity was observed in the cells deprived of serum (Figure 4) and in the cells treated with dibutyryl cyclic AMP (Figure 5). This effect was dependent upon the presence of the ATGCTAATGAGAT Brn-3 binding site since it was not observed with the parental pBL CAT 2 plasmid alone. Apart from Brn-3, the only other POU factors expressed by ND7 cells are Oct-1 and Oct-2 (10). We have previously shown that the neuronal form of Oct-2 cannot bind to the target site used here (28) whilst although Oct-1 can

bind, it has no significant effect on promoter activity (our unpublished data) consistent with its ability to act as only a very weak transactivator (30).

Hence Brn-3a and Brn-3b are the only proteins present in ND7 cells which can influence the activity of the target site in the promoter we use. This indicates that effectors which produce a change in the ratio of endogenous Brn-3a and b can indeed modulate the activity of a target promoter which is activated by Brn-3a and repressed by Brn-3b in co-transfection experiments. Moreover, the binding site for Brn-3a and Brn-3b can act as a response element modulating gene expression in response to serum or dibutyryl cyclic AMP.

DISCUSSION

Both the Brn-3a and Brn-3b transcription factors have previously been reported to be expressed in neuronal cells within the developing rat (10), mouse (11, 12) and human (12, 14) nervous systems. Here we show that the levels of the mRNAs encoding these two proteins are regulated in opposite directions by treatment of ND7 cells with dibutyryl cyclic AMP and serum. Moreover, a binding site for these two factors can modulate promoter activity in opposite directions in response to addition of dibutyryl cyclic AMP or serum to neuronal ND7 cells. Interestingly these effects may be cell type specific since Turner *et al.*, (31) observed only a slight decrease in the mRNA encoding Brn-3b (which they refer to as Brn-3.2) in response to cyclic AMP treatment of F9 embryonal carcinoma cells with a slight increase being observed following similar treatment of N18 neuroblastoma cells. Similarly they observed only a slight rise in the mRNA encoding Brn-3a (which they refer to as Brn 3.0) following cyclic AMP treatment of N18 cells (31). Since N18 cells represent one of the two parents of the ND7 hybrid cell line (9), this suggests that the strong response of the Brn-3a and Brn-3b mRNAs to cyclic AMP which we observe is a property derived from the primary dorsal root ganglion neuron fusion partner, although further experiments will be required to confirm this.

Regulation by cyclic AMP treatment has previously been reported to play a critical role in the functioning of the POU factors Pit-1 and SCIP (32, 33) neither of which are expressed in ND7 cells (10). Synthesis of SCIP (also known as Oct-6 or Tst-1) is stimulated by cyclic AMP treatment and is thought to be involved in the modulation of gene expression in developing glial cells (32, 33). Similarly, the synthesis of the pituitary specific POU factor Pit-1 is enhanced by cyclic AMP treatment (34). In this case however, the activation of protein kinase A by cyclic AMP also leads to the phosphorylation of Pit-1 allowing it to bind to non-canonical DNA binding sites in the thyrotrophin β-subunit gene and thereby stimulate gene expression (35).

It is likely that the regulation of Brn-3a and b mRNA levels by dibutyryl cyclic AMP similarly plays a critical role in regulating the expression of their, thus far unidentified, target genes in neuronal cells. This case differs from the others however, in that the synthesis of these two related mRNAs is regulated in opposite directions by the same exogenous stimulus. Moreover serum factors appear to have an effect opposite to dibutyryl cyclic AMP since removal of serum enhances Brn-3a mRNA levels and lowers the Brn-3b mRNA (11). This effect can be reversed by readdition of foetal calf serum to serum starved ND7 cells (V B-M and DSL unpublished observations). Interestingly it is known that cyclic AMP can antagonise the effect of growth factors in many cell types (36) and this has recently been show to involve an inhibition by cyclic AMP of Ras-depender activation of Raf kinase activity (37). When taken together wit the antagonistic effects of Brn-3a and b on gene expression thi suggests that the ratio of Brn-3a to Brn-3b in a specific cell i critical for the regulation of gene expression and represents mechanism for modulation of target gene activity by specifi cellular signalling pathways.

The antagonistic effects of Brn-3a and Brn-3b are reminscer of the closely related *Drosophila* POU factors I-POU and twi of I-POU (16, 17). In this case twin of I-POU binds to DN and activates transcription (17) whereas I-POU acts as a repress since it prevents the DNA binding of the activating POU facto CF1a (16). It is possible therefore that Brn-3b acts similarly b preventing the DNA binding of Brn-3a or other activating PO proteins via a protein–protein interaction. Alternatively, sinc the isolated POU domains of both Brn-3a and Brn-3b have bee shown to bind to the DNA target sequence used here (11), the opposite activities may differ from those of I-POU and twin c I-POU in being dependent on an ability of Brn-3a and not Brn-3 to activate transcription following DNA binding.

This antagonistic activity of two factors which can both bin to DNA could lead to competition between the factors for bindin sites in any common target genes. Similarly in view of the abilit of POU factors to heterodimerize with one another (38) it possible that Brn-3a and b can interact to form heterodimer In both these cases, the precise ratio of Brn-3a to Brn-3b in th cell would play a critical role in the level of target gene activity This point is emphasized by our finding that Brn-3b can prevei the stimulatory effect of Brn-3a on target gene activity in c transfections (Figure 3b) and can also inhibit the ability of Brn-3 to co-operate with Ha-ras in the transformation of primary cel (11).

In contrast to I-POU and twin of I-POU which are alternativel spliced products of the same gene and differ by only two amin acids (16, 17), Brn-3a and Brn-3b differ by seven amino acic in the POU domain (10), show extensive differences outside th POU domain (11, 13) and are encoded by two distinct genes (11 These differences may result in only Brn-3a containing functional activation domain allowing it to directly stimulate ger transcription following DNA binding. Interestingly however, th only amino acid difference between Brn-3a and b in the highl conserved POU homeobox is at position 22 in the first helix whic has been shown to be critical in controlling the protein–protei interactions of the POU factors. Thus substitution of the alanir found at this position in the Oct-2 POU factor with the glutam acid found in the Oct-1 factor confers on Oct-2 the ability heterodimerize with the herpes simplex virus trans-activat Vmw65 which is normally an exclusive property of Oct-1 onl (39). Hence Brn-3a and b may differ in their ability to intera with other proteins allowing Brn-3a but not Brn-3b to functic indirectly by recruiting an activating factor to the gene promote

In agreement with this idea we have shown that the exchang of the POU domains between Brn-3a and b confers upon Brn-3 the ability to act as an activator of the promoter used he (unpublished observations). This is in agreement with the findir that both the long and short forms of Brn-3a can trans-activa the target promoter used here indicating that this effect is n dependent upon the N-terminus of the protein. In contrast th transforming activity of Brn-3a does appear to be dependent c the N-terminal region since it is a property only of the long form of the protein (11).

Interestingly a form of Brn-3b with additional N-terminal sequences has recently been detected in the retina (14) and in the CNS (31) although it was absent in the spinal cord (11) raising the possibility that two tissue-specific forms of Brn-3b may exist. Moreover, both Brn-3a (40) and this longer form of Brn-3b (31) have been shown to activate a promoter containing a non octamer motif from the corticotrophin releasing hormone promoter which binds Brn-3. Hence different regions of the Brn-3 proteins may be important for transactivation via different DNA binding sites in different genes with activation via some sites being dependent on the POU domain present in all forms of a specific factor whilst activation via other sites is dependent upon the N-terminal region which is present only in the longer forms of each factor.

Although it remains to be determined whether the Brn-3a and Brn-3b factors modulate gene expression directly or indirectly, it is already clear that these factors are regulated in opposite directions by specific cellular signalling pathways and can have antagonistic effects on the activity of a target promoter. The relative levels of these factors, perhaps in conjunction with the level of a third recently identified member of the Brn-3 family Brn-3c (41) are therefore likely to play a critical role in regulating the activity of specific genes in neuronal cells.

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