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Expression & Role of Brn3 Family Of Transcription Factors In Human Neuroblastomas

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A thesis submitted for the degree of Doctor of Philosophy

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December 2004

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Abstract

Brn-3a and Brn-3b are members of the POU-domain containing transcription factors. Both are found in specific neurons of the CNS and PNS. In mouse and human neuroblastoma cell line models, high levels of Brn-3a are found in differentiating neurons whereas high levels of Brn-3b are present in actively proliferating cells. The reciprocal expression of these two transcription factors in neuroblastoma cells is paralleled by their differential regulation on gene promoters. While Brn-3a activates several gene promoters whose products are associated with neuronal differentiation, Brn-3b has repressive effects on the activity of most of these promoters.

Stable cell line systems were developed to test the effects of these factors in human neuroblastoma cell line, IMR-32, where the levels of Brn-3a and Brn-3b have been manipulated. While results from IMR-32 constitutively over-expressing Brn-3a remain to be validated, manipulation of Brn-3b showed profound effects on the growth characteristics of these cells. Thus, over-expression of Brn-3b caused increased growth rate, saturation density, proliferation, anchorage independent colonies, and enhanced migration compared to the mock transfected cells. Furthermore, cells with elevated levels of Brn-3b exhibited a stronger ability to form tumours in xenograft mouse models compared to controls. IMR-32 cells with reduced Brn-3b levels exhibited a significant decrease in growth and proliferation parameters. Moreover, Brn-3b over-expressing cells failed to respond to differentiation stimulus such as all-trans-retinoic-acid and continued to proliferate in its presence, while empty vector control and cells with reduced Brn-3b levels ceased to proliferate when grown in the presence of this differentiation stimulus.

The levels of cell cycle regulator protein, cyclin D1 were found to be elevated in Brn-3b over-expressing IMR-32 cells, with a concomitant decrease in cells with reduced levels of this protein. Using transactivation studies, we have shown for the first time that Brn-3b is able to activate the cyclin D1 gene promoter (5'-A TTT CTA TGA-3'), and this activation is dependent on a specific octamer sequence located between positions -240 and -231 from the transcriptional start site in this promoter. Taken together these studies are of fundamental importance in devising targeted therapeutic strategies for aggressive stages of human neuroblastomas.

For my Daddy who taught me to dream

&

my Deedly who kept me from flying away too soon

ACKNOWLEDGEMENTS

It is finally a pleasure to thank the many people who made this thesis possible. I would first like to express my gratitude to my supervisors, Dr. Budhram-Mahadeo and Prof. David Latchman, for allowing me to undertake this PhD project in their lab, and for their guidance and valuable advice throughout the course of this study. Special thanks to Jonathan Dennis for taking me under his wing and guiding me patiently through the early dark and bewildering days of my PhD.

The support and encouragement of my family, both in Kashmir and the UK, lies behind the completion of this thesis. A special thank you to my grandparents, Tataji, Bubajee and Ama, for including the welfare of my cells into their daily prayers! I owe a debt of gratitude to Chota Uncle for meticulously proof reading the thesis. Also, a huge thanks to Iku Baya, Obi, Shaya and, especially Sheebly and Fais, my super-heroes, for helping with the editing of the thesis in the last week of submission. It could not have happened without your help. Thanks also to my little Welsh cousins, Furat and Uwais, for conveying their support from a distance. Finally, I need to mention Baya for being incredibly supportive and understanding during some difficult periods in the last few years. Once again, thank you, all you guys.

Without the company of some other fabulous people, my journey through this degree would have been much impoverished. I am indebted to the members of my department (MMBU, ICH) for providing a stimulating and fun environment in which to learn and grow. In particular, I would like to thank Alex and Taz for starting this journey together with me, and appreciating my joys and woes as a student more than anyone else; also a big thank you to Cristina for being a fantastically level-headed and supportive friend; thanks also to Misbah for being so wonderfully warm and patient; thanks to Christopher for making me laugh in the past four years; thanks also to Duncan and Francesca for their sound advice in both professional and personal matters; a big hug of gratitude to Sonia for being incredibly generous for trusting *me* with her brand new computer for a few weeks; and, I would also like to thank Miratul for his sincerity as a friend and colleague, and for sharing all those late night samosas and pizzas in the office over discussions of how Kashmir is better than Bangladesh – I sure will miss that in the future.

I also wish to thank my non-PhD friends (Alison, Hannah, Elvira, Joby, James and Liz) for being so understanding about my commitment to the PhD. I am greatly indebted to Vish for his help in formatting this thesis. A big thank you to Olga, my Greek 'Ta-ran-tu-la', for being a fantastic friend during the frustrating period of writing up this thesis. Thanks also to Cherrene for her support and selfless friendship of 8 years. I also want to thank Mattia for being the most special friend during my PhD years and for making me understand a little more about courage, courtesy and character. Thank you.

Finally, and most importantly, I would like to thank the most valuable person in my life, my mum. Deedly, thanks for being a pillar of support and an inspiration to me. I dedicate this thesis to you and Daddy.

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* This special footnote has been inserted subsequent to the submission and acceptance of this thesis.

LIST OF FIGURES & TABLES

ABBREVIATIONS

BDNF	Brain-Derived Neurotrophic Factor
cAMP	Adenosine 3', 5'-cyclic monophoshate
CAT	Chloramphenicol acetyltransferase
CDK	Cyclin-dependant kinase
CDKI	Cyclin-dependent kinase inhibitor
CHIP	Chromatin immunoprecipitation
Cisplatin	Cis-Platium(II)diamminedichloride
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
EC	Embryonic carcinoma cell
ECL	Enhanced chemiluminescent reagent
EFS	Event-free survival
EMSA	Electrophoretic mobility shift assay
ER	Oestrogen receptor
ERE	Oestrogen receptor element
ES	Embryonic stem cell
FCS	Foetal calf serum
GH	Growth hormome
^{3}H	Tritium
^{3}H-thymidine	Tritiated thymidine
HBSS	Hank's Balanced Salt Solution
HD	Homoedomain
HLH	Helix-loop-helix
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HTH	Helix-turn-helix
IgH	Immunoglobulin heavy

JNK	Jun kinase
MAP-2	Microtubule Associated Protein-2
MoMuLV	Moloney murine leukaemia virus
NAG	Neuroblastoma Amplifier Gene
NF	Neurofilament
NFH	Neurofilament heavy
NFM	Neurofilament medium
NFL	Neurofilament light
NGF	Nerve growth factor
NMR	Nuclear magnetic resonance spectroscopy
P1	Post-natal day 1
PCNA	Proliferating Cell Nuclear Factor
PI	Propidium iodide
PIC	Pre-initiation complex
PNS	Peripheral nervous system
POU	Pit-1, Oct-1/2, Unc-86
POU_{hd}	POU-homeodomain
POU_s	POU-specific domain
PRL	Prolactin
PS	Phospholipid phosphatidylserine
pTF	Precursor terminal protein
RA	All-trans-retinoic acid
RASSF1A	Ras-association domain family 1A
pRB	Retinoblastoma protein
RGC	Retinal ganglion cells
mRNA	Messenger ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNAP-25	Synaptosomal-associated protein 25
SRO	Shortest regions of overlap

TAP73	Transactivator form of p73
TBP	TATA box binding protein
TEMED	N', N', N', N'-Tetramethylethylenediamine
TG	Trigeminal ganglia
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Trk	Tyrosine receptor kinase
TSHβ	Thyroid stimulating hormone beta sub-unit
URR	Upstream regulatory region
UKCCCR	United Kingdom Coordinating Committee on Cancer Research
INSS	International Neuroblastoma Staging System
Lac Z	B-galactosidase reporter gene
HSV	Human simplex virus
TFII	General transcription factor II
snRNA	Small nuclear RNA
BDNF	Brain-Derived Neurotrophic Factor

LIST OF PUBLICATIONS

Shazia Irshad, R.Barbara Pedleys, John Anderson, David Latchman and Vishwanie Budhram-Mahadeo (2004). The Brn-3b Transcription Factor regulates the growth, behaviour, and invasiveness of Human Neuroblastoma cells in vitro and in vivo. The Journal of Biological Chemistry, Vol 279, No.20, Issue of May14, pp.21617-21627

Budhram-Mahadeo V, Morris P, Ndisang D, **Irshad S,** Lozano G, Pedley B, Latchman. The Brn-3a POU family transcription factor stimulates p53 gene expression in human and mouse tumour cells. Neurosci Lett. 2002 Dec 6;334(1):1-4.

CHAPTER 1

Introduction

INTRODUCTION

1.0 Transcriptional regulation in eukaryotes

In eukaryotes formation of a zygote and the subsequent appearance of morphologically and functionally distinct cells during development and their maintenance in adult life requires a precise and tightly regulated gene expression. The importance of aberrant gene expression has been highlighted in a number of diseases, including cancers. Most cancers arise from abnormalities in the expression of genes that control growth and differentiation (increase in proto-oncogenes or decrease of tumour suppressor genes). Various metabolic disorders and perturbed hormone function are also the consequence of aberrant gene expression.

The control of tightly regulated expression and function of mammalian genes is maintained by a number of processes. These include pre-transcriptional control of chromatin remodelling; gene transcription, which involves the synthesis of mRNA from DNA; post-transcriptional control determining the stability of the mRNA transcript; translation which involves the synthesis of proteins; and, finally post-translational modifications required for producing a functionally active or inactive protein.

1.1 Pre-transcriptional control of gene expression

Transcriptionally silent genes are condensed and organized into chromatin in the eukaryotic nucleus (for review see Latchman, 2002; Felsenfeld and Groudine, 2003). The term chromatin refers to the organization of DNA in an intricate, dynamic nucleoprotein assembly, accomplished by a remarkable feat of biological engineering. Chromatin is composed of repeating units called nucleosomes. The nucleasome core is composed of an octamer of four highly folded proteins, H2A, H2B, H3, and H4 (two of each protein), and of 147 base pairs of DNA wrapped around the octamer.

This chromatin structure imposes barriers for each step in eukaryotic transcription, primarily by preventing entry of the transcriptional machinery to the gene regulatory sequences. Therefore, the first step in activation of a gene requires remodelling of the chromatin to allow entry of transcriptional regulators (Kornberg and Lorch, 1999). Two major classes of multiprotein complexes regulate accessibility of the template to DNA binding factors. The first class involves ATP-dependent complexes which use ATP hydrolysis to 'slide' DNA with respect to the histone octamer, and thereby expose or occlude DNA sequences (Meersseman et al, 1992). The other class is composed of protein complexes that covalently modify nucleosomes by adding or removing many chemical moieties in the histone amino termini. The best characterized of these complexes are those that cause either hyperacetylation (histone acetyltransterase complexes, HAT) or hypoacetylation (histone deacetylase complexes, HDAC) of lysines in the amino-terminal tails of the core histones (Roth et al, 2001; Vogelauer et al, 2000). It is thought that targeting of HAT and HDAC complexes to promoter regions creates specific patterns of hyper- and hypoacetylation in a backbone of global acetylation that correlate with transcription activation and repression, respectively. Other modifications, such as methylation, ubiquitination, and phosphorylation are also crucial in regulation of transcription (for review Berger, 2001).

1.1.1 Eukaryotic transcription

Gene expression is primarily controlled at the level of transcription. Eukaryotic transcription is the synthesis of RNA from a DNA template by RNA polymerase (RNA Pol). There are three classes of eukaryotic RNA Pols: I, II and III, each comprising two large subunits and 12-15 smaller subunits. RNA polymerase II is involved in the transcription of all protein genes and most snRNA genes. The other two classes transcribe only RNA genes. RNA Pol I is located in the nucleolus, transcribing rRNA genes except 5S rRNA. RNA Pol III is located outside the nucleolus, transcribing 5S rRNA, tRNA, U6 snRNA and some small RNA genes.

The basal transcription complex includes the RNA polymerase and additional proteins (discussed in section 1.4) that are necessary for correct initiation and

elongation of RNA synthesis. RNA Pol II is not able to bind the DNA directly. Hence, following chromatin remodeling, DNA becomes available to other proteins which results in the formation of the pre-initiation complex. RNA Pol II then initiate its function by directing 3'-hydroxylation of the RNA chain on a ribonucleoside triphosphate, leading to extension of the chain in the 5' -> 3' direction (for review see Crammer et al, 2001).

1.1.2 DNA sequence elements regulate transcription

The molecular basis for the transcriptional regulation of gene expression is the binding of trans-activating proteins (transcription factors) to cis-regulatory DNA response elements (Ptashne, 1988; Latchman, 1990). The presence of cis-acting DNA sequences establishes the binding sites for transcription factors at locations from which they can interact, either to form the transcription initiation complex (TIC - a macromolecular assembly of RNA polymerase II and associated protein cofactors) or to interact with the TIC to modulate transcription.

Several types of cis-active elements exist, including promoters, enhancers, upstream promoter elements (UPEs) and response elements. Promoter sequence is usually located at the 5' end relative to the coding sequence. Analysis of many eukaryotic promoters has revealed some of the sequence motifs that are present within the proximal promoter elements of genes that are required for transcription at initiation. These include the A/T rich sequence element (TATA box) and initiator (Inr) elements, which are present in many, but not all, RNA polymerase II transcribed genes. The TATA box is located 25 to 30 base pairs 5' to the transcriptional start site and is recognized by the TBP (TATA binding protein). In general, it is thought that genes containing a TATA box usually initiate transcription from a single downstream site, whereas some housekeeping and tissue-specific genes lacking a TATA box often initiate transcription from initiator elements (Y2CAY5 where Y= pyrimidine) that may be found at a distance of -3 to +5 from the transcriptional start site (Zawel and Reinberg, 1993). Hence, promoter sequences establish the site of transcription initiation and the direction of transcription.

Upstream promoter elements (UPEs) are other regulatory elements that are found in a wide variety of genes with different patterns of expression (Goodwin, 1990). These sequences are bound by specific transcription factors and play a role in stimulating the constitutive basal activity of a promoter. The Sp1 box (a GC rich sequence) and the CCAAT box (sequence implied in the name) are both UPEs, and promoters may contain one or both in single or multiple copies (Latchman, 1998b).

Enhancers are DNA sequences that are located 5' or 3' to the coding sequence of a gene or within an exon or intron of the gene. In contrast to the promoter, enhancer function is independent of position and orientation (Thompson and McKnight, 1992). Enhancers have a modular structure with each module representing a transcription factor binding site. It is the juxtaposition of binding sites for multiple factors in close proximity to one another that is the basis for enhancer function, as individual modules have little or no enhancing activity. This requirement for proximity suggests cooperativity of function, at the level of DNA binding and/or transactivation. Enhancer sequences seem to be less protected by histones and other chromosomal proteins as they often demonstrate a hypersensitivity to digestion by DNase I within nuclei isolated from specific cells. This allows greater accessibility to DNase I *in vitro* and to transcription factors *in vivo.* Enhancers also exhibit the ability to function at distances of 10 kb or more at 5' or 3' from the transcription initiation site. It appears that the formation of a DNA loop allows the general transcription factors of the transcription initiation comlex to interact with factors bound both 5' and 3' to the transcription initiation site.

While transcription factors that bind to these enhancers often activate gene expression, there are also some negative regulatory elements (silencers) (for review by Renkawitz, 1990), such as the sequence which is recognized by the neuron-restrictive silencer factor to inhibit transcriptional activity (Schoenherr and Anderson, 1995). Furthermore, sequences which act as positive enhancer in some cells may be negative regulators in other cell types depending on the position of its binding site in the promoter as well as other transcription factors which may be co-expressed in the cell.

Finally, DNA sequences that enhance activation of a promoter in response to exposure to different stimuli are termed response elements. These include the *H*eat *S*hock *E*lement (HSE), *G*lucocorticoid *R*esponse *E*lement (GRE), O*E*strogen *R*esponse *E*lement (ERE) and *M*etal *R*esponse *E*lement (MRE). These elements are bound by specific transcription factors induced by a particular stimulus and are capable of being induced in response to specific stimulus even when cloned into a heterologous promoter. This is exemplified by HSE of the Hsp70 gene cloned into the promoter of the thymidine kinase gene, which when exposed to an increase in temperature caused thymidine kinase production (for review see Latchman, 1997).

1.1.3 The process of transcription: a *rendezvous* between RNA polymerase II and transcription factors

Transcription factors are proteins that bind to cis-DNA elements and play a pivotal role in the regulation of gene expression. They include an array of proteins such as activators, co-activators, architectural factors, repressors and co-repressors, chromatin remodeling factors and transcription elongation factors. Eukaryotic transcription factors can be divided into two main groups, namely general transcription factors and, gene specific transcription factors. The mechanism by which these proteins act will be discussed briefly.

As RNA polymerase is unable to bind the DNA directly, it is recruited to the accessible promoter as a part of a complex with general transcription factors. On many promoters this process occurs by the step-wise assembly of general transcription factors (TFIID, IIB, IIA, IIE, IIF and IIH) that form the pre-initiation complex (PIC) (Drapkin et al, 1993). On a TATA containing promoter, the initial binding of TFIID, consisting of TATA box binding protein (TBP) and the TBP associated factors (TAF), to the TATA elements appears to facilitate the assembly of other transcription factors to the complex. Moreover, binding of TFIID stabilizes the nucleosomes in the promoter region and therefore encourages PIC formation (White and Jackson, 1992, Buratowski, 1994). Thus, binding of TFIID to the DNA is one of the rate-limiting steps of transcription and the TBP component of this complex has been shown to be one of the core proteins with which transcriptional

activators interact to modulate gene expression (Chatterji and Struhl, 1995). Another key rate-limiting step for transcription is the recruitment of TFIIB. In fact, it is the binding of TFIIB to the TFIID/TFIIA/DNA complex that recruits RNA polymerase II to this complex (Lin and Green 1991, Choy and Green, 1993). TFIIB most likely acts as a bridge between TFIID/TFIIA/DNA and RNA polymerase II. Following binding of RNA polymerase to the PIC, other general transcription factors are recruited to this site where they exert their effects to ensure transcription of the target gene. These include TFIIE, a DNA-dependent ATPase, which provides the energy for transcription, TFIIF which unwinds the DNA, and TFIIH which exhibits enzymatic activity required to initiate transcription (Gilbert, 1994).

While general transcription factors are common to all RNA polymerase II transcribed genes, independent of cell type, gene specific transcription factors are responsible for the activation or repression of specific genes. These ensure tightly controlled temporal and/or tissue-specific expression. Binding of these specific transcription factors at short specific sequences, referred to as response elements, mostly enhance but may repress the formation of PIC and, thus modulate the relative efficiency of transcriptional initiation by activation or repression. The specificity of function of this class of transcription is exemplified by the MyoD transcription factor which is synthesized exclusively in skeletal muscle cells. Upon artificial expression of MyoD in non-muscle cells such as fibroblasts, it is converts these cells into muscle cells, indicating that MyoD activates transcription of all the genes necessary to produce a differentiated muscle (Olson, 1990). Hence, gene specific transcription factors ensure tight control of transcription in a tissue- or time specific manner, hence allowing for proper development and maintenance of different cell lineages.

Regulation of transcription by specific transcription factors may be controlled at different levels. Some transcription factors are activated to bind their DNA elements on target promoters after post-translational modifications such as phosphorylation, acetylation and/or ubiquitination. For example, tumour suppressor protein p53 and its negative regulator Mdm 2 are both subject to phosphorylation, and present an excellent case for positive and negative effects of post-translational

modifications. While p53 is subject to hyperphosphorylation following DNA damage and becomes active and stable by this change, Mdm 2 also becomes phosphorylated but this change results in its inactivation. It is believed that phosphorylation of these two factors causes weaking of the p53-Mdm 2 association so that p53 is released and free to bind the DNA and controll the target gene expression (Hirao et al, 2000; Mayo et al, 1997). Similarly, other inducible transcription factors are activated by hormones or growth factors such as the steroid and thyroid hormones that combine with intracellular receptors to form active transcription factors.

1.1.4 Families of transcription factors

The ability of these factors to modulate transcription is due to their modular structure in which distinct regions of the protein mediate its association with the DNA and factors involved in the regulation of a gene (Brent and Ptashne, 1985; Johnson and McKnight, 1989). Specific domains of eukaryotic transcription factors can adopt highly ordered conformations for specific requirements in terms of binding to the DNA or other proteins. The presence of domains that allow interactions with other proteins that may be expressed at a specific time or in response to specific stimulus, provides a mechanism by which the expression of a diverse range of genes can be regulated by a relatively limited number of transcription factors (Struhl, 1991; Hershlag and Johnson, 1993). Modular structure of transcription factors is exemplified by the estrogen and glucocorticoid nuclear receptors, where different domains are interchangeable so that the DNA-binding or ligand-binding can be altered by switching of specific domains with resultant changes in activation and function (see Hollenberg and Evans, 1988).

On the basis of common evolutionary conserved DNA binding motifs, most transcription factors have been grouped into families. These include the zinc finger motif, the basic leucine zipper motif, the helix-loop-helix motif and the related helix-turn-helix (HTH) motif (for review see Patikoglou and Burley, 1997; Latchman, 1990a; 1990b). However, there are several other interesting and important DNA-

binding proteins that do not fall into any of the classes listed above. An example of this is the tumour suppressor p53 protein (Cho et al, 1994).

The zinc finger motif was originally recognized in factor TFIIIA, which is required for RNA polymerase III to transcribe 5S rRNA genes. It has since been identified in several other transcription factors (and presumed transcription factors). A distinct form of the motif is found also in the steroid and thyroid receptors. Retinoic acid and vitamin D receptors are also found in this family of transcription factors. A single amino acid change within one of the zinc fingers of the vitamin D receptor has been found to be a cause of hereditary vitamin D-resistant rickets (Mechica et al, 1997). Transcription factors with a zinc motif have also been implicated in several malignant diseases. Mutations in steroid hormone receptors such as oestrogen receptors have been reported in some breast cancers (Webster et al, 2001; Wang et al, 2004). The oncogene bcl-6, which has an important role in large-cell lymphoma, is a zinc-finger protein (Ye et al, 1993). Another zinc-finger protein, shown to have clinical implications, is Wt-1, a tumour-suppressor protein that is inactivated in children with Wilm's tumours (Schneider et al, 1993).

Leucine zippers consist of a stretch of amino acids with a leucine residue in every seventh position. A leucine zipper in one polypeptide interacts with a zipper in another polypeptide to form a dimer. Adjacent to each zipper is a stretch of positively charged residues that is involved in binding to DNA. The leucine-zipper motif underlies the interactions between the proto-oncogene products Jun and Fos, which constitute the heterodimeric transcription factor, Ap-1, and are implicated in signal-dependent processes that determine cell lineages and growth, and hence carcinogenesis (van Dam and Castrillazzi, 2001).

The amphipathic helix-loop-helix (HLH) motif has been identified in some developmental regulators and in genes coding for eukaryotic DNA-binding proteins. Each amphipathic helix presents a face of hydrophobic residues on one side and charged residues on the other side. The length of the connecting loop varies from 12-28 amino acids. The motif enables proteins to dimerize, and a basic region near this motif contacts DNA. While involved in lymphocyte, muscular, neuronal, adipocyte, and epithelial homeostasis (Coppe et al, 2003), aberrant

transcriptional activity of this group of factors are also implicated in malignant disorders such as the aberrant expression of HLH HASH-1 protein in neuroblastomas (for review see Axelson, 2004).

The closely related HTH motif of transcription factors was originally identified as the DNA-binding domain of phage repressors. One alpha-helix lies in the wide groove of DNA; the other lies at an angle across DNA. A related form of the motif is present in the homeodomain, a sequence first characterized in several proteins coded by genes concerned with developmental regulation in *Drosophila*. It is also present in genes for mammalian transcription factors. Some of these regulatory proteins are essential for the differentiation of hematopoietic precursor cells, and have been implicated in the development of breast cancers where constitutive expression of a novel gene product with HTH motif (termed candidate of metastasis 1) has been shown to increase metastatic potential (Hansen Ree et al, 1999).

As this thesis primarily deals with the effect of Brn-3a and Brn-3b which are members of the POU-domain of transcription factors, which contain the HTH that facilitates DNA binding, the general properties of this family of proteins will be discussed in some detail (section 1.2), followed by a detailed account of Brn-3a and Brn-3b in section 1.3.

1.2 The POU domain family of transcription factors

In 1988, three mammalian transcription regulators were independently isolated and characterized. They included the ubiquitously expressed Oct-1 (Sturm et al, 1988), the largely B-cell restricted Oct-2 (Muller-Immergluck et al, 1988; Clerc et al, 1988; Ko et al, 1988; Scheidereit et al, 1988), and the pituitary-specific Pit-1 (Bodner et al, 1988; Ingraham et al, 1988). The subsequent comparison of their nucleotide and amino acid sequences with the *Caenorhabditis elegans* developmental regulator, Unc-86 (Finney et al, 1988), identified a region of high homology encoding a conserved structural component that was common to all these factors. Hence, a new family of transcriptional regulators, commonly referred

by the acronym POU (derived from the first letter of Pit-1, Oct-1/2 and Unc-86) emerged. Since their initial discovery, several other proteins have been added to the family based on the homology shared within the evolutionary conserved POU domain. All members have been shown to play important roles in ontogenetic development and cellular differentiation in specific cell types of the nervous system (for reviews see Anderson and Rosenfeld, 2001; Rosenfeld, 1991; Wegner et al, 1993).

1.2.1 The structurally conserved POU domain

The POU domain is a region of high homology that is unique to all the members of the POU-domain protein family. This domain, which varies in length from 147 to 156 amino acids, comprises two structurally independent sub-domains, the amino-terminal POU-specific domain (POU_s), of 70 – 80 amino acids, and the carboxyl-terminal POU-homeodomain (POU_{hd}), of 60 amino acids (Herr et al, 1988). The two sub-domains are linked via a poorly conserved linker region of 14-25 amino acids in length (Fig. 1. 2.1).

The sequence homology within the POU domain, including the poorly-conserved linker region, has allowed the known mammalian POU domain proteins to be further classified into six groups I-VI (for reviews see Andersen and Rosenfeld, 2001; Rosenfeld, 1991; Treacy and Rosenfeld, 1992). Outside the POU domain, there is significant divergence in the sequence and structure of POU-proteins.

Despite the structural independence of the two sub-domains (Botfield et al, 1992), they function as a single functional unit. Mutagenesis studies involving POU proteins, Pit-1 and Oct-1, have clearly shown that the two sub-domains are required together to allow high-affinity, sequence-specific binding to the appropriate DNA response elements (Sturm and Herr, 1988; Ingraham et al, 1990; Klemm et al, 1994; Kristie and Sharp 1990; Aurora and Herr, 1992; Verrijzer et al, 1990, 1992).

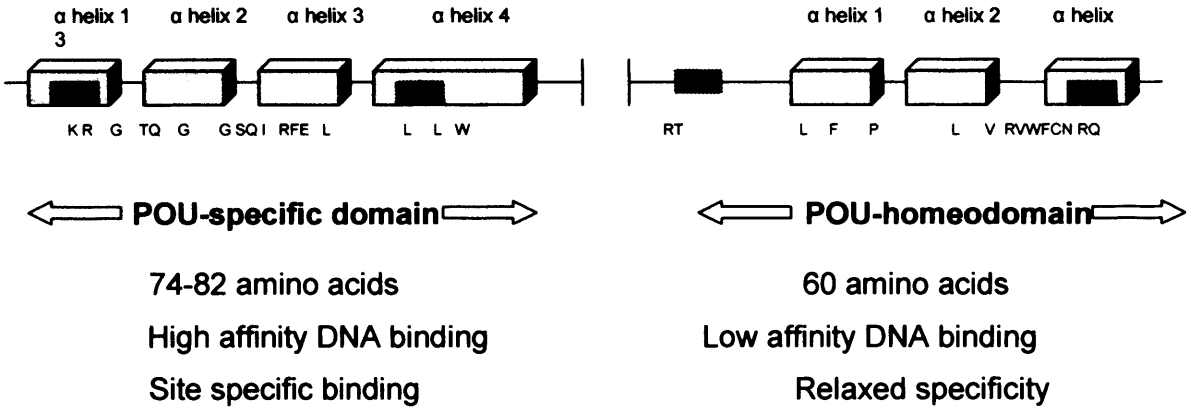


Fig 1.2.1: Schematic representation of the secondary structural elements of the POU domain

The POU domain can be subdivided into the highly conserved, POU specific domain (POU_s) and the POU homeodomain (POU_{hd}) separated by a linker region that varies in length (14-25 amino acids). The POU_s domain consists of 75-82 amino acids, and is composed of four α - helices. The POU_{hd} domain consists of a region of 60 amino acids and has three α - helices. Conserved regions of basic amino acids are found in both POU_s and POU_{hd} (indicated by ■). Highly conserved amino-acids among POU domain proteins are written underneath in the 1-letter code. (Adapted from Wegner, 1993)

1.2.1.1 POU-Specific domain (POU_s)

The POU specific domain (POU_s) is unique to the POU-domain proteins and is highly conserved among all members of the POU proteins. Nuclear magnetic resonance spectroscopy (NMR) analysis on the solution structure of POU_s of Oct-1 has been carried out and taken to represent the general structure of POU proteins. This has shown that the POU_s domain consists of two long and two shorter α-helices (Klemm et al, 1994). The second and the third helix form a helix-turn-helix (HTH) structure that binds to the major groove of the DNA (Fig. 1.2.1.1). This HTH motif is similar to that found in the DNA binding domains of the bacteriophage λ repressor protein (Dekker et al, 1993; Assa-Munt et al, 1993). The marked similarity between the two HTH α-helices in the POU_s and bacteriophage DNA-

binding domains may be suggestive of evolutionary conservation due to the ability of this structure to bind DNA efficiently.

1.2.1.2 POU homeodomain (POU_{hd})

The POU homeodomain (POU_{hd}) shows homology with the homeodomain of classic homeobox genes and is also highly conserved in POU proteins. The crystal structure of the Oct-1 POU domain bound to a DNA sequence containing an octamer site has revealed that the POU_{hd} consists of three α-helices that form a compact globular structure, with helix 2 and 3 forming an HTH unit (Klemm et al, 1994). In this Oct-1/DNA structure it is helix 3 that makes several contacts with the the 3' half of the octamer site (Fig 1.2.1.1).

1.2.1.3 The linker region

The linker element in the POU domain is a poorly conserved spacer region of 14 – 25 amino acids in length that is found between the POU_s domain and the POU_{hd}. Analysis of the crystal structure of Oct-1 POU domain has suggested that the linker region is a flexible, disordered structure in POU proteins (Klemm et al, 1994). The precise function of the linker region remains to be elucidated. However, proteolysis studies have ruled out the possibility of the linker being directly bound to the DNA since this region is easily accessible to the proteases (Botfield et al, 1992; Aurora and Herr, 1992). At present, the linker is simply regarded as a flexible tether that covalently connects the POU_s domain to the POU_{hd}, with a role in facilitating the orientation of these two sub-domains on the DNA.

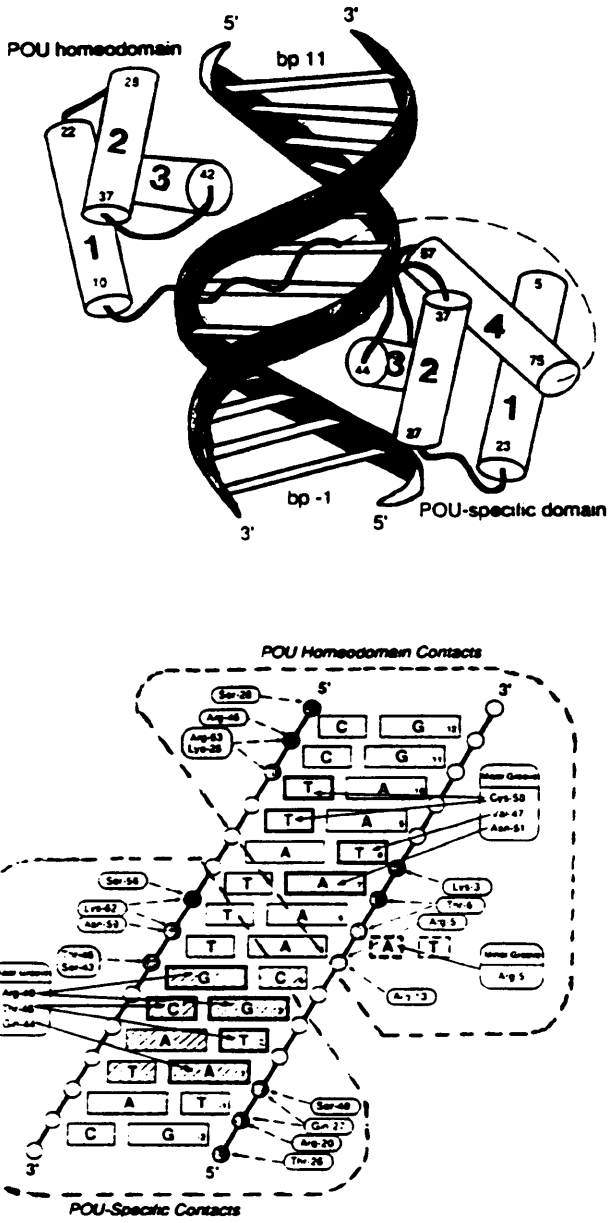


Fig 1.2.1.1: (A) Schematic representation of the contacts made by the POU-specific and POU- homeo domains upon binding to the DNA recognition site. Cylinders indicate the positions of the α-helices. The flexible linker which connects the two domains are indicated by the solid and dotted lines. (B) Sketch summarizing all the DNA contacts made by the POU domain of Oct-1 on its DNA recognition sequence. The DNA is represented as a cylindercal projection and the Atgc and AAAT subsites recognised by the POU-specific and POU- homeo domain, respectively, are highlighted (Taken with permision from Klemm et al, 1994).

1.2.2 DNA response elements recognized by the POU domain factors

The categorisation of the POU domain proteins into six distinct sub-classes is based on similarities in their genetic structure and expression, as well as cis-acting DNA response elements that are recognised by members within each class. Each POU transcription factor family exhibits preferential binding to a specific DNA sequence. For example, the class I Pit-1 protein binds most effectively to the consensus sequence 5'-ATGNATAWW-3' (where N= any base and W=A/T) and was first identified as the transcription factor that bound a regulatory region common to the growth hormone and prolactin genes (Bodner et al, 1988; Ingraham et al, 1988). Class II proteins bind to the octamer motif 5'-ATCGAAAT-3' with the maximum affinity (Aurora and Herr, 1992; Verrijzer et al, 1991; 1992). However, the proteins of this class also have the ability to bind other non-canonical sequences, but with a commensurate reduction in the binding affinity (Verrijzer et al, 1991; 1992; Aurora and Herr, 1992; Baumruker et al, 1988). The class III and class IV POU protein bind with the highest affinity to sites with the consensus sequence $CAT(N)_nWAAT$ (where N=any base, W=A/T, class III n=0, 2, or 3, class IV n=3) (Li et al, 1993). Sequences flanking the core recognition site confer a moderate but significant influence on the binding affinity of POU-domain proteins with the DNA, hence allowing for some degeneracy within the DNA binding motif recognised by these proteins (Baumruker et al, 1988). In summary, members of the same sub-class of POU domain factors exhibit similar DNA-binding preferences, and all DNA binding sites recognized by POU transcription factors are A/T rich sequences and lable sequence similarities.

1.2.3 Bipartite DNA binding by POU domain proteins

POU domain proteins interact with DNA via their unique POU-domains. X-ray crystallography and NMR imaging have resolved several structures of POU family of transcription factors on their DNA response elements. These analyses have revealed in atomic detail the great flexibility allowed by the bipartite DNA binding POU domain (Klemm et al, 1994; Jacobson et al, 1997). The two structurally independent sub-domains of the POU domain, Pou_s and POU_{hd}, both contain HTH

DNA binding motifs which make sequence specific DNA contacts in a cooperative manner (Klemm et al, 1994).

The isolated POU-specific domain binds to the DNA recognition sites with high sequence specificity but the binding affinity is low (Strum and Herr, 1988; Ingraham et al, 1990; Verrijzer et al, 1992; Aurora and Herr, 1992). *In vitro* binding assays including electromobility shift assay (EMSA) with normal and mutated octamer binding sites as well as using ultraviolet cross-linking have shown that the POU-specific domain of Oct-1 recognises and binds to the 'left side' of the octamer motif (Verrijzer et al, 1990,1992; Aurora and Herr, 1992). In agreement, crystallography studies of the POU domain of Oct-1 bound to an octamer sequence, ATGCAAAT, have clearly highlighted that the POU_s domain recognises ATGC sequence on the 5' half of the POU domain binding site (Klemm et al, 1994). It is, in fact, the third α-helix of the POU_s domain that binds this sub-site in the major groove of the DNA (Fig. 1.2.1.1). This high specificity binding of the POU_s domain to the DNA-recognition site causes bending in the DNA, which may be critical for conformational changes to allow transcriptional complex assembly or protein-protein interactions, necessary for the POU domain proteins to function (Dekker et al,1993). Moreover, POU_s has been implicated in DNA replication function by Oct-1, and it is thought that the unwinding of the DNA by the torsion at the bend may also facilitate DNA replication machinery to bind to the single-stranded, unwound DNA (Verrijzer et al, 1990, 1991).

The POU_{hd} of Oct-1 has been shown to bind AAAT subunit at the 3' ends portion of the octamer sequence (Klemm et al, 1994). The first nine residues of the POU_{hd} form an extended N-terminal arm that fits into the minor groove of the DNA. It is the third helix that makes base contacts in the major groove of the DNA and is referred to as the recognition helix of WFC region. This region contains the invariant RVWFCN residues in all known members of the POU domain proteins and makes contact with the TAAT sub-site of the recognition sequence. The importance of this helix is exemplified by the mutations in the Pit-1 POU_{hd} where the conversion of a trytophan residue at position 7 to a cysteine results in the loss of binding to the recognition motif (Li et al, 1990). Both the amino- and carboxyl-

terminals of the POU_{hd} contain a set of basic amino acids, critical for their ability to bind DNA (Treacy and Rosenfeld, 1992).

Upon binding the consensus octamer motifs, the two sub-domains are oriented on the opposite sides of the DNA, with no contacts between them (Fig 1.2.1.1) (Dekker et al, 1993; Klemm et al, 1994). It appears that the POU_s domain exerts a stronger preference for binding to the cognate DNA sequences and, therefore, dictates the overall binding of the POU proteins (Verrijzer et al, 1992a, 1992b). In addition, the POU_s domain stabilizes the contacts made by the adjacent POU_{hd}. Hence, the two sub-domains of the POU domain acting in concert confer the high-affinity, sequence specific binding to the appropriate recognition sites of the DNA.

1.2.4 Different classes of the POU family members

Following the initial identification of four members of the POU domain proteins, an additional twenty two POU domain gene products have been identified in mammals, *C.elegans*, *Drosophilia*, and *Xenopus*. These known POU domain family members have been subdivided into six distinct classes: I-VI (Table 1.2.4). Originally this subdivision into groups was based on the similarities shared within the POU_s and linker region. However, additional features are conserved within these classes. For example, the mammalian class III POU domain genes are intronless and have homologous regions of various homo- and poly-meric residues at their amino-terminal (for review see McEvilly and Rosenfeld, 1997). Some members of the class IV POU domain genes contain a conserved region referred to as the POU IV box in the amino terminus of the protein that shares homology with the mammalian gene MYC-N (discussed in section 3) (Veenstra et al, 1997).

Class	Factor	Chromosome Localization	Expression		Knock-out Phenotype
			Embryo	Adult	
I	Pit-1(GHF-1, PUF-1, Pou1f1)	Mouse 16	Neural tube	Pituitary	Dwarfism
II	Oct-1(OTF-1, NF-A1, NFIII,OBP100,Pou2f1)	Mouse 1 Human 1	Ubiquitous	Ubiquitous	Not reported
	Oct-2(OTF-2, NF-A2, POU2f2)	Mouse 7 Human 19	Neural tube, Brain	Lymphoid cells, Nervous system, Intestine, Testis, Kidney	Aberrant B-cell maturation;
	Skn-1a/i(Oct-11, Otf-11, Epoc-1, POU2f3)	Mouse 9 Human 11	Developing Epidermis	Epidermis	Abnormal terminal differentiation of epidermal keratinocytes
III	Brn-1(Otf-8, POU3f3)	Mouse 1	Developing nervous system	CNS, Kidney	Not reported
	Brn-2(N-Oct3,N-Oct5, Otf-7, POU3f2)	Mouse 4 Human 6	Developing nervous system	CNS, Glioblastoma, Neuroblastoma	Defects in the differentiation and migration of hypothalamic neurons
	Brn-4(RHS2, N-Oct4, Otf-9, POU3f4)	Mouse X	Neural tube	CNS (forebrain)	Defects in the inner ear development manifested as deafness
	Tst-1(Oct-6,SCIP, Otf-6, Pou3f1)	Mouse 4	Blastocyst, ES/EC cells, Brain	Nervous system, Testis Myelinating glia	Defective myelination; abnormal phrenic nucleus
IV	Brn-3a(Brn-3.0, RDC-1, Pou4f1)	Mouse 14 Human 13	Brainstem Retina, Spinal cord Sensoryganglia Neuroepithelioma	Nervous system, Retina, Sensory ganglia, Ewing's sarcoma	Lack of specific sensory and autonomic neurons
	Brn-3b(Brn-3.2, Pou4f2)	Mouse 8 Human 4	Developing brain, Sensory neurons, Retina	Nervous system, Sensory ganglia, Retina, Cervix, Ovary	Loss of retinal ganglon cells resulting in blindness
	Brn-3c (Brn-3.1, POU4f3)	Mouse 18 Human	Sensory neurons, neurons, DRG, Trigeminal ganglia	Sensory DRG, TG	Loss of inner hair cells, resulting in deafness
V	Oct3/4(Oct-5, Otf-3, NF-A3, POU5f1)	Mouse 17 Human 6	ES/EC cells Primordial germ cells Testis, Ovaries	Oocytes	All cells in the early embryo become trophoblasts
VI	Brn-5(Emb, mPOU, TCFβ, Pou6f1)	Mouse 15 Human 12	Developing brain	Skeletal muscles, Brain, Heart, Lung	Not reported
	RPF-1(POU6f2)	Mouse 13 Human 7	Developing brain, Lung, Spinal cord	Brain, Heart, Adrenal anterior pituitary	Not reported

Table 1.2.4: The six classes of POU transcription factors (adapted from Andersen and Rosenfeld, 2001; NCBI Entrez Gene)

The alternative names by which these factors may be referred to are also given. The chromosomal localization of the genes and their expression pattern during development and in adult life are indicated. The phenotypes of the knock-out mouse models are also briefly discussed. (ES-embryonic stem cell, EC- embryonic carcinoma cell, CNS- central nervous system, TG-trigeminal ganglia, DRG-dorsal root ganglia)

1.2.5 Expression and function of POU domain proteins

Ontogenic studies of the POU-domain gene family have revealed that specific POU-domain proteins are expressed temporally in different cells of the developing nervous system. In adults, the POU domain transcription factors exhibit a more spatially restricted pattern of expression, with the exception of the ubiquitously expressed Oct-1 which is thought to be involved in the regulation of expression of a number of housekeeping genes such as the histone H2B and snRNA genes (Hinkley and Perry, 1992; Mattaj et al, 1985). Subsequent studies using developmental mutants or genetic analysis, in which specific POU domain genes were mapped to the loci associated with developmental disorders, have further highlighted an important role for POU proteins either during early embryogenesis or in the terminal differentiation of specific cell types (see review, Rosenfeld, 1991; Verrijzer and van der Vilet, 1993; Wegner et al, 1993; Latchman, 1999).

For instance, the class I POU protein, Pit-1, is expressed in the anterior pituitary and regulates the expression of the prolactin and growth hormone genes (Nelson et al, 1988). Pit-1 is critical for the normal development of the anterior pituitary gland and mutations that result in its inactivation have resulted in congenital dwarfism in both mice (Li et al, 1990) and in humans (Radovick et al, 1992). Loss of Pit-1 is associated with the absence of pituitary hormones prolactin (PRL), growth hormone (GH) and thyroid stimulating hormone (TSHβ) all of which are crucial for normal growth. Besides its role in the regulation of these terminal differentiation marker genes (GH, PRL, and TSHβ), Pit-1 stimulates cell proliferation of pituitary cells, as evidenced in the Pit-1 deficient *Snell* and *Jackson* dwarf mice, which exhibit a severe failure in the expansion of somatotropes, lactotrophes and thyrotropes (Li et al, 1990). Moreover, reduction in the levels of Pit-1 using antisense strategy results in decreased cell proliferation of somatotroph cells (Castrillo et al, 1991). Pit-1 provides an underlying theme of the involvement of POU-domain proteins in the coordination of cell proliferation and orderly progression of cellular differentiation.

Oct-2 is expressed in B lymphocytes as well as in specific neuronal cells in the developing and adult nervous system (Hatzopoulos et al, 1990; He et al, 1989). Oct-2 exhibits different functions in the two cellular systems such that in B lymphocytes, it activates the expression of immunoglobulin genes whose promoters contain an octamer motif (Singh et al, 1986; Wirth et al, 1987). In contrast, in neuronal cells, Oct-2 protein essentially inhibits expression of certain genes such as the herpes simplex virus immediate-early genes (Lillycrop et al, 1991) as well as the cellular tyrosine hydroxylase gene promoter (Dawson et al, 1994). These differences in activity between Oct-2 in neuronal and B-cells result from protein variants being produced in the two cell types. Alternative splicing of the primary transcript of the Oct-2 gene gives rise to different forms of the mRNA encoding different Oct-2 proteins (Oct 2.1 to Oct 2.8), with different transactivation function (Wirth et al, 1991; Lillycrop and Latchman, 1992). These different forms of Oct-2 are differentially expressed in B-cells and neuronal cells, with the activator Oct-2.1 being the predominant form in B-cells whilst repressor isoforms, Oct 2.4 and Oct-2.5, are more abundant in neuronal cells (Lillycrop and Latchman, 1992). Knock out Oct-2 mice show a failure of the mutants to feed properly and die shortly after birth, supporting a critical role for this transcription factor in the normal development of the nervous system (Corcoran et al, 1993; Ninkina et al, 1995).

Other POU factors such as Oct-3/4 and SCIP/Oct-6/Tst-1 are expressed in very early developmental stages. Oct-3/4 expression is characteristic of a pluripotent undifferentiated cell phenotype and is detected in unfertilized oocytes as well as in the fertilized ova. The levels of Oct-3/4 expression are downregulated upon differentiation of the blastocyst (Scholer et al, 1990; Rosner et al, 1990). The SCIP/Oct-6/Tst-1 gene is expressed in pre-implantation embryos and later on in the developing brain and specific neuronal cells, but once these cells have differentiated and exited the cell cycle, expression of this transcription factor drops dramatically (Suzuki et al, 1990; Monuki et al, 1990). Hence, Oct3/4 and SCIP/Oct-6/Tst-1 are associated with a proliferative, pluripotent state of the cell.

Similarly, expression of members of the POU IV family of transcription factors are also developmentally regulated. For instance, the nematode gene product, Unc-86, is developmentally regulated in sensory neurons and is critical for the normal

development of these cells (Finney et al, 1988; Finney and Ruvkun, 1990; Xue et al, 1993). Thus, mutations in Unc-86 gene, which is normally needed for the commitment of several sensory neuroblast lineages as well as the specification and maintenance of particular neural phenotypes, results in the absence of specific sensory neurons (Desai et al, 1988; Finney and Ruvkun, 1990). Members of the Brn-3 sub-family of POU IV proteins are also expressed in specific cells of the mammalian developing nervous system, with their expression becoming more restricted in adults. Inactivation of any of the three closely related POU factors Brn-3a, Brn-3b, and Brn-3c results in the absence of specific neuronal cell types (discussed in detail in section 3). Similarly, mutations in the gene encoding the Brn-4 factor, which result in frameshift and premature stops in the translation of this gene or non-conserved amino acid mutations in the POU domain of the protein, have been shown to be associated with the most common form of deafness in humans (de Kok et al, 1995).

Studies of the expression pattern of several POU domain genes have highlighted some features common to these factors. Firstly, mRNA encoding many POU proteins have been widely detected in the developing nervous system and this suggests a function for POU domain transcription factors in the development and maintenance of the nervous system. Secondly, many POU domain factors appear to be critical for the normal development of specific cell types as demonstrated by *in vitro* and *in vivo* deletion studies. A summary of the expression and function of some POU domain proteins during development and in adult tissue is given in Table 1.2.4.

1.2.6 Phosphorylation and POU protein function

Phosphorylation, as a mechanism of regulation of proteins at a post-translational level, is a recurrent theme in several biochemical pathways. A number of POU proteins undergo such modification and this has been shown to directly modulate their DNA binding activity and hence their function as transcription factors (for review see Jackson, 1992; Caelles et al, 1995). For example, the Oct-1 is phosphorylated (possibly on the Ser^{385} residue in the POU-homeodomain) during

mitosis and is thereby prevented from binding the H2B response element (Segil et al, 1991). This phosphorylation of Oct-1 is reversed as the cells exit mitosis (Segil et al, 1991; Roberts et al, 1991). Therefore, while the protein levels of Oct-1 remain constant during the cell cycle, its function changes. Similarly, phosphorylation of Oct-2 is a prerequisite for the activation of transcription of the β-globin gene promoter (Tanaka and Herr, 1990).

Phosphorylation of Pit-1 protein alters its DNA-binding ability as well as its dimerization potential depending on the recognition site. *In vitro* protein kinase A assays have shown that the phosphorylation of a threonine^{220} residue in the POU_{hd} of Pit-1 decreases its binding to the growth hormone response element, while increasing its binding affinity on the proximal prolactin promoter and has no effect on binding of Pit-1 to its own promoter (Kapiloff et al, 1991). Jun kinase (JNK) / stress-activated protein kinase binds the activation domain of Brn-5 and phosphorylates its POU domain on sites that are distinct from the aforementioned Oct-1/Pit-1 phosphorylation sites. This phosphorylation results in increased binding to DNA and enhanced transactivation (Kasibhatla et al, 1999).

1.2.7 Protein-protein interaction by POU domain proteins

In addition to their ability to bind DNA, POU proteins can also mediate interactions with other proteins to regulate transcription. Since several members of the POU domain family are co-expressed during development, the protein-protein interactions of different POU proteins may provide an additional control for the differential regulation of distinct genes by changes in the levels of different POU proteins. This interaction of POU proteins with co-activators in specific cells at different developmental stages might explain the highly restricted tissue specificity during development by these proteins.

Proteins that interact with POU factors can be classified into four classes: DNA-binding proteins transcriptional activators, co-regulators, basal transcription factors, and replication factors. Interaction of POU proteins with each class is discussed in

the following sections and Figure 1.2.7 gives a schematic representation of a few POU domain proteins interacting with other proteins on some target promoters.

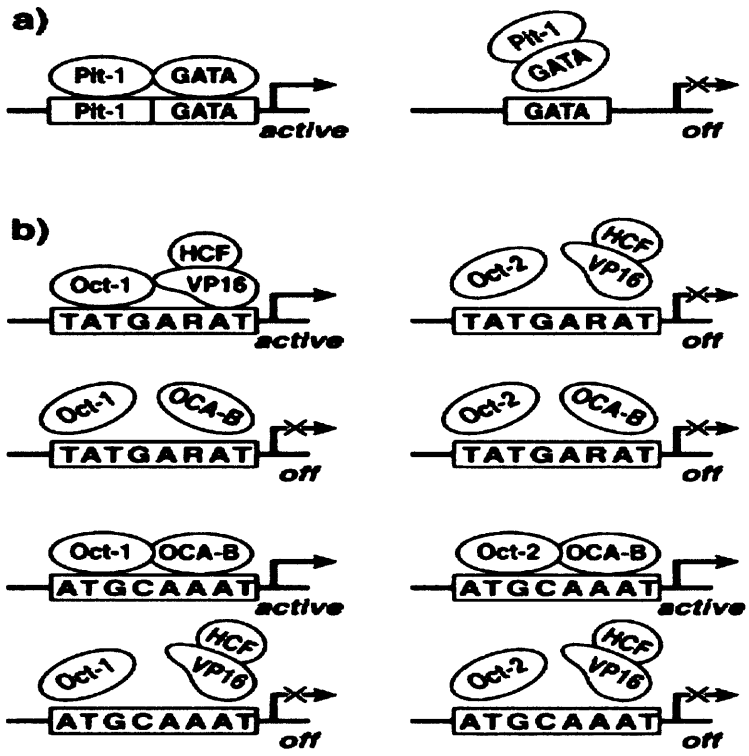


Fig 1.2.7: Protein-protein interactions of POU domain factors.

Interactions of POU domain factors with other proteins are effected by the amino acid sequence of the POU domain and the sequence of the DNA binding site.

a)Pit-1 interacts with GATA transcription factors. On promoters containing binding sites for both Pit-1 and GATA, the interaction seems to be cooperative and results in transcriptional activation (left panel). On promoters containing GATA-binding sites, but lacking Pit-1 binding sites, Pit-1 can inhibit GATA binding to DNA and transactivation (right panel).

b)The cofactors VP16 and OCA-B interact selectively with Oct-1 and Oct-2. VP16 interacts with Oct-1 on TATGARAT DNA sites, with VP16 contacting the GARAT part of the site. This interaction is stabilized by HCF. Oct-1 and VP16 do not interact on a classical octamer site and VP16 is incapable of interacting with the highly related Oct-2, even on TATGARAT sites, indicating that minor differences in the sequence of the POU domain can direct the specificity of protein-protein interactions. In contrast, OCA-B can interact both with Oct-1 and Oct-2. However, this interaction is restricted to sites related to the classical octamer sequence and

does not form on TATGARAT sites (Taken with permission from Andersen and Rosenfeld, 2001).

1.2.7.1 Interactions with other DNA binding proteins

The complexity of regulatory effects by POU proteins is demonstrated by their ability to bind as monomers or dimers (either homo- or heterodimer). For example, Pit-1 and Oct-2 have been shown to be largely monomeric in solution (Lebowitz et al, 1989; Poellinger and Roeder, 1989; Igraham et al, 1990), but these proteins also exhibit cooperative or synergistic interactions with other transcription factors on the target gene promoters in order to maximize their transactivation potential. In this regard, homodimerisation of Pit-1 is required for the maximal stimulation of the prolactin gene. DNA-dependent cooperative binding interactions are encouraged by the presence of four Pit-1 recognition sites within the promoter and four more sites within the enhancer (Nelson et al, 1988). Most of these sites are able to accommodate Pit-1 dimers, allowing a cooperative Pit-1-dependent, cell-specific expression of the prolactin promoter (Crenshaw et al, 1989). Similarly, multiple binding sites for Pit-1 have also been shown to be present in the promoters of the growth hormone gene and the Pit-1 gene itself (Chen et al, 1990; McCormick et al, 1990).

Similarly, cooperative interactions have been shown between two Oct-2 proteins on the immunoglobulin heavy (IgH) chain promoter which contains a heptamer element, adjacent to the natural response octamer motif for this protein (LeBowitz et al, 1989). The heptamer motif enhances activation of the IgH promoter by up to 2-5 fold. Both sites are generally occupied within the IgH promoter so facilitating the cooperative interactions between the POU domains of the bound Oct-2 proteins (Kemler et al, 1989; LeBowitz et al, 1989; Poellinger et al, 1989). Mutations in either the canonical octamer site or the adjacent heptamer site results in complete loss of transactivation suggesting that interaction of POU domain proteins is not restricted to bases within the octamer sequence elements but is also influenced by sequences flanking this octamer motif and hence, contributes to the specificity and activity of these proteins (Poellinger and Roeder, 1989).

POU proteins can also form heterodimers with other POU transcription factors. An example of this heteromeric complex formation between two different POU proteins is provided by interactions between Pit-1 and Oct-1 to mediate synergistic induction of the prolactin promoter (Voss et al, 1991). Oct-1 has also been shown to heterodimerise with Oct-3, Oct-2 and SCIP/Oct-6/Tst1 (Vigano et al, 1996; Verrijzer et al, 1992).

Furthermore, POU proteins can also bind other classes of transcription factors as heterodimers to enhance transcription. For example, Pit-1 can interact with the oestrogen receptor, and together the two transcription factors are required for the maximal stimulation of the prolactin gene expression (Simmons et al, 1990). Similarly, Pit-1 can interact and cooperate with the thyroid hormone receptor of the growth hormone gene promoter (Schaufele et al, 1992), and the GATA-2 transcription factor on the thyrotrope-specific TSHβ promoter (Gordon et al, 1997; Dasen et al, 1999). The members of the POU IV sub-family can also interact with other transcription factors and modify expression of target genes. For example, Brn-3a interacts with p53, which results in differential effects on Bax and $p21^{CIP1/WAF1}$ expression (Budhram-Mahadeo et al, 2002; Perez-Sanchez et al, 2002), whereas Brn-3b interacts and cooperates with the oestrogen receptor (Budhram-Mahadeo et al, 1998). These interactions of Brn-3 proteins are discussed in section 1.3 of this chapter.

Heterodimeristion by POU domain proteins does not only act to enhance transactivational effect but, depending on the target gene promoters, some act as repressors to reduce or prevent transcription. This is exemplified by the heterodimerization between two co-expressed *Drosophila* proteins, I-POU and Cfla, an association which prevents Cfla homodimerisation, needed for binding to and activation of the DOPA decarboxylase gene (Treacy et al, 1991). It appears that the affinity of I-POU for Cfla is much higher than the affinity of the latter for its cognate DNA recognition sequence (Treacy et al, 1991). The biological significance of this association is provided by the detection of co-expression of these two proteins in a sub-set of developing neurons during *Drosophila*

development. Therefore, levels of different proteins that are co-expressed will influence the effects on transcription of specific genes.

The mammalian POU domain protein, Oct-1, provides another example of transcriptional repression achieved by interactions with other proteins. The interaction between the nuclear hormone receptor, retinoic acid receptor (RXR), and Oct-1 causes transcriptional repression of the thyroid hormone response elements (Kakizawa et al, 1999). Oct-1 represses transcription by sequestering RXR and inhibits it from forming active dimers with other members of the nuclear hormone receptor family.

1.2.7.2 Interaction with co-regulator proteins

POU domain proteins have also been shown to interact with co-regulators proteins that fail to bind the DNA directly. One of the most extensively studied examples is the interaction between Oct-1 and co-regulator VP16/Vmw65/αTIF, a viral protein which regulates the expression of HSV immediate-early (IE) genes. VP16 does not bind DNA directly, but is recruited to IE promoters by interacting with Oct-1 (Kristie et al, 1989; Stern et al, 1989; Stern and Herr, 1991). This complex is stabilized by the host cell factor (HCF/C1) (Xiao and Capone, 1990). Oct-1/VP16/HCF complex is recruited to modified octamer sites (5'-TAATGARAT-3') of immediate early genes, ICPO and ICP4. The specific amino acids in the Oct-1 POU domain that are important for this interaction have been located on the surface of the second helix of the POU_{hd} (Lai et al, 1992; Pomerantz et al, 1992). Another transcriptional regulatory region found adjacent to the IE enhancer is that for the cellular transcription factor GABP (Triezenberg et al, 1988). Vogel and Kristie (2000) have shown Oct-1, GABP and VP16 complex is recruited by HCF/C1 to this enhancer site, where HCF/C1 acts as a co-activator of GABP mediated transcription.

Similarly, both Oct-1 and Oct-2 have been shown to transactivate several lymphoid-specific promoters but only after associating with the B-cell specific co-activator, OCA-B (also referred to as OBF-1 and Bob-1) (Luo et al, 1992; Strubin et al, 1995). By interacting with the POU domains of Oct-1 and Oct-2, OCA-B is

recruited to a subset of octamer sites where it serves as a coactivator for transcription (Babb et al, 1997; Mattias, 1998). In addition to making contacts with the POU domains of Oct-1 and Oct-2, it appears that OCA-B also makes direct contacts with the DNA backbone at the centre of the octamer site (Chang et al, 1999; Chasman et al, 1999). A model has been proposed where OCA-B clamps the two POU sub-domains together and simultaneously bridges the DNA within the octamer motif at several positions. Hence, OCA-B seems to help organize the conformation and stabilization of Oct POU domain on DNA (Babb et al, 1997).

1.2.7.3 Interactions with basal transcription factors

Oct-1 and Oct-2 also provides a model of how POU domain proteins are able to interact with the basal transcriptional machinery. The POU domains of Oct-1 and Oct-2 interact with the TATA box binding protein (TBP) *in vitro*. This interaction has been confirmed *in vivo* by co-immunoprecipitation, and co-transfection experiments have shown that introduction of both Oct-2 and TBP gives rise to synergistic activation of transcription at an octamer-containing promoter (Zwilling et al, 1994). Deletion studies have demonstrated this interaction to be mediated via the POU domain of Oct-2 and C-terminal domain of the general transcription factor, TFIID (Zwilling et al, 1994). Moreover, Oct-2 seems to be required continuously at the promoter for multiple rounds of transcription (Arnosti et al, 1993).

In addition, Oct-1 has also been shown to play a critical role in pre-initiation complex recruitement at some promoters that lack the TATA element. For example, interaction of Oct-1 with transcription factor II B (TFIIB) stimulates the lipoprotein lipase promoter containing an octamer motif (Nakshatri et al, 1995). Therefore, in certain cases, Oct-1 appears to functionally substitute for TBP.

1.2.7.4 Interactions with replication factors

The POU domain of Oct-1 interacts with the precursor terminal protein (pTP), a viral protein required for adenoviral replication (de Jong and van der Vliet, 1999;

Botting and Hay, 1999). The Oct-1 interaction affects the conformation of pTP and this interaction may activate the DNA polymerase and initiate viral DNA replication (Botting and Hay, 1999). However, more extensive studies are required to establish the role of POU factors in DNA replication.

1.2.8 Activation and repression domains of POU domain proteins

Whilst the POU domain is involved in DNA binding, the major regulatory domain(s) of known POU proteins have been mapped outside their DNA binding domain at the amino- and carboxyl- terminus of the proteins. These domains are poorly conserved between different POU domain factors, reflecting the specificity of action of different factors which often bind to the same DNA response elements.

The N- and C- terminal transactivation domains of Oct-2 have been extensively characterised (Tanaka and Herr, 1990; Tanaka et al, 1992; 1994; Muller-Immergluck et al, 1990). Various isoforms of Oct-2 have different activation domains which may be associated with the different transcriptional effects. Thus, whereas Oct-2.1 has two activation domains located in the amino and carboxyl terminals, Oct-2.5 lacks the carboxyl-terminal activation domain due to post-transcriptional processing (Schreiber et al, 1988; Hatzpoulos et al, 1990; Wirth et al, 1991). The significance of these differences in the activation domains is seen in the ability of Oct-2.1 to activate all octamer containing promoters while Oct-2.5 has a generally repressive effect on transcription (Lillycrop et al, 1994; Dent et al, 1991; Lillycrop and Latchman, 1992). The identification of other domains such as the leucine zipper domains in Oct-2.1 protein may also contribute to protein-protein interactions, suggesting that interaction between different factors achieves the resultant transcriptional effect of POU proteins (Lillycrop and Latchman, 1992).

SCIP/Oct-6/Tst-1 has its activation domain located at the amino-terminus of the protein. When this domain is coupled to the heterologous Gal4 DNA-binding domain, the hybrid protein acts as a strong transactivator of Gal4 target constructs in Schwann cells but not in Hela cells. This suggests that SCIP/Oct-6/Tst-1interacts with a cell-specific co-activator that is only present in Schwann cells and

this transactivation domain may determine the target regulated by SCIP/Oct-6/Tst-1. SCIP/Oct-6/Tst-1 acts as a transcriptional activator on some genes, such as immunoglobulin promoter (Suzuki et al, 1990) or as a repressor of genes encoding myelin specific protein (Monuki et al, 1990). The mechanism by which the repression is achieved has not yet been determined.
In general, POU domain proteins contain one or more regulatory domains, and the requirement of other cell-type-specific co-factors suggests a complex interaction of a number of factors to achieve strong transcriptional effects reported on various target gene promoters controlled by these proteins.

1.3 POU IV subclass of POU domain proteins

Following the identification of the four POU-domain containing proteins, He and colleagues (1989) subsequently found other novel proteins from different tissues within this family. They utilised a technique in which reverse transcriptase/polymerase chain reaction strategy was carried out using degenerate primers designed from each end of the highly homologous POU domain. These primers were used to amplify cDNA made from RNA extracted from human and rat brain and testis. One factor isolated from brain cDNA using this approach was called Brn-3 (now referred to as Brn-3a, Brn-3.0, or POU4F1). A similar approach was utilized by Lillycrop and colleagues (1992) using cDNA prepared from the ND7 cells, a cell line derived from the fusion of mouse neuroblastoma cell line and sensory neurons derived from dorsal root ganglia (DRG) (see later section 1.3.6.1.2). Results of these studies identified a protein with high homology to Brn-3a in the POU domain, and this is now referred to as Brn-3b (Brn-3.2 or POU4F2) (Lillycrop et al, 1992). Similarly, another member of this sub-family, Brn-3c (Brn-3.1 or POU4F3) has been isolated from a cDNA library prepared from DRG and spinal cord (Ninkina et al, 1993).

The mammalian family of Brn-3 proteins together with nematode Unc-86 and *Drosophila* I-POU have been categorised into POU-IV sub-family of POU domain proteins. The proteins of this class share a particularly high homology in the POU domain. Some members of this family also have a highly conserved region in the

N-terminal domain referred to as the POU-IV box (Gerrero et al, 1993; Theil et al, 1993).

As this thesis primarily deals with Brn-3a and Brn-3b, the focus in the next sections will be on these two transcription factors in terms of their expression and functional roles in different cell types.

1.3.1 Chromosomal localization of Brn-3a and Brn-3b

Brn-3a and Brn-3b proteins are encoded by different genes and localized on different chromosomes. While Brn-3a is found on mouse chromosome 14 (14E1-3) (Xia et al, 1993; Theil et al, 1994), its human homologue has been mapped to chromosome 13 (13q14-q22) (Collum et al, 1992). This is in agreement with the evolutionary conserved relationship between human chromosome 13 and mouse chromosome 14 (Lyon and Kirby, 1993).

Fluorescent in situ hybridization has mapped Brn-3b gene to human chromosome 4 (4q31.2) (Xiang et al, 1993) and on chromosome 8 in the mouse (NCBI data base).

1.3.2 Isoforms of Brn-3a and Brn-3b

Sequence analysis of Brn-3a and Brn-3b has revealed two distinct isoforms of these transcription factors that differ in the amino terminal region (Fig. 1.3.2) (Theil et al, 1993; Theil and Moroy 1993; Gerrero et al, 1993; Turner et al, 1994). Analysis of the genomic sequence for Brn-3a reveals that it is encoded by two exons separated by an intron.

Brn-3a locus has two start sites of transcription located about 500 base pairs apart in the gene sequence. As a consequence of alternative promoter usage, two distinct mRNAs are generated that differ at their 5' end (Fig. 1.3.2) (Theil et al, 1993; Theil and Moroy, 1994). Hence, two distinct Brn-3a proteins are produced that vary in size and have distinct amino terminal domains. The longer Brn-3a

cDNA [Brn-3a(l)] is generated from the upstream transcription start site and has an additional exon at the 5' end, which is not present in the cDNA of the short isoform [Brn-3a(s)]. This additional exon gives rise to an mRNA with a coding potential of 421 amino acid residues, and results in a protein with a molecular weight of 43 KDa. The shorter intronless Brn-3a(s) cDNA has an open reading frame of 1011 nucleotides and encodes a protein of 337 amino acids, with a predicted molecular weight of approximately, 33.5 KDa. Brn-3a(s) protein lacks approximately 80 amino terminal residues and so does not have the POU-IV box found in Brn-3a(l).

Brn-3b has a similar genomic organization with the gene being encoded by two exons separated by an intron. The two isoforms of Brn-3b are also generated as a result of usage of alternative translation start sites. The larger protein [Brn-3b(l)] consists of 411 amino acid residues, giving rise to a protein of 42 KDa (Turner et al, 1994). The shorter isoform [(Brn-3b(s)] consists of 322 residues, with a predicted molecular weight of 35 KDa (Theil et al, 1993). Due to the different site of exon/intron boundaries in the Brn-3b gene, Brn-3b(s) protein has nine amino-terminal residues that are not present in Brn-3b(l) (Fig. 5). However, this shorter isoform also lacks the POU IV box found in Brn-3b(l). The effects of these differences are still to be determined.

1.3.3 The POU IV box

Outside the highly homologous POU domain that is present at the carboxyl terminus of all Brn-3a and Brn-3b proteins, the longer isoforms Brn-3a(l) and Brn-3b(l) proteins share another region of significant homology at the amino terminus, referred to as the POU-IV box (Fig. 1.3.2; Gerrero et al, 1993; Theil et al, 1993). The POU IV box contains forty amino acids which are well conserved in many members of the POU-IV proteins. Comparison of the sequence of the POU-IV box shows significant homology to the highly conserved region in the N-terminus of the c-Myc family members (Collum et al, 1992). In the c-Myc proteins, this region is modified by the microtubule associated protein 2 (MAP-2) kinase for transactivation of these proteins (van-Beneden et al, 1986) but its function in POU IV proteins remains to be elucidated.

The functional significance of this additional domain in the longer Brn-3a and Brn-3b proteins has yet to be ascertained fully. However, recent studies with Brn-3a suggest that this domain but may be required for the increased survival associated with Brn-3a (Faulkes et al, 2004). Even though Brn3a(s) and Brn-3b(s) bind DNA, they are not capable of this function as they lack the amino-terminal POU-IV box. Therefore, the POU IV domain may represent an interface for interacting with other co-activators to modify transcription of specific target genes.

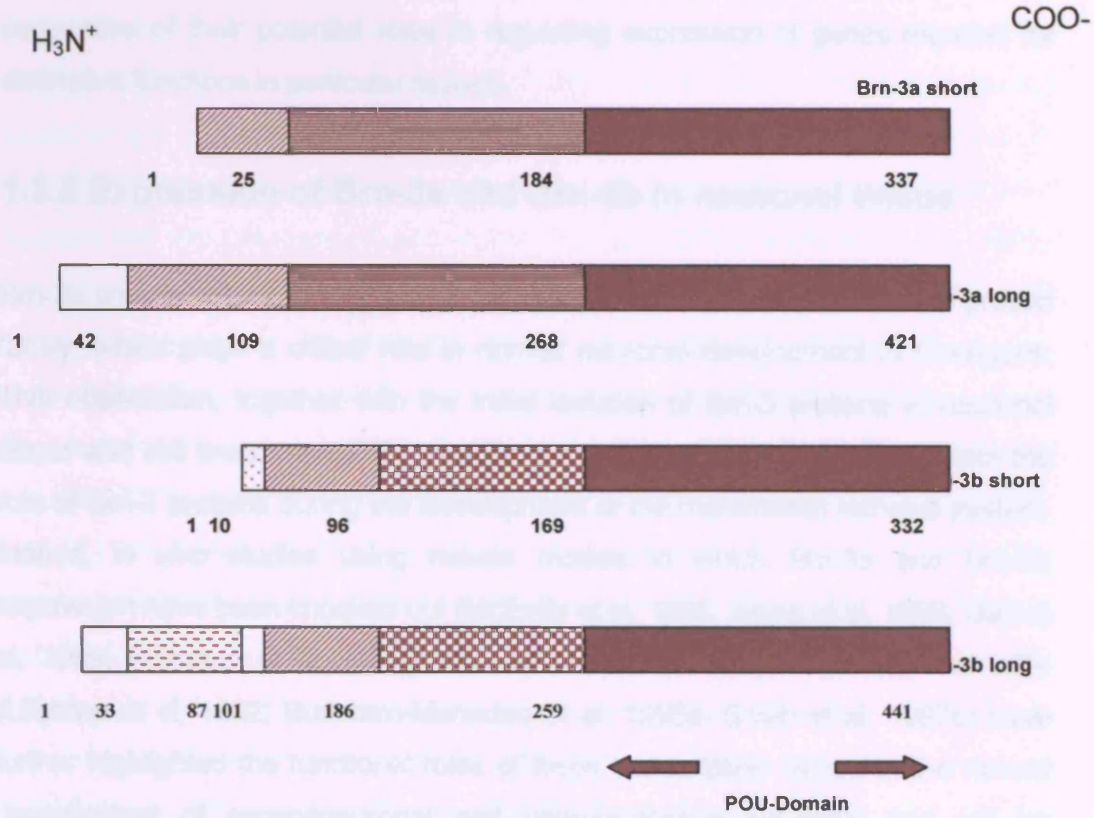


Fig 1.3.2 Schematic representation of the different domains of Brn-3a and Brn-3b
The long isoform [Brn3a(l), Brn-3b(l)] of both transcription factors is due to an extra domain at their N-terminal region. The POU IV box is a highly conserved region found only in the longer isoforms of both proteins, Brn-3a(l) and Brn-3b(l), while the POU domain is found in both forms of each proteins. Regions of high homology are represented by the same pattern. The numbers below each isoform is the positions of the amino acids found at the borders of the subdomains. (Adapted from Theil et al, 1994)

1.3.4 Expression and functional role of the Brn-3a and Brn-3b

Analysis of the expression of Brn-3a and Brn-3b during development in the mouse has shown overlapping expression of these two factors in the central and peripheral nervous systems (Gerrero et al, 1993; Turner et al, 1994; Fedtsova and Turner, 1995; Xiang et al, 1993). Brn-3a and Brn-3b have also been identified in other non-neuronal tissues including the cervix of uterus (Ndisang et al, 1998), breast and ovaries (Budhram-Mahadeo et al, 1998). The expression pattern of Brn-3 proteins in specific subset of cells during development and in adult tissues is suggestive of their potential roles in regulating expression of genes required for distinctive functions in particular tissues.

1.3.5 Expression of Brn-3a and Brn-3b in neuronal tissue

Brn-3a shows high homology to Unc-86, another member of class IV POU protein family, which plays a critical role in normal neuronal development in *C.elegans*. This observation, together with the initial isolation of Brn-3 proteins in neuronal tissue and cell lines, paved the way for extensive investigations to elucidate the role of Brn-3 proteins during the development of the mammalian nervous system. Indeed, *in vivo* studies using mouse models in which Brn-3a and Brn-3b expression have been knocked out (McEvilly et al, 1996; Xiang et al, 1996; Gan et al, 1999; Erkmann et al, 1996), as well as *in vitro* analyses in neuronal cells (Lillycrop et al, 1992; Budhram-Mahadeo et al, 1995a; Smith et al, 1997c) have further highlighted the functional roles of these transcription factors in the normal development of sensorineuronal and neuroendocrine sysytems and will be discussed in detail in the following sections. Table 1.3.5 summarises the expression of these two genes in the developing and adult nervous system.

1.3.5.1 Brn-3a expression

Brn-3a is the most widely expressed of the Brn-3 proteins in the nervous system. The expression pattern of Brn-3a in the peripheral nervous system (PNS) is distinct from that observed in the central nervous system (CNS). In the PNS, Brn-3a expression is first detected in the neuronal precursor cells, immediately before the cells exit the cell cycle, as well as in differentiated sensory neurons. In the CNS, Brn-3a expression is observed only in post-mitotic neurons (Gerrero et al, 1993; Turner e al, 1994; Fedtsova and Turner et al, 1995; Xiang et al, 1995).

Analysis of the expression and localization of Brn-3a in the PNS during development showed that Brn-3a is first detected at embryonic day 9.5 (E9.5) in the migrating neural crest cells, destined for the sensory lineage, just before the neurons exit the cell cycle (Fedtsova and Turner, 1995; Greenwood et al, 1999). Precursors arising from the neural crest can give rise to both sensory and autonomic neurons of the PNS (for review see, LeDouarin et al, 1994). In this respect, Brn-3a is expressed in cells destined for sensory neurons but not in those of autonomic neuronal lineage. By E9.5, Brn-3a is detected in the dorsal root ganglia (DRG) and the spinal cord, when some cells are still dividing (Fedtsova and Turner, 1995). It then continues to be expressed in sensory neurons throughout development when the cells have become post-mitotic, and remains high in trigeminal ganglia (TG) and DRG postnatally (Turner et al, 1994; Fedtsova and Turner, 1995; Greenwood et al, 1999). The early expression of Brn-3a in precursors cells destined for sensory neurons suggests a role for this factor in specification of these cells that is analogous to the role of Unc-86, which is activated before the cessation of cell division in the neuronal precursor cells in the nematode, and plays a critical role in the commitment of dividing neuronal precursors (Finney and Ruvkun, 1990).

In the CNS, Brn-3a is detected at high levels in developing mid-, and hind-brain and in the spinal cord, and continues to be expressed in specific regions of adult brain. During development of the CNS, Brn-3a is detected at E8.5 in occasional cells near the midline of the rostral mesencephlic tectum, prior to the closure of the

anterior neuropore (Fedtsova and Turner, 1995). Unlike Brn-3a expressing cells in the PNS, these cells do not express PCNA and have therefore already exited the cell cycle. By E9.5, Brn-3a can be clearly seen in mesencephalic neurons. Between E12.5 and E15.5 a strong hybridization signal is observed in the inferior olivary complex of the hindbrain, the outer layer of post-mitotic cells in the tectum of the midbrain, and in the interpeduncular nucleus of the midbrain. Low level expression is also observed in the superior and inferior colliculus, the medial habenula and the brainstem. Around this time, Brn-3a expression is also observed in the inner retinal neuroblastic layer of the developing eye, when the ganglion cells are undergoing their differentiation and axonal outgrowth (Gerrero et al, 1993; Turner et al, 1994). By E17.5, Brn-3a RNA is only detected in the medial habenula of the diencephalon in the mid-brain (Gerrero et al, 1993; Ninkina et al, 1993; Xiang et al, 1993; Turner et al, 1994; Xiang et al, 1995). Hence, during CNS development, Brn-3a expression is restricted to post-mitotic neurons in specific regions of the brain.

In adult tissues, Brn-3a mRNA is detected in similar areas of the brain, but in a more restricted manner, compared to the pattern seen during developmental stages. Hence, high levels of Brn-3a have been detected in specific regions of the hind-brain such as the medial habenula, the nucleus ambiguus and the inferior olivary complex. Moderate levels are detected in the region of the midbrain such as the red nucleus, the mesencephalic nucleus and the superior colliculus. Brn-3a is also detected in the adult retina. Moreover, even though present at lower levels, Brn-3a is also found in the adult heart (personal communication S. Farooqui) and lungs (Budhram-Mahadeo et al, 2001). Brn-3a mRNA is not detected in other tissues such as adult liver, kidney, spleen and adrenal glands, suggesting that this factor is more characteristic of neuronal cells and hence most studies have characterized its expression and function in this cell type (Ninkina et al, 1993; Gerrero et al, 1993; Turner et al, 1994; Fedtsova and Turner, 1995).

1.3.5.2 Brn-3b expression

Brn-3b expression during development and in adult tissues shows considerable overlap with Brn-3a. Table 1.3.5 gives a summary of the expression of these two genes in the developing and adult nervous system.

Brn-3b expression is first detected in mouse at E10.5 in the embryonic body, followed by expression in the head at E11.5 compared with the earlier onset of Brn-3a expression observed at E9.5 in similar structures. There is a decrease in the mRNA levels encoding Brn-3b post-partum (Turner et al, 1994).

Detailed analysis for Brn-3b expression in the CNS of the developing mouse revealed its localization in the hindbrain and midbrain, with some similarity to Brn-3a expression. Brn-3b is first detected in these regions at E13.5. By E15.5 positive signals for Brn-3b are also observed in the postmitotic neuronal cell population of inferior olivary complex of the hindbrain, paralleling the Brn-3a expression. By E17.5, Brn-3b, but not Brn-3a, is uniquely detected in the interpeduncular nucleus of the midbrain, where its expression persists in the adult brain (Turner et al, 1994). Protein localisation studies at post-natal day 1 (P1) using Brn-3b antibodies have confirmed the expression of Brn-3b protein in the cerebullum, inferior colliculus and peri-aquaductal gray of the midbrain. In addition, Brn-3b protein levels were also detected in the brain stem, the pons and medulla of the hindbrain (Xiang et al, 1993).

In the retina, Brn-3b mRNA is first detected in the innermost layer of ganglion precursor cells at E11.5 but is absent from the neuroblast layer of cells (Turner et al, 1994). By E17.5, this expression is restricted to the post-mitotic layer. In relation to expression in the retina, it is interesting that Brn-3b preceeds Brn-3a so that whilst Brn-3b is detected by E11.5, Brn-3a is not detected until two days later. The relative expression of both factors peaks at birth but gradually decrease by p30 (Turner et al, 1994).

In marked contrast in the PNS, Brn-3b is detected by E13.5 in dorsal root ganglia (DRG) and trigeminal ganglia (TG), two days later than the onset of Brn-3a expression in these neurons. By E15.5, Brn-3b mRNA is also detected in the cochlear and vestibular regions of cranial nerve ganglion VIII, and in the glossopharyngeal nerve IX ganglion. In the spinal cord, Brn-3b is widely distributed at E12.5 but by E15.5, its expression is restricted to the intermediate gray region (Gerrero et al, 1993; Turner et al, 1994).

In the adult mouse and rat neuronal tissues Brn-3b expression is restricted to specific regions of the brain and PNS. In particular, it is expressed in the superior colliculus, the lateral interpeduncular nucleus of the midbrain and in pontine central gray. It is also expressed in the dorsal root and trigeminal ganglia (Turner et al, 1994). However, Brn-3b expression is also observed in adult heart and the reproductive tract, but not in the liver, spleen, kidney, lung, pituitary gland and thymus (Turner et al, 1994; Budhram-Mahadeo et al, 2001).

	Brn-3a		Brn-3b		Localization
	e 15.5	Adult	e 15.5	Adult	
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 (& related)					
- red nucleus	++	++	-	-	
Other Neurons					
Retina	++	++	++	+	post mitotic ganglion cells
Sensory neurons					
- Trigeminal ganglia	+++	+++	++	+	
- Dorsal root ganglia	+++	+++	++	+	
Spinal cord	++	++	++	+	Brn-3a in dorsal gray Brn-3b in intermediate gray.

Table 1.3.5: Expression pattern of Brn-3a and Brn-3b in the developing and adult nervous system

(Reproduced from a PhD thesis, titled Regulation and Function of POU domain transcription factors, Brn-3a and Brn-3b, UCL, 1995, with permission from the author, Dr. Budhram-Mahadeo)

- indicates no detectable expression
+/- equivocal signal
+ weak signal
++ moderate signal
+++ strong signal
n indicates nucleus

1.3.6 Brn-3a function in neuronal cells

Extensive *in vivo* and *in vitro* studies have shown that Brn-3a proteins play a critical role in the survival and differentiation of particular neuronal cells. The following sections discuss the role of Brn-3a in these specific cell types.

1.3.6.1 Brn-3a knock-out mouse model

Studies in which the Brn-3a allele was deleted by homologous recombination have suggested a distinct developmental role for this factor in the maintenance and terminal differentiation of specific neuronal cell types. Brn-3a homozygous mutant mice (Brn-3a-/-) show gross phenotypic abnormalities which result in ineffective swallowing, uncoordinated limb and trunk movements. Consequently, these mutant mice do not survive beyond 24 hours postpartum.

Histological and hybridization studies of the CNS of Brn-3a-/- have shown a loss of neurons at a number of sites that may contribute to some of the observed phenotypes. For instance, there is a significant reduction in the number of neurons in the caudal region of the inferior olivary nucleus of the hind-brain. Similarly, regions in the midbrain show a striking loss of red nucleus neurons, the nucleus ambiguus and absence of the motor neurons which innervate oesophagus (McEvilly et al, 1996; Xiang et al, 1996).

The red nucleus functions to modulate information from the cerebellum and the cerebral cortex to the spinal cord via the rubrospinal tract (for review see Paxinos, 1995, Academic, San Diego). Therefore, its absence could cause aberrant spinal reflexes manifested in the abnormal limb and trunk movement of Brn-3a-/- mice. An added factor contributing to the abnormal locomotion and posture in these animals could be due to loss of cells in the caudal region of the inferior olivary nucleus, which normally integrates sensory information and sends its output to the cerebellular Purkinje cells (for review see Paxinos, 1995). The loss of the motor neurons which normally innervate oesophagus would result in abnormal peristaltic swallowing reflex, accounting for the inability of Brn-3a-/-mutants to suckle.

From the observations within the CNS of Brn-3a-/- embryos, two possible functional roles for Brn-3a have been suggested. Firstly, Brn-3a may be required for the response to, or synthesis of cell survival neutrophic factor, at sites such as the red nucleus, where extensive cell death occurs between E16.5 and P0.5 in Brn-3a-/- embryos. The emergence of the red nucleus neurons and migration to appropriate positions even in Brn-3a-/- mice excludes the role of Brn-3a in these earlier events. Secondly, the distortion of specific subunits in the inferior olive and nucleus ambiguus due to a failure of cellular migration suggests a role for Brn-3a in migration of compact formation neuron. Therefore, Brn-3a may be required to control survival and differentiation events in specific neuronal cells in the CNS.

In the PNS of Brn-3a null mutant mice, there is a significant reduction in the biochemical markers that characterise sensory neurons (McEvilly et al, 1996; Xiang et al, 1996). Eng and colleagues (2001) generated a transgenic mouse in which they cloned a Lac Z reporter, into the Brn-3a locus. This facilitated analysis of cells that would normally express Brn-3a. Crossing this strain with a Brn-3a knock-out therefore allowed the analysis of specific neurons where Brn-3a would normally be expressed. This study has shown that during early development of the PNS the absence of Brn-3a results in abnormal axon bundling in the TG, with premature branching of nerves at the opthalmic and maxillary divisions. Furthermore, Brn-3a-/- TG neurons do not effectively innervate their peripheral targets, particularly obvious in the whisker follicles. However, during late development, there is a striking loss of neurons by apoptosis in the dorsal division of the anteromedial lobe of the TG. Hence, Brn-3a appears to control survival and normal differentiation/ path-finding of specific neurons.

Analysis of target gene expression showed in Brn-3a-/- embryos, there is a decrease in the number of Trk A and Trk B expressing neurons. Moreover, Trk C is not detected in TG of Brn-3a -/- mutants, suggesting that Brn-3a is critical for its expression (Huang et al, 1999, McEvilly et al, 1996; Xiang et al, 1996). Despite the expression of Brn-3a in proliferating precursors of TG, loss of Brn-3a does not result in a reduction in the number of these precursor cells, and therefore is not needed for the emergence of these cells (Huang et al, 1999).

The developmental defects observed in TG of Brn-3a-/- embryos have highlighted possible functions for this regulator in specific neurons. Evidence so far has demonstrated that Brn-3a can have direct and indirect effects on the survival and differentiation of specific neurons during development. While it has been shown to regulate genes associated with apoptosis (activation of anti-apoptotic Bcl-2 and Bcl-X_L, and repression of pro-apoptotic Bax and Noxa – discussed later in section 1.3.7), it has quite profound effects on neurotrophin receptors. Firstly, the essential role of neurotrophins and their receptors in sensory neuronal development is well documented. For example, absence of NGF or its receptor Trk-A results in approximately 75% loss of sensory neurons. Similarly, absence of BDNF or its receptor Trk-B causes loss of 30% of neurons while null mutations in Trk-C receptor results in 20% of neuronal loss (for review see Farinas and Reichardt, 1997; Tessarollo et al, 1997). The timing of the initiation of apoptosis in Brn-3a-/- TG provides a vital clue as to the role of Brn-3a in Trk- expressing neurons. A major wave of neuronal death in Brn-3a-/- TG occurs at E15.5, after Trk-A and Trk-B receptors have decreased below the minimal level required for neuronal survival. This suggests that Brn-3a, normally expressed at E9, is not required for the initiation, but rather for the maintenance of Trk-A and Trk-B expressing neurons. Conversely, the failure of Trk-C expressing neurons to emerge in Brn-3a-/- mice, implies that Brn-3a is required for their induction (Huang et al, 1999). However, the effects of Brn-3a are complex and its regulation of Trk- receptors is perhaps only one mechanism of a complex network that is regulated by Brn-3a to mediate survival and differentiation of these cells. Another study has also shown the loss of Trk-A expression in Brn-3a-/- in the absence of cell death by utilizing double knock-out mice of Brn-3a in pro-apoptotic Bax-null background (Ma et al, 2003). The requirement for Trk A in these neurons does not affect survival but rather it is thought to control appropriate differentiation.

The aberrant axonal guidance observed in TG of Brn-3a-/- mice suggests a role for this factor in the development of functional axons. A ligand semaphorin 3A (Sema3A) and its receptor Npn-1 have been shown to partly regulate trigeminal axon guidance (Koodkin et al, 1997). Interestingly, Npn-1-/- mice show aberrant TG branching which strongly resembles the TG axonal abnormality in Brn-3a-/- mutants (Kitsukawa et al, 1997; Eng et al, 2001). However, Brn-3a-/- appear to

express normal levels of Npn-1 in PNS, and in the CNS areas innervated by ganglia. Although, this rules out a direct regulation of Brn-3a on Npn-1, it is still possible that the absence of a critical co-factor for Npn-1, required for the formation of a receptor complex, may account for the abnormal axon pathfinding in Brn-3a-/- mice (Eng et al, 2001). To date other regulatory targets through which Brn-3a might exert its effect in sensory axonal formation have not been identified.

Brn-3b expression is altered in Brn-3a knock-out mice in the PNS but not in the CNS, suggesting that there is no difference in the number or location of normally Brn-3b expressing neurons within the superior colliculum, dorsal central gray, or interpeduncular nucleus of the midbrain. Therefore, Brn-3b expression is not dependent on Brn-3a expression in these regions. However, there is evidence for the loss of Brn-3b in the DRG, suggesting that in the PNS cells normally expressing Brn-3b need Brn-3a for survival or possibly, that Brn-3a regulates Brn-3b expression in these cells.

1.3.6.1.1 *In vitro* studies to analyse Brn-3a function

To understand how Brn-3a mediates its effects in neuronal cells, cell lines and primary cultures have been utilized along with *in vivo* models as they provide systems that can be more readily manipulated. Initial studies were carried out in the ND7 cell line which was derived by fusing neurons derived from non-dividing rat dorsal root ganglion cells with the C1300 mouse neuroblastoma cell line (Wood et al, 1990). One resultant clone, called ND7, could proliferate indefinitely in full growth medium, behaving like the parental neuroblastoma cells. However, these cells can be induced to differentiate upon serum withdrawal and addition of defined medium supplemented with growth factors or cyclic AMP analogues. Upon differentiation, these cells undergo cell cycle arrest and morphological changes with extensive neurite outgrowth, accompanied by movement of secretory granule component and synaptic vesicle component to the apex of processes, mimicking the events associated with differentiation of sensory neurons (Suburo et al, 1992). As such it has provided a good model for the analysis of changes in gene expression as cells transit from a proliferating phenotype to a differentiative state.

Brn-3a mRNA and protein levels are found at very low levels in actively proliferating ND7 cells. However, upon differentiation of these cells, Brn-3a levels are significantly elevated (Lillycrop et al, 1992; Budhram-Mahadeo et al, 1995a). Therefore, Brn-3a appears to be associated with neuronal differentiation in this cell model. In support of this Smith and colleagues (1997) have shown that artificial over-expression of Brn-3a in ND7 cells is sufficient to cause increased (60%) greater neurite outgrowth even when cells are grown in full serum medium that would not normally promote process formation (Smith et al, 1997c). Over-expression of Brn-3a in ND7 cells causes an upregulation of at least four proteins that are involved at various stages of the synaptic vesicle cycle, a process of multiple steps involved in docking, exocytosis, endocytosis and vesicle recruitement (Smith et al, 1997a; for review see Sudhof, 2004). The first of these is a presynaptic membrane component, the synaptosomal-associated protein (SNAP-25), which is involved in vesicle exocytosis and regulates axon outgrowth (for review see Bark and Wilson, 1994). The other proteins elevated in Brn-3a over-expressing ND7 cells are: synaptophysin (involved in docking with the membrane), synaptotagmin I (allowing fusion and exocytosis) and synapsin I (well known to be responsible for regulation of trafficking) (for review see Sudhof, 1995).

The correlation of expression of neurofilament protein and Brn-3a in early post-mitotic neurons in the CNS (Fedtsova et al, 1996) led Smith and colleagues (1997b) to investigate a possible link between these factors. The neurofilament heavy (NFH), neurofilament medium (NFM) and neurofilament light (NFL) form the major intermediate filament component of the neuronal cytoskeleton and are partly responsible for maintaining the axon diameter and axonal transport (for review see Lee and Cleveland, 1996). All three neurofilaments were elevated at both mRNA and protein levels in ND7 cells over-expressing Brn-3a protein (Smith et al, 1997b). Moreover, reduction of Brn-3a levels in ND7 cells using anti-sense strategy results in failure of extension of neurites even when cells were grown in optimal differentiation conditions (Lakin et al, 1995). Taken together, these results support a role for Brn-3a in the transition from proliferating ND7 cells to quiescent differentiated neurons.

In addition to its effects on neuronal differentiation, Brn-3a is also implicated in survival of neurons. High levels of Brn-3a protect ND7 cells as well as primary cultures of trigeminal and dorsal root ganglia from apoptotic stimuli such as the withdrawal of nerve growth factor (NGF) from the medium (Smith et al, 1998b; Ensor et al, 2001). Similarly, increasing Brn-3a expression protects dorsal root ganglia following sciatic nerve lesion (Smith et al, 2001). Conversely, increased apoptosis is observed in ND7, TG and DRG neurons in cultures where endogenous Brn-3a expression is decreased using an antisense strategy (Ensor et al, 2001; Smith et al, 1998b).

Subsequently, anti-apoptotic genes associated with survival in these cells were shown to be regulated by Brn-3a. Brn-3a has also been shown to strongly and directly activate the Bcl-2 P2 promoter (the predominant promoter that is active for this gene in neuronal cells) (Smith et al, 1998a; 1998c). Interestingly, a concomitant increase in the expression of the anti-apoptotic protein, Bcl-2, has been observed in over-expressing Brn-3a cells (Smith et al, 1998a; Ensor et al, 2001). Similarly, decrease in the levels of Bcl-2 expression is evident in Brn-3a anti-sense cells. Recent studies have shown Brn-3a can also regulate another anti-apoptotic member of the Bcl family, Bcl-X_L, which shares high homology to Bcl-2 (Boise et al, 1993). Interestingly, phenotypically Bcl-X_L knock-out shows some similarity to Brn-3a homozygous mutants in terms of its requirement for normal neuronal development (Parsadanian et al, 1998). Hence, this supports the hypothesis of these factors being in a similar pathway that is required for protection *in vivo*. Moreover, exogenous introduction of Brn-3a into DRG and TG by transfection results in a clear elevation in the endogenous mRNA and protein levels of Bcl-X_L (Smith et al, 2001). Conversely, use of antisense to decrease levels of Brn-3a show a concomitant decrease in Bcl-X_L levels. Furthermore, Brn-3a activates the Bcl-X_L promoter in primary sensory neurons and ND7 cells (Smith et al, 2001).

Hence, the protective effect of Brn-3a against apoptosis seems to be mediated partly via its regulation of Bcl-2 gene, which is known to enhance survival in both CNS and PNS neurons against a variety of apoptotic stimuli (Allsopp et al, 1992). However, the direct control of Bcl-X by Brn-3a might be more relevant in protection

of neurons during development and has been shown to protect sensory neurons against apoptosis in NGF-dependent and BDNF-dependent sensory neurons against apoptosis (Gonzalez-Garcia et al, 1995). Interestingly, Brn-3a null mutant mice and Bcl-X_L homozygous mice exhibit similar loss of neurons in terms of time of death and the population of neurons affected in the DRG and TG (Motoyama et al, 1995; McEvilly et al, 1996; Xiang et al, 1996).

Another possible mechanism by which Brn-3a might enhance the survival of neurons during the normal apoptotic period of embryonic development is via its regulation of Trk-receptors. In primary cultures of cells prepared from TG, Brn-3a seems to exert a more profound effect on the survival of neurons derived from later developmental periods (i.e. between E13 and E14, and not at E12 and E11) (Ensor et al, 2001). This temporal effect of Brn-3a on survival during development is of particular interest as extensive loss of Trk A- and Trk B- expressing neurons in Brn-3a null mice occurs at E13.5 (Trk A) and E12.5 (Trk B), while Trk-C expressing neurons never appear (Huang et al, 1999). Interpretation of these observations suggests that Brn-3a is either involved in regulating the expression of Trk A-, Trk B- and Trk-C or in maintaining Trk A- and Trk B- expressing neurons and in the generation of Trk C- expressing neurons. Brn-3a has been shown to directly regulate Trk A- promoter but Trk-B and Trk-C promoters have not yet been analysed (Huang et al, 1999).

1.3.6.2 Brn3b function in neuronal cells

In vivo and *in vitro* studies aimed at elucidating the role of Brn-3b have revealed a complex role for this protein that is highly dependent on cell types and possibly other cell specific factors (discussed in detail in the following sections).

1.3.6.2.1 Brn-3b knock out mouse models

Extensive studies using mice models in which Brn-3b gene has been deleted have highlighted the role for this protein during development. In 1996, two independent groups developed homozygous null Brn-3b mice and showed that these mice

display normal gross morphology but are blind (Gan et al, 1996; Erkmann et al, 1996). Detailed analysis of the eye of these mutants at a cellular level, revealed loss of ~70% of their retinal ganglia cells between post-natal days 17 and 20. Although Brn-3b is also expressed in other regions of the CNS and PNS (as discussed in section 1.3.5.2), there were no apparent defects in these structures, suggesting that the absence of Brn-3b is compensated for by other factors such as Brn-3a due to redundancy.

The significance of the changes in Brn-3b -/- retinal ganglion cells is demonstrated by its high expression in these cells. Brn-3b is expressed earlier than Brn-3a in these cells, and is first detected at E11.5 and peaks between E13.5 and E16.5. This expression coincides with the emergence of post-mitotic ganglion cell precursors en route to the ganglion cell layer (Turner et al, 1994; Xiang 1998). This spatio-temporal correlation between Brn-3b expression and generation of ganglion cell suggests that Brn-3b may be involved in the initial and /or terminal differentiation of retinal cells.

Another elegant study in which the Lac Z gene was inserted upstream of the Brn-3b translational initiation codon at the Brn-3b locus, allowed an in depth analysis of the specific subset of cells in the retina that express the Brn-3b gene (Gan et al, 1999). The progressive loss of Lac Z positive retinal ganglion cells (RGC) after birth suggests firstly, that Brn-3b is not involved in the initial cell fate determination of retinal ganglion cells and, secondly and more importantly, a possible role for Brn-3b in completing the normal differentiation of RGCs.

During normal development, all retinal cell types emerge from a limited population of actively dividing neuroblasts that commit to their fate, independently of cell lineages, in a temporal manner (Cepko et al, 1996). RGCs are the first cell types to commit and migrate from the proliferative zone to the innermost layer of the retina (Alexiades and Cepko, 1997). RGCs extend their axons towards the optic disc and establish connections in the CNS (Tessier-Lavigne and Goodman, 1996). Brn-3b is among one of the earliest markers of RGC formation (Xiang et al, 1993; 1995). Interestingly, mutants with no expression of Brn-3b exhibit distinct morphological differences in the RGC bodies compared to the wild-type mice. The processes

projecting from the mutant cells are smaller in diameter, and in fact, form numerous dendrites at the expense of mature axon formation. Furthermore, the disorganised neurites of RGCs, which fail to fasciculate efficiently in the absence of Brn-3b, give rise to thinner optic nerves that fail to make contacts with the optic chiasm and subsequently undergo apoptosis (Gan et al, 1999; Wang et al, 2000; Erkman et al, 2000). Hence, Brn-3b is critical for regulating genes that allow normal ganglion cell differentiation. In fact, *in vitro* studies have identified Pax-6, a master regulator which is involved in the regulation of some of the inductive events that occur during the formation of the eye, including axon guidance (Ziman et al, 2001), as a direct downstream target of Brn-3b in neuroretinal cells (Plaza et al, 1999).

In contrast to retinal ganglial cells, most cells of the peripheral sensory ganglia undergo their final mitoses as early as embryonic day 13 (E13) (Altman and Bayer, 1982). In view of the observation of the onset of Brn-3b expression by E13.5 in sensory ganglion cells, it is likely that this protein exerts its effects predominantly, if not exclusively, in post-mitotic, terminally differentiated neurons (Turner et al, 1994). It is possible that cell specific factors in retina may be important in determining the effect of Brn-3b on a distinct subset of genes and hence its effects compared with other neurons, especially sensory neurons. Given the lack of gross abnormalities in the neurons of other regions of the CNS and PNS in Brn-3b knock-out mice, it has been difficult to elucidate its function in these areas and would require inducible model to study.

1.3.6.2.2 *In vitro* Brn-3b studies

The effects of Brn-3b appear to be complex. Whilst Brn-3b appears to be required for the survival and terminal differentiation of retinal ganglion cells, its effects in other cell types suggest a very complex role for this factor in cell fate determination. This was demonstrated in studies undertaken in cell lines and primary tissue materials which have attempted to highlight the role of Brn-3b in different cell types (see section 1.3.6.3).

Studies using the ND7 cell line have shown that actively proliferating cells grown in full growth medium express high levels of Brn-3b. Upon induction of differentiation by growth in defined medium (e.g. removal of serum or addition of cAMP), Brn-3b levels are dramatically decreased (Lillycrop et al, 1992; Budhram-Mahadeo et al, 1995a). Similarly, studies using cell lines derived from human neuroblastoma tumours (IMR-32, SK-N-SH, SK-N-MC) have confirmed this change in Brn-3b levels, with higher levels detected in proliferating cells compared to the low levels detected after cells are induced to differentiate (Smith and Latchman, 1996). Interestingly, Brn-3b demonstrates a reciprocal expression pattern compared to the levels of Brn-3a in these cells. These findings suggest that Brn-3b is associated with a proliferation in these cells.

Further evidence for the role of Brn-3b in cellular proliferation has come from studies in which the expression of Brn-3b has been manipulated. Over-expression of Brn-3b in ND7 cells results in failure of neurite outgrowth even when the cells are grown in differentiation medium (Smith et al, 1997a). Interestingly, there is no effect on the number of cells bearing processes indicating that Brn-3b may act by inhibiting the neurite extension rather than their initial formation. The complexity of effects mediated by Brn-3b was also demonstrated by Ensor and colleagues (2001) who showed that its effects are altered depending on the time of expression during development. For example, over-expression of Brn-3b in trigeminal ganglia cultures taken from E11 and E12 embryos results in increased survival upon NGF withdrawal but this was not seen in cultures taken from older embryos (Ensor et al, 2001). Hence, it is possible that Brn-3b can substitute for Brn-3a during early trigeminal ganglia development but not at later stages, when there is significant loss of Brn-3a expression (Fedtsova and Turner, 1995). Moreover, Brn-3b represses many neuronal target genes associated with differentiation so its effects may change depending on target genes as well (discussed later in section 1.3.7). Therefore, the effects of Brn-3b expression are likely to depend on its temporal expression as well as the specific cell types.

Taken together the results using neuronal cell line models showed the opposite expression and sometimes anatagonistic effects of Brn-3b to Brn-3a in ND7 cells.

This may suggest that there two factors are involved in a fine balance between proliferation and differentiation of neuronal cells depending on cell type.

1.3.6.3 Expression and function of Brn-3a and Brn-3b proteins in non-neuronal tissue and cancers

Even though Brn-3a and Brn-3b were originally detected in the developing and adult nervous system where they play a critical role in neural development, subsequent studies have demonstrated expression of both these transcription factors in non-neuronal cells. Hence, Brn-3a and Brn-3b have been detected in cervical epithelial cells (Lillycrop et al, 1992; Ndisang et al, 1997), in the testis (Budhram-Mahadeo et al, 2001), in human breast adenocarcinoma cell line, MCF7 (Budhram-Mahadeo et al, 1998). Moreover, both Brn-3a and Brn-3b are expressed in primary breast tissue including the normal mammary cells, benign tumours, but high levels of Brn-3b has been detected in a subset of malignant tumours (Budhram-Mahadeo et al, 1999).

1.3.6.3.1 Cervical tissue

Reverse-transcriptase polymerase chain reaction (RT-PCR) assay has detected low levels of Brn-3a and Brn-3b mRNA expression in human cervical biopsies from individuals with no histologically detectable cervical abnormality, as well as in high grade cervical intraepithelial neoplasia type 3, CIN3, samples (Ndisang et al, 1997). More importantly, the mean level of Brn-3a expression is elevated 300-fold in CIN3 tissue compared with normal cervical tissue. In contrast, Brn-3b levels do not change significantly in these cells. Hence, there is a very large elevation in the Brn-3a:Brn-3b ratio in CIN3 compared with normal samples (Ndisang et al, 1997; 2000). This finding is particularly interesting in view of the ability of Brn-3a to bind and activate the upstream regulatory region (URR) of human papilloma viral genome (Morris et al, 1994). Conversely, Brn-3b also binds the same octamer motif in the URR of HPV but represses URR-driven transcription and negates activation by Brn-3a (Morris et al, 1994). HPV types 16 and 18 (HPV-16 and HPV-18) encode specific proteins, E6 and E7, which are able to transform cells to a malignant phenotype and it is now generally accepted that the production of these

oncogenic proteins contribute to the development of cervical neoplasia (Schiffman et al, 1983; Burghardt E, 1984). Thus, the changes in the ratio of Brn-3a:Brn-3b in CIN3 cervical tissue may represent a critical role for these cellular regulatory factors, with Brn-3a, in particular, regulating the URR activity and, in turn, the progression of cervical cell transformation.

1.3.6.3.2 Testis

Immunocytochemical studies and in situ hybridisation studies in adult rat testis has demonstrated distinct expression patterns for Brn-3a and Brn-3b expression at specific stages of germ cell development. Thus, whilst Brn-3a is detected predominantly in spermatogonia around the periphery of the seminiferous tubules, high Brn-3b expression is observed in post-meiotic mature spermatids located centrally in the tubule (Budhram-Mahadeo et al, 2001). Interestingly, Brn-3a expression during germ cell development correlates with BRCA-1 expression in the testis and may represent one of the potential target genes regulated by Brn-3a in this tissue. In agreement with this hypothesis, BRCA-1 promoter has been shown to be activated by Brn-3a in the testis derived GH cell line. Hence, Brn-3a and Brn-3b may be important in specific stages of germ cell development in the testis. Interestingly, Brn-3b is found in more mature spermatids, suggesting a role in maturation, similar to its role in the retinal ganglion cells in the retina. However, confirmation of the precise role for Brn-3 protein in the testis requires analysis of testis development in mice lacking Brn-3b and in conditional/ inducible knock-out of Brn-3a models, where, at present, neonatal lethality of the knock-out animals has hindered such as a study.

1.3.6.3.3 Prostate

Both Brn-3a and Brn-3b has been detected in human prostrate tissue and cell lines. However, the expression of Brn-3a has been more intensively studied as it is expressed in the weakly invasive LNCAP cells but is found at lower levels in more metastatic cells PC-3 or PC-M cells (Diss J, personal communication). Moreover, overexpression of the short isoform of Brn-3a significantly increases the growth of

PC-3 cells but reduces their invasiveness *in vitro*. More importantly, in primary prostate tissues, Brn-3a expression is significantly decreased in > 50% of prostrate cancers, hence paralleling the functional observed *in vitro,* and may play a role in growth and progression of prostate cancer.

1.3.6.3.4 Breast tissue

Brn-3b has been detected in breast epithelial cells with localization to ductal epithelium in sections of normal breast. However, it is also expressed in the MCF7 human breast adenocarcinoma cell line and its expression is increased in patients with breast cancers (Budhram-Mahadeo et al, 1998). Its relevance to this disease is highlighted by a number of findings. Firstly, Brn-3b functionally interacts with and enhance the transcriptional effect of oestrogen receptor (ER), which is associated with growth and proliferation in breast tissue (Budhram-Mahadeo et al, 1998; Endoh et al, 1999). Whereas both Brn-3a and Brn-3b interact with the ER, only Brn-3b can increase ER-mediated transcriptional activity on an oestrogen receptor element (ERE) containing promoter such as the vitellogenin gene promoter and an artificial ERE-containing promoter upon stimulation with the ligand 17-β-estradiol. In contrast, Brn-3a represses the ER mediated activation under similar conditions (Budhram-Mahadeo et al, 1998). The physiological relevance of this interaction is further confirmed by co-immunoprecipitation experiments using cellular extracts taken from rat brain and ovary which express both Brn-3b and ER, and in which Brn-3b and ER are found complexed. Furthermore, electromobility shift assays (EMSA) have shown that ER associates more readily with the ERE in the presence of Brn-3a and Brn-3b proteins.

Both these Brn-3 proteins have been detected in primary breast tissue but interestingly, the levels of Brn-3b, but not of Brn-3a, are elevated in tumours compared to the levels in normal mammary tissue (Budhram-Mahadeo et al, 1999). Extensive studies have subsequently highlighted an important role for Brn-3b in breast tumourigenesis. In breast tumours, Brn-3b shows an inverse correlation with the levels of the breast cancer susceptibility gene, BRCA-1 (Budhram-Mahadeo et al, 1999). In addition, artificial over-expression of Brn-3b

with the BRCA-1 promoter in co-transfection assays produces a strong inhibitory effect on the BRCA-1 promoter, repressing its activity by approximately 20 fold. This suggests a direct regulation of BRCA-1 expression by Brn-3b (Budhram-Mahadeo et al, 1999). Being a tumour suppressor, BRCA-1 acts to halt cell proliferation in normal epithelial cells and therefore repression of such a protein by Brn-3b may enhance cell proliferation during transformation. Interestingly, BRCA-1 mutations have been identified in a number of familial breast cancers (for review see Casey, 1997) but recent studies have shown decreased expression of the BRCA-1 gene both at mRNA and protein levels in non-familial sporadic cancers lacking BRCA-1 mutations (for review see Mueller and Roskelley, 2002). Thus, its inactivation either by mutations or repression by proteins such as Brn-3b may contribute to increased cellular proliferation. To date, Brn-3b is the first transcription factor reported to regulate BRCA-1 expression, supporting an important role for Brn-3b in regulating cellular proliferation.

To further explore the role of Brn-3b in breast cancer, Dennis and colleagues (2001) established stable cell lines that either over-expressed Brn-3b or had reduced expression of endogenous Brn-3b by using an anti-sense strategy. Results of these studies have demonstrated that over-expression of Brn-3b in MCF7 cells consistently increases their growth and proliferation rate compared to the vector control transfected cells which grow slower. In addition, Brn-3b over-expressing cells exhibit increased growth under anchorage independent conditions, suggesting that high levels of Brn-3b provides an advantage for tumour growth in vivo, compared to the control cells or cells with reduced levels of Brn-3b protein (Dennis et al, 2001). These results strongly support a role for Brn-3b in regulating mammary cell growth and proliferation, and suggest its elevation in breast cancer to be of functional significance.

Furthermore, Brn-3b has been shown to correlate positively with the cyclin-dependent kinase (CDK4) in human breast cancer cell lines and in tumour samples (Samady et al, 2004). Further analysis showed that Brn-3b could transactivate the CDK4 promoter. Since CDK4 is required for the G1 to S transition of the cell cycle, the ability of Brn-3b to activate its transcription suggests another mechanism by how this transcription factor gives rise to its effects of increased

proliferation breast cells. Recently, Brn-3b has also been shown to repress the adhesion associated protein, plakoglobin, which might help to explain the elevated migration of breast cell with increased Brn-3b levels (personal communication, Dr. L. Samady, MMBU, ICH, UCL)

1.3.7 Promoters regulated by the Brn-3a and Brn-3b proteins

Alterations in the proliferation and/or differentiation status as demonstrated by Brn-3a and Brn-3b in different cell types require the reprogramming of gene expression that is predominantly brought about by the activity of transcriptional regulators within a cell. Transcription factors mediate their effects by associating with other proteins in transcriptional complex. As such factors that are co-expressed in specific cell types are likely to have significant impact on the effects of particular transcription factors.

A number of target gene promoters regulated by the Brn-3 transcription factors have been identified and characterized. The distinct roles demonstrated for Brn-3a and Brn-3b factors in neuronal cells *in vivo* and results showing differential expression of Brn-3a and Brn-3b in proliferating versus differentiated neuroblastoma cells is of particular interest since this reciprocal expression is paralleled by their antagonistic effect on several different gene promoters. Table 1.3.7 gives a summary of the various target gene regulated by Brn-3a and Brn-3b. This inverse correlation in the expression pattern of these two proteins is associated with their functional antagonism on many target promoters. Hence, a minimal promoter containing an octamer-related upstream elements was stimulated by Brn-3a whereas Brn-3b represses the transcription of this promoter and even inhibits the Brn-3a-mediated transcription (Gerrero et al, 1993; Budhram-Mahadeo et al, 1994; Morris et al, 1994). In general, Brn-3a seems to be involved in transactivation of genes associated with neuronal differentiation and cell cycle arrest, which are repressed or unaffected by Brn-3b and will be discussed in the following sections (Budhram-Mahadeo et al, 1995; Morris et al, 1996; Smith et al, 1997b).

The expression of Brn-3a in the earliest post-mitotic neurons of the CNS and in adult sensory ganglia of the PNS as well as the promotion of neurite extension by this factor in ND7 cells suggests a role for Brn-3a in the differentiation of neurons. In depth analysis of the precise mechanisms by which Brn-3a mediates differentiation in neuronal cells has demonstrated that this transcription factor can regulate a number of genes whose protein products are critical for neuronal differentiation. For instance, Brn-3a directly activates the promoters of genes whose protein products are required at various stages of synaptic vesicle cycle and have been shown to be upregulated in Brn-3a overexpressing cells (Smith et al, 1997a). These include the SNAP-25, synaptotagmin 1 and synapsin I. Extensive promoter analyses confirm direct effects of Brn-3a on their regulation rather than a secondary consequence of differentiation events occurring in neuronal cells (Lakin et al, 1995; Morris et al, 1996). Subsequent search for other neuronal differentiation associated genes as possible targets for Brn-3a led to the identification of another gene, whose protein product plays a critical role in the radial growth of axons. The promoter of α-internexin gene is strongly activated by Brn-3a in co-transfection experiments in ND7 cells (Budhram-Mahadeo et al, 1995b). Moreover, Brn-3a is able to directly transactivate the promoters of all three neurofilament genes (NFH, NFM, NFL) whose proteins products have also been elevated in Brn-3a over-expressing cells (Smith et al, 1997b). This regulation of neurofilament genes by one transcription factor is interesting in view of the fact that neurofilament are obligate heteropolymers (Ching et al, 1993), and any decrease (or increase) in the expression of individuals subunits results in aberrant axonal size and transport (Eyer and Peterson, 1994; Sakaguchi et al, 1993). Hence, a common regulatory mechanism, allowing simultaneous expression of the three neurofilament genes in a spatio-temporal manner may be of critical importance for their correct functioning, making Brn-3a an attractive master regulator for controlling the expression of these in neurons which express this factor.

Interestingly, Brn-3b represses these Brn-3a target genes that are associated with differentiation of neurons, including SNAP-25 (Morris et al, 1996), the neuronal intermediate filament gene product α-internexin (Budhram-Mahadeo et al, 1995), and promoters of all three neurofilament genes (Smith et al, 1997c). However, Brn-

3b does activate the promoter of synapsin 1, in a manner similar to Brn-3a. This suggests that Brn-3b is able to compensate for Brn-3a in terms of transactivation of some but not all target genes in neuronal cells (Morris et al, 1996).

Brn-3a and Brn-3b have also been shown to regulate some non-neuronal target genes. For example, CDK4 is transcativated by Brn-3b in breast cancer cells while plakoglobin and BRCA 1 are repressed by Brn-3b, providing a mechanism by which Brn-3b promotes cellular proliferation and invasion in these cells (see earlier section 1.3.6.3.4). A member of the heat shock family of proteins, HSP 27, has also been shown to be transactivated by both Brn-3b and Brn-3a in both neuronal and breast cancer cells (Farooqui, S and Budhram-Mahadeo personal communication). Therefore, a theme that emerges from these studies of various gene targets of Brn-3a and Brn-3b is the complexity of effects on different targets, such that Brn-3a and Brn-3b can be an activator or repressor but effects depend on the specific target in specific cell types.

Studies using chimaeric constructs encoding different regions of Brn-3a and Brn-3b have identified two activation domains present in the longer Brn-3a (l), which regulates the ability of this protein to activate specific promoters (Budhram-Mahadeo et al, 1995; 1996; Morris et al, 1994). Thus, activation of a test promoter containing a Brn-3 binding site is dependent on the DNA-binding POU domain of Brn-3a as the isolated Brn-3a POU domain is enough for activation and so Brn-3a(s) and Brn-3a(l) can transactivate such promoters (Budhram-Mahadeo et al, 1995; 1996; Morris et al, 1994). In contrast, the activation of α-internexin promoter requires the second activation domain located at the N-terminus of the protein and present only in Brn-3a(l) (Budhram-Mahadeo et al, 1995). Whilst Brn-3a(l) can activate the α-internexin promoter, neither the isolated POU domain nor the shorter Brn-3a(s) isoform were capable of transctivating this promoter (Budhram-Mahadeo et al, 1995). Similarly, Brn-3a(l) but, neither Brn-3a(s) nor isolated POU domain, can transactivate the Bcl-2 promoter (Smith et al, 1998). Interestingly, although the α-internexin promoter contains several Brn-3 consensus binding sites, Brn-3a is able to activate a minimal α-internexin promoter, lacking any sequence closely related to the octamer motif (Budhram-Mahadeo et al, 1995).

Similar effects are also observed on $p21^{CIP1/WAF1}$ promoter (discussed later in this section). This suggests that Brn-3a may associate with the basal transcription machinery to mediate its effects.

Test promoters responsive to the isolated POU domain of Brn-3a are not activated by the corresponding POU domain of Brn-3b (Budhram-Mahadeo et al, 1995). Interestingly, this differential effect is associated with a single amino-acid difference found at position 22 of the POU homeodomains which is a valine residue in Brn-3a and an isoleucine residue in Brn-3b. This difference is located in the first α-helix of the POU homeodomain and is critical for the transcriptional effects on the target genes dependent on the POU domain of Brn-3a and Brn-3b. Thus, substitution of the valine residue at position 22 in Brn-3a with the isoleucine found in Brn-3b results in transcriptional repression of the promoters that are normally activated by wild-type Brn-3a (Smith et al, 1997). Conversely, substituting an isoleucine in Brn-3b with a valine residue at this position converts it into an activator of some target promoters tested (Morris et al, 1997; Dawson et al, 1996). Thus, the residue at position 22 may be critical for recruitement of a transactivator in the case of Brn-3a or a transcriptional co-repressor by Brn-3b on some neuronal target genes and alteration at position 22 may affect the protein-protein interactions by these factors (Dawson et al, 1998).

In addition to the direct transcriptional regulation of target genes by Brn-3a and Brn-3b, recent studies in our laboratory have also shown that these proteins can interact with other proteins, thus providing another mechanism by which these proteins can regulate gene transcription of diverse target genes. A well established phenomenon of POU family members is the ability to activate their target promoters via cooperative binding as homodimers and hetero-dimers with other proteins (Voss et al, 1991; Poellinger et al, 1989; Herr and Cleary, 1995). Budhram-Mahadeo and colleagues have shown strong interaction of Brn-3a and Brn-3b with other cellular proteins that can modulate transcription. *In vitro* and *in vivo* studies have shown a direct interaction of both Brn-3a and Brn-3b with the tumour suppressor p53. Interestingly, interaction of wild type p53 with Brn-3a antagonises the ability of Brn-3a to transactivate the Bcl-2 promoter (Budhram-

Mahadeo et al, 1999). However, when tested on p53 target promoters the interaction of Brn-3a with p53 gives rise to differential effects on gene expression. Thus, Brn-3a cooperates with p53 to increase expression of the endogenous $p21^{cip1/waf1}$ protein levels (Budhram-Mahadeo et al, 2002; Perez-Sanchez et al, 2002), but decreases the expression of pro-apoptotic p53 targets Bax and Noxa (Budhram-Mahadeo, 2003). In support of this, co-transfection studies show that Brn-3a cooperates with p53 on $p21^{cip1/waf1}$ promoter but antagonizes p53 mediated transcription on Bax and Noxa promoters. Consequently, co-expression of Brn-3a and p53 results in enhanced survival and cell cycle arrest in G0/G1, resulting in cell cycle arrest/differentiation (Budhram-Mahadeo et al, 2002; Perez-Sanchez et al, 2002). These results support the role of Brn-3a in cell survival. Conversely, Brn-3a antagonises the p53-mediated effect on Bax promoter, thus enhancing cell survival. In support of this, analysis of Noxa and Bax protein levels in embryonic brain and spinal cord showed elevated Bax and Noxa in Brn-3a knock-out cells compared to the wild-type (Budhram-Mahadeo et al, 2003; Hudson et al, 2004). Therefore, these findings indicate that Brn-3a can mediate its effects in cell survival and differentiation, both directly and indirectly.

Another factor that associates with Brn-3a and Brn-3b is the proliferation-associated estrogen receptor (ER), which transactivates estrogen responsive element (ERE) containing promoter (Budhram-Mahadeo et al, 1998). In breast cancer cells Brn-3b/ER interaction results in greater induction of ERE containing promoters compared with Brn-3b alone. In contrast, upon binding of Brn-3a to ER there is a mild repression, suggesting a negative role for Brn-3a in cellular proliferation in these cells compared to the enhanced proliferative effect of Brn-3b in breast cancer cells (Budhram-Mahadeo et al, 1998).

Therefore, a number of different studies to characterize the effects of Brn-3a and Brn-3b on target gene promoters have highlighted the functional complexity of these transcription factors. It appears that cell specific factors and tissue of expression dictates the target and the nature of regulation by these factors. In addition, they can have a direct effect on gene regulation by binding to cis-regulatory elements of the target gene or associate with other proteins to exert their effect.

Promoter	Brn-3a	Brn-3b	Domain
α-internexin	+	-*	N-term
SNAP-25	+	-*	POU
Synaptotagmin 1	+	-	POU
Synapsin 1	+	+	POU
NFH	+	-*	POU
NFM	+	-*	POU
NFL	+	-*	POU
Bcl-2	+	0	N-term
Bcl-x	+	0	POU
$p21^{cip1/waf1}$	+	N.D.	POU
Bax	-	N.D.	POU
Noxa	-	N.D.	POU
CDK4	N.D.	+	N.D.
Plakaglobin	N.D.	-	N.D.
BRCA1	-	- -	N.D.
Vitellogenin	0	+	POU

Table 1.3.7: Promoter regulation by Brn-3a and Brn-3b transcription factors

Some of the known promoters regulated by Brn-3a and Brn-3b are given above. Activation of a promoter is indicated with a "+", repression of a promoter is indicated with a "-", no effect on the promoter is indicated with a 0. An "*" indicates that Brn-3b not only represses the promoter, but also negates activation by Brn-3a. "Domain" indicates the Brn-3 protein domain responsible for the transcriptional effect.

1.3.8 Regulation of Brn-3 proteins

While Brn-3 proteins control downstream targets, the factors that alter their expression in specific cells are also important. Regulation of Brn-3a has been better characterized than Brn-3b. Expression of Brn-3a appears to be controlled by two distinct promoters (Frass et al, 2002). The first more distal upstream promoter is TATA-less and contains two bona fide mRNA start sites spanning over 500 base pairs 5' of the ATG translation initiation and directs the expression of the long isoform of Brn-3a [Brn-3a(l)]. The second promoter, also TATA-less, has been identified in the first intron of the Brn-3a gene and has been shown to control the expression of the shorter isoform of Brn-3a [Brn-3a(s)], specifically in neuronal cells but not in epithelial cells. The existence of two different promoters may help to explain the differential expression of the Brn-3a gene with respect to cell specificity and developmental stage.

Analyses using a number of techniques have identified Brn-3a binding sites within the Brn-3a locus itself (Trieu et al, 1999). The distal binding site is found at -9 kb from the start site of transcription, and the proximal binding site is located at approximately at 5.4-5.6 kb. The proximal binding site can more effectively transactivate Brn-3a expression compared with the distal region. Thus, it appears that autoregulation plays an important role in maintaining Brn-3a expression, and hence provides a mechanism for the maintenance of differentiated neuronal phenotypes.

The co-expression of Brn-3a with Brn-3b in several sub-sets of neurons has led to a study determining the possible role of Brn-3b in regulating Brn-3a. Indeed, Brn-3b does exert a positive transcriptional effect on Brn-3a autoregulatory sequences, to similar levels to Brn-3a itself (Trieu et al, 1999). This suggests a convenient regulatory mechanism for the expression of Brn-3a in specific neurons where Brn-3b expression precedes that of Brn-3a such as in the retina, the inferior olivary and interpeduncular nuclei. Moreover, if this ability of Brn-3b to activate Brn-3a is mirrored during development, it may partly explain the severity of the retinal defects in Brn-3b null mutant mice, where the absence of Brn-3b results in the loss

of Brn-3a expression (Erkman et al, 1996; Xiang et al, 1998), and effectively results in a mouse with defective expression of both Brn-3 factors.

At present not much is known about the regulation of Brn-3b expression but ongoing studies in our laboratory aim to characterize the Brn-3b promoter and determine whether it is regulated in a similar manner to Brn-3a.

1.3.9 Brn-3 expression in neuroblastomas

Even though no work to date has been carried out in primary neuroblastoma tumours to determine the expression levels of Brn-3a and Brn-3b, studies carried out in neuroblastoma cell lines have provided some clue as to the possible function of these neuronal transcription factors in this cancer. As such, mouse cell line, ND7, and human neuroblastoma cell lines IMR-32, SK-N-MC, SK-N-SH have shown Brn-3a mRNA to be present to varying degrees in these cell lines, but is dependent on the cellular status (Smith and Latchman, 1996; Budhram-Mahadeo et al, 1995; Lillycrop et al, 1992). Interestingly, a clear pattern emerges when Brn-3a levels are compared to the levels of Brn-3b mRNA in each cell lines studied. Despite the variation in Brn-3a or Brn-3b levels, all nine proliferating neuroblastoma cell lines tested have a much higher Brn-3b to Brn-3a ratio than the rabdomyosarcoma and melanoma control cell lines. Moreover, Brn-3a levels rise dramatically following differentiation of these cells with different stimuli (Smith and Latchman, 1996). Hence, Brn-3a and Brn-3b expression appears to be associated with the different cell fate in of neuroblastoma cells, with Brn-3a being strongly associated with differentiation as cells withdraw from the cell cycle and enter a quiescent state. Conversely, high levels of Brn-3b in actively proliferating neuroblastoma cells which have lost their growth restraints, indicating that it may be more associated with of cancer cells. Clearly, more research needs to be carried, especially in primary human tumour samples, in determining the precise role of these proteins in tumourigenesis.

The work described in this thesis is aimed at studying the role of Brn-3a and Brn-3b in human neuroblastoma cells in terms of initiation and progression of the tumourigenic characteristics of these cells.

1.4 Neuroblastoma

Neuroblastomas are malignant tumours of poorly differentiated neuroectodermal cells thought to be derived from the neural crest which are destined for the adrenal medulla and the sympathetic nervous system. Neuroblastoma represents the most common intra-abdominal malignancy of infancy and the most common extracranial solid tumor of childhood. It accounts for 8-10% of all paediatric malignancies, with an annual incidence of 1 per 7,000 live births. The median age at diagnosis is about 18 months, of which 40% of cases present by 1 year of age, 75% by 4 years and 98% by 10 years (Grovas et al, 1997; Miller et al, 1995; Brodeur and Maris, 2002).

The multiple anatomic sites where these tumours occur parallel the origin and migration pattern of neuroblasts during fetal development. For instance, 40% of tumors occur in the adrenal medulla, whereas 25% of tumours arise in paraspinal ganglia. Other primary sites of origin include the thoracic (15%), pelvic (5%) and cervical regions (3%). Metastases occurs in nearly 70% of patients, and the most common sites for distal spread being the bone marrow, bone, liver and skin. Secondary spread to lungs and brain has also been reported (Kellis et al, 1991; Shaw et al, 1992; Watts et al, 1996). The primary tumour site, as well as the pattern and frequency of metastatic spread, appears to be dependent on age. In infants the tumours usually arise in the thoracic and cervical areas, whereas older children frequently present with abdominal tumours.

Neuroblastoma tumours show remarkable clinical heterogeneity, ranging from life-threatening malignant tumours, to less aggressive tumours that mature to ganglioneuroblastoma or ganglioneuroma, and some more benign growth that show spontaneous regression of tumours. Highly malignant neuroblastomas are undifferentiated tumours, consisting of small, round cells called neuroblasts.

However, ganglioneuroblastomas do exhibit moderate differentiation while ganglioneuromas are a sub-set of well-differentiated benign tumours that consist of clusters of mature neurons surrounded by stromal Schwann cells (for review see Brodeur, 1990, 2003; Westermann and Schwab,2002 Schwab et al, 2003).

An international staging system (INSS) for neuroblastoma patients has been developed and is clinical factors such as sites of origin, other metastasis and histology of the tumour. The staging system allows clinical risk groups to be identified upon which other biologically-based risk factors can be determined. Together, these staging systems allow for precise diagnosis, prognosis and for rapid and efficient therapy to be administered (Brodeur et al, 1993). High-risk tumours, classed as INSS stages 3 and 4, include disseminated disease or bulky tumours with gross genetic abnormalities, such as MYCN amplification. Patients within this group have a 5-year event-free survival (EFS) of 25-30%, despite multi-modality treatment including myelo-ablative chemotherapy. The intermediate risk group patients (INSS stages 2b-3) have characteristic large, unresectable localized tumours without structural genetic alterations. These patients have 5-years EFS of (60-80%) and show a more favourable outcome when treated with a combination of surgery and (neo-) adjuvant chemotherapy. INSS stages 1 and 2a represent the low risk patients with small tumours, which can be clinically managed by surgery alone, with 5 year EFS of greater than 90%. Finally, infants under 1 year of age at diagnosis, with small primary tumours and disseminated disease, represent INSS stage 4s. These patients have an excellent 5- year EFS of 70-90%, and exhibit a high frequency of spontaneous regression (van Noesel et al, 1997). Table 1.4 summarises the main characteristics of different neuroblastoma risk groups.

For reasons unknown at present, the age of the patients at diagnosis of neuroblastoma tumour also strongly influences the clinical prognosis and outcome of neuroblastoma treatment (Carlsen et al, 1986; Evans et al, 1987; Jereb et al, 1984). Generally, infants diagnosed before 1 year of age and/or with localized disease are curable with surgery and little or no adjuvant therapy. Conversely, 55% of neuroblastomas in older children (<I year) frequently have extensive hematogenous metastases at diagnosis, and most of them die from disease progression despite intensive chemotherapy.

Tumour characteristics	Low-risk	Intermediate-risk	High-risk
Clinical			
Age	Typically <1 year	Typically >1 year	Typically >1 year
INSS Stage	1, 2, 4s	3, 4	3, 4
5-year survival	95%	50%	25%
Genetic			
Ploidy	3n	2n/4n	2n/4n
1p loss	Rare	Rare	Frequent
11q loss	Rare	Frequent	Rare
14q loss	Rare	Frequent	Rare
17q gain	Rare	Frequent	Frequent
MYCN	Normal	Normal	Amplified
Molecular			
TrkA	High	Low	Low
TrkB	Low	Low	High
TrkC	High	?	Low
Cyclin D1	?	?	?
NM23	Low	?	High

Table 1.4: Characteristics of neuroblastoma risk groups

Trk, tyrosine receptor kinase; International Neuroblastoma Staging System (adapted from reference Brodeur, 2003)

1.4.1 Genetics of neuroblastoma

Most neuroblastomas occur spontaneously. Although the precise causes of tumour initiation and progression are still unclear, multiple somatic defects, such as gain of alleles, activation of oncogenes, loss of alleles or alterations in tumour- cell ploidy, have so far been identified in many neuroblastoma tumours. Several of these changes correlate with the different clinical manifestations of the disease. This has allowed a more accurate stratification of tumours at presentation, thereby allowing an appropriate selection of therapeutic strategies in individual patients (Table 4.0). Important genetic aberrations shown to contribute to the development of neuroblastoma are discussed in the following sections (for reviews, see Westermann and Schwab, 2002; van Noesel and Versteeg, 2004).

1.4.1.1 Changes in ploidy and chromosomal abnormalities

1.4.1.2 Ploidy

Aneuploidy (loss or gain of one or more chromosomes of a diploid genome) is frequent in neuroblastoma. The ploidy status of neuroblastoma is associated with the clinical heterogeneity (Look et al, 1991; Kaneko and Knudson, 1990). Cytogenetic analyses have distinguished four ploidy levels: near-diploid, near-triploid, near-tetraploid, and near-pentaploid tumours (Kaneko et al, 1987). The near-diploid and near-tetraploid tumours are observed in patients over 1 year of age, with structural chromosomal abnormalities such as the deletion of chromosome 1 and amplification of MYCN gene (discussed later in section 4.2.1). These genetic aberrations are associated with advanced stages of the disease, with rapid tumour progression and poor prognosis. The near-triploid tumours are characterized by three almost complete haploid sets of chromosomes, with few structural abnormalities, and are predominantly detected in patients under the age of 12 months (INSS stage 1, 2, and 4s), with favourable clinical outcome. Similarly, patients with near-pentaploid tumours have a good prognosis and prolonged disease free survival (Look et al, 1991; Kaneko et al, 1990; Oppedal et al, 1989; Kaneko et al, 1999; Cohn et al, 1990).

The genetic mechanisms leading to aneuploidy in neuroblastoma still remains to be determined conclusively. However, it has been suggested that aneuploid tumour cells in neuroblastoma might arise from tetraploidization and subsequent bipolar, tripolar, or tetrapolar divisions (Kaneko and Knudson, 2000). Increase in the number of centrosomes and aberrant spindle formation, resulting in aberrant chromosome segregation and multipolar cell divisions, has been implicated in the generation of aneuploid cells in many cancers, including neuroblastoma (for review see Brinkley, 2001; Sugihara et al, 2004). The incomplete segregation of chromosomes in a tetraploid cell undergoing a tripolar division could result in one near-triploid and one near-pentaploid daughter cell. In fact, some neuroblastoma tumours comprising of more than one tumour cell clone, have been shown to have both near-pentaploid and near-triploid cells.

1.4.1.3 Trisomy of 17q

Trisomy for the long arm of chromosome 17 (17q) has been shown to occur in more than 80% of neuroblastomas, making it the most prevalent genetic abnormality in this disease. This trisomy can result from the gain of 17q in an unbalanced translocation, most commonly with chromosome 1p, followed by 11q, or as part of whole chromosome gain (Caron et al, 1994; Savelyeva et al, 1994; Lastowska et al, 1997; Meddeb et al, 1996; Plantaz et al, 1997; Stark et al, 2002). Trisomy 17 has been seen in near-triploid tumours with favourable outcome. In contrast, selective gain of the long arm of chromosome 17 is associated with advanced stage disease, in tumours from children aged over 1 year, and correlates with 1p loss and MYCN amplification (Lastowska et al, 1997; Bown et al, 1999).

The poor prognosis of neuroblastoma with an unbalanced translocation of 17q could result either from the fusion of a gene localised at the breakpoint, or caused by a dosage effect of gene(s) within the additions 17q region. The identifications of at least seven different breakpoints, but preferential gain of a region from 17q21.3-17qter, strongly favours the possibility of a dosage effect of one or more genes localized distal to the breakpoint, rather than interruption of just a single gene, that

may be responsible for the aggressive nature of these tumours (Lastowska et al, 1997; 1998; 2002). Several genes have been proposed to be responsible for tumour growth characteristics in the common translocated 17q segment, but the most prominent candidates are survivin, NM23-H1 and NM23-H2.

Survivin is an anti-apoptotic protein which negatively regulates caspase 9 expression. The gene for survivin has been mapped to 17q25, which is within the minimal region of chromosomal gain in neuroblastomas and, has been shown to correlate with unfavourable histology, adverse clinical factors, such as age and stage, and outcome (Adida et al, 1998). Over-expression of survivin in a neuroblastoma cell line results in inhibition of retinoic acid induced apoptosis, suggesting a functional role for this protein (Islam et al, 2000).

NM23-H1 maps to 17q22 which is within the common amplified region of 17q which correlates with poor prognosis and increased aggressiveness in non-Hodgkin's lymphomas and other haematological malignancies (Aryee et al, 1996; Niitsu et al, 2000). Over-expression of NM23-H1 is observed in aggressive neuroblastomas. The closely related NM32-H2 co-localises at 17q22. Both NM23-H1 and NM23-H2 are up regulated by MYCN in neuroblastoma, suggesting that in tumours with MYCN amplification and gain of 17q, a synergistic mechanism may exist that leads to over expression of NM23-H1 and –H2. These two factors belong to a metastasis gene family. NM23-H1 promotes metastasis of neuroblastoma cell lines (Almgren et al, 2004). However, their precise pathogenetic role in tumourigenesis of unfavourable neuroblastoma remains to be fully established.

1.4.1.4 Deletion of 1p

Deletion of the short arm of chromosome 1 (1p) has been identified in 30-35% of neuroblastomas (White et al, 2001; 1995). Most studies have identified the smallest region of overlapping deletion (SRO) on chromosome 1 at 1p36 but as yet there is no agreement about the proximal and distal break points (Ejeskar et al, 2001; Maris et al, 2001; Ohira et al, 2000). Loss of 1p is frequently associated with an unbalanced translocation with 17q t(1;17). However, no genes have been

identified at the chromosomal break-points, as the breakpoints are highly variable on both chromosomes, and are dispersed throughout a large genomic region (Laureys et al, 1995; van der Drift et al, 1995). This implies the existence of more than one tumour suppressor gene at 1p. In the absence of genetic mutations, epigenetic silencing of potential tumour suppressor genes at 1p36 provides an alternative mechanism in neuroblastoma progression but this has not yet been investigated. Hence, due to the genomic complexity of the region and the large size of the deletions it has been difficult to elucidate the biological significance of 1p loss in neuroblastoma.

Cytogenetic and molecular analyses of large neuroblastoma tumour cohorts and cell lines have identified at least two different regions of overlapping deletion (SRO) and one region of homozygous deletion (HD). In one cell line, the region of HD has been mapped to chromosome 1p36.2, spanning approximately 500 kb. To date, no oncogenic mutations in this region have been identified (Ohira et al, 2000). In tumours with MYCN amplification, loss of heterogeneity of 1p seems to affect larger regions, often spanning to 1p32. The SRO for MYCN amplified tumours has been defined to 1p35-1pter (Caron et al, 1995; Schleiermacher et al, 1996; Spieker et al, 2001). This deletion correlates with grim prognosis. In contrast, the SRO for 1p deletions in MYCN single copy tumours is smaller, and defined at 1p36.3 (Caron et al, 2001; Bauer et al, 2001; White et al, 2001). These tumours are also often near-triploid and associated with a better outcome. These different regions of deletion exhibiting varied biological outcome suggest that there may be more than one tumour suppressor genes localized to chromosome 1p.

The significance of del 1p as an independent predictor for survival is still debatable. Deletion of large regions of 1p is usually found in patients with advanced disease (INSS stages 3 and 4) and is strongly associated with MYCN amplification, di- and tetraploidy, and gain of chromosome 17q (Caron et al, 1995; Spieker et al, 2001; Maris et al, 2000)). However, loss of 1p alone is not an independent prognosticator (Gehring et al, 1995).

1.4.1.5 Deletion of 11q

Genetic aberrations at chromosome 11q include balanced translocation involving 11q21 and 11q22, deletion of 11q23-ter, inversion 11q21-q23 and more frequently allelic loss (Koiffmann et al, 1995; Bown et al, 1993; Vandesompele et al, 1998). Loss of whole chromosome 11 (19% of neuroblastoma tumours) is associated with near-triploid low stage tumours whereas unbalanced translocations (22% of neuroblastoma tumours) are associated with patients over 1 year, stage 4 disease and unfavourable histology (Plantaz et al, 2001; Maris et al, 2001; Guo et al, 2000; Spitz et al, 2003; Guo et al, 1999; 2000). In many cases, unbalanced 11q deletions correlate with stage 4 neuroblastomas without MYCN amplification, and hence define a distinct sub-set of stage 4 tumours. This sub-group of patients also show gain of 17q, and losses of 3p, 4p and 14q, while exhibiting an inverse correlation with 1p deletion (Plantaz e al, 2001; Vandesompele et al, 2001).

Tumours without complete loss of chromosome 11 often demonstrate a common deleted segment between 11q14-23 (Maris et al, 2001). This suggests that one or more tumour suppressor gene(s) are possibly located at this position on chromosome 11, but these have yet to be elucidated. This hypothesis is supported by evidence showing that transfer of intact chromosome 11 into neuroblastoma cell line NGP lacking this chromosome, result in restoration of differentiation of the cells (Bader et al, 1991).

1.4.1.6 Other chromosomal deletions

Allelic losses in other chromosomes such as at 2p, 3p, 9p and 14q have been observed with variable frequency in neuroblastoma patients. Loss at 2q33 and is often associated with loss of expression of the caspase-8 which is localized to this chromosomal locus (Teitz et al, 2000; Takita et al, 1997). Capsase-8 is a member of the caspase family of proteolytic enzymes activated during apoptosis, thus its inactivation would lead to survival advantage (Teitz et al, 2000). Indeed, tumours cells with the loss of caspase-8 expression do not respond to tumour necrosis

factor-related apoptosis (TRAIL) mediated apoptosis (Eggert et al, 2001; Fulda et al, 2000; Teitz et al, 2000). Loss of caspase-8 mRNA expression and protein levels has been observed in 13 out of 21 neuroblastoma cell lines studied and this correlates with a resistance to apoptosis which may be important for disease initiation, progression and resistance to chemotherapeutic agents. In primary patient samples, the loss of caspase-8 has been detected in 25-35% of high-risk tumours, and correlates with amplified MYCN (Teitz et al, 2000 van Noesel et al, 2002). Some tumour cells also exhibit hypermethylation of caspase-8 gene, resulting in its inactivation (Banelli et al, 2002). Interestingly, treatment of hypermethylated caspase-8 tumours with demethylating agent, such as 5-aza-2' deoxycytidine (5-AZA), reverts cellular responsiveness to TNF with resultant apoptosis (Fulda et al, 2001).

Loss of 3p25.3-p14.3, and is often found with loss of 11q in localized tumours with MYCN single copy (Ejeskar et al, 1998; Spitz et al, 2003). As such these tumours are often associated with a poor clinical outcome. RASSF1A (Ras-association domain family 1) is a strong candidate gene believed to be lost by this deletion as it localizes to chromosomal band 3p21.3. RASSF1A is regarded as a tumour suppressor gene since re-expressing this gene in cancer cell lines decreases proliferation of the cells. Moreover, RASSF1A counteracts the oncogenic HRAS GTPase activity (Dammann et al, 2003). Moreover, RASSF1A also appears to be inactivated by hypermethylation in 86% of neuroblastoma cell lines and 40-55% of tumours (Astuti et al, 2001; Harada et al, 2002).

The loss of chromosome 9p occurs in a small number of tumours (Takit et al, 1995). The tumour suppressor gene CDKN2A (encoding both $p16^{INK4A}$ and $p14^{ARF}$) is localised to 9p21. Homozygous deletion of CDKN2A has been detected in 4 out of 46 neuroblastoma cell lines (Thompson et al, 2001). To date, the relevance of this chromosome change is unclear.

Delection of 14q23-32 occurs in 23% of localized tumours with a single copy of MYCN and is often associated with deletion 11q (Thompson et al, 2001).

Therefore, a number of chromosomal abnormalities, resulting from loss or gain of genetic material, are found in neuroblastoma. Whilst some have diagnostic and prognostic values, the precise mechanism by which these lead to development and progression of neuroblastoma are still not fully understood.

1.4.2 Changes in gene expression associated with neuroblastoma

Aberrant gene expression in a normally tightly regulated cellular system will inevitably affect cellular growth and behaviour leading to tumourigenesis. Neuroblastomas exhibit alterations in gene expression and these are discussed in the following sections.

1.4.2.1 Amplification of MYCN oncogene

Amplification of the proto-oncogene, MYCN, is one of the most prominent abnormalities found in a sub-set of neuroblastomas. It occurs in 20-25% of all neuroblastomas but accounts for 50% of tumours classified as stage 3 and 4 tumours (Brodeur et al, 1984; Caron et al, 1996). No mutations in the MYCN coding sequence has been detected in neuroblastomas. High levels of MYCN mRNA and protein expression are detected in MYCN-amplified tumours (Schwab et al, 1984; Wenzel et al, 1991). Since MYCN is recognized to induce neoplastic phenotype in embryonic rat cell cultures (Small et al, 1987), it is likely that a similar mechanism is operative in human neuroblastoma cells. This implies that the biological effects of MYCN in neuroblastoma tumours results from an increased dosage effect which contributes to tumourigenesis.

Patients with tumours that lack MYCN amplification have a 60% survival at five years, whereas only 10% of patients with highly amplified MYCN (greater than ten copies) survive for 1-year period (Seegar et al, 1985). Hence, a strong correlation exists between MYCN amplification and advanced stage disease and aggressiveness of tumours (Nakagawara et al, 1987; Brodeur et al, 1986; Berthold et al, 1997).

The significance of the amplification of MYCN in terms of oncogenic effect has been shown in various experimental systems. For instance, transgenic mice engineered to over-express MYCN in neural crest-derived tissues develop neuroblastoma (Weiss et al, 1997). Similarly, overexpression of MYCN in neuroblastoma cells and post-mitotic neurons promotes S phase entry of cells (Lutz et al, 1996; Wartiovaara et al, 2002). In agreement, with these findings decreasing the levels of MYCN using anti-sense strategies reduces proliferation rates and/or induces differentiation of human neuroblastoma cell line (Whitesell et al, 1991; Schmidt et al, 1994). Moreover, over-expression of MYCN also over-rides G1 arrest after gamma-radiation in *ex vivo* primary untreated neuroblastoma tumours (Tweddle et al, 2001), and abrogates p53-mediated cell cycle arrest in MYCN amplified neuroblastoma cell lines (Tweddle et al, 2001). Recently, MYCN amplification in neuroblastoma cells has been shown to cause centrosome hyperamplification, which could result in increased frequency of multipolar mitotic spindles and imbalanced segregation of chromosomes into daughter cells, a hallmark of cancer cells (Sugihara et al, 2004).

The region of DNA that is amplified in MYCN-amplified tumours can range from 100kb to >1 Mb, with a core region of 100-200kb domain, which encompasses the MYCN gene (Amler et al, 1989). Since the amplified region can often be much larger than the actual size of the MYCN gene, other genes co-amplified have been identified. For instance, the DDX1 gene, which maps to within 400kb upstream of MYCN, has been found to be co-amplified in approximately 50-70% of MYCN amplified tumours (Amler et al, 1996; Squire et al, 1995). Another gene, neuroblastoma amplified gene (NAG), is also co-amplified in 70% of MYCN-amplified neuroblastomas (Wimmer et al, 1999). Amplification of these two genes has not been detected without MYCN amplification, suggesting that MYCN maintains the 2p24-amplified DNA.

MYCN is a nuclear phosphoprotein that functions as a transcription factor (for review see Wenzel and Schwab, 1995). A direct transactivational target of MYCN is MCM7, which has been shown to be overexpressed both at mRNA and protein level in MYCN amplified neuroblastoma tumours and cell lines (Shohet et al, 2002). MCM7 belongs to a family of DNA-binding proteins with a vital function in

'licensing' DNA synthesis during transition from G1 to S phase of the cell cycle (Takisawa et al, 2000). Even though the precise role of MCM7 in neuroblastoma cells has not been determined, the transcriptional activation of this gene by MYCN oncoprotein could be a significant component of MYCN-induced tumourigenesis.

1.4.2.2 Expression of neurotrophin receptors

Neuroblastomas are thought to originate from neural crest precursor cells that normally mature and differentiate into sympathetic ganglia or the adrenal medulla. Development of the sympathoadrenal cell lineage requires the activation of specific signaling pathways linked to survival and differentiation which partly depends on neurotrophins that activate or interact with their tyrosine kinase receptors, Trk A, Trk B, and Trk C (for review see Patapoutian and Reichardt, 2001).

In the normal sympathetic nervous system, Trk B, and Trk C, are expressed at an earlier stage of neuronal development, whilst later on there is predominantly Trk A expression (Birren et al, 1993). Trk A is a transmembrane receptor that binds nerve growth factor (NGF) and is expressed at high concentration in mature neurons of the normal sympathetic ganglia. Nakagawara and colleagues (1993) studied the relationship between Trk A mRNA expression and patient survival in a series of neuroblastomas and ganglioneuromas. It appears that high expression of Trk A correlates with younger age of disease detection, early stage of disease and no MYCN amplification, and is therefore associated with a favourable outcome (Nakagawara et al, 1993; Suzuki et al, 1993; Kogner et al, 1993). By contrast, reduced Trk A expression is associated with poor prognosis and MYCN amplification (Kogner et al, 1993; Nakagawara et al, 1993).

In contrast to Trk A, the expression of TrkB receptor is strongly associated with higher stage tumours and MYCN amplification. These tumours often also express the Trk B ligand (BDNF), and this might represent an autocrine or paracrine loop, that enhances cell survival and confers a growth advantage to the cells (Matsumoto et al, 1995). High expression Trk B/BDNF is associated with

angiogenesis and drug resistance (Eggert et al, 2000; Nakagawara et al, 1994; Ho et al, 2002).

In a cohort of 55 tumours, Trk C receptor were detected in 25% of neuroblastomas, with high levels found in stages 1, 2, and 4s and correlate negatively with MYCN amplified tumours. The exact function and role of Trk C in neuroblastomas remains to be elucidated. However, *in vitro* studies demonstrate that transfection of Trk C into neuroblastoma cells induces differentiation and this has suggested that, like Trk A, this neurotrophin receptor may also be associated with a favourable outcome in neuroblastomas (Yamashiro et al, 1996).

1.4.2.3 Expression of anti-apoptotic genes in neuroblastomas

Increased levels of anti-apoptotic gene BCL-2 and BCL-X_L have been detected in nearly 82 % of neuroblastoma tumours and majority of neuroblastoma cell lines tested (Ikeda et al, 1995; Ikegaki et al, 1995). High BCL-2 expression is associated with poor prognosis and correlates with MYCN amplification (Castle et al, 1993). Studies in neuroblastoma cell lines to analyse the mechanism by which BCL-2 acts in these cells have shown that over-expression of BCL-2 can block TRAIL-induced apoptosis (Fulda et al, 2002). Similarly, over-expression of both BCL-2 and BCL-X_L has been shown to block apoptosis in neuroblastoma cell lines exposed to chemotherapeutic agents such as cisplatin, doxorubicin and etoposide. The ability of these proteins to protect neuroblastoma cells from apoptosis may explain the drug-resistance that is exhibited by high-risk neuroblastoma tumours (Dole et al, 1995; Fulda et al, 1997). No structural abnormalities in BCL-2, Bcl- X_L or any other genes involved in intrinsic apoptotic pathway have been detected in neuroblastomas (Teitz et al, 2002; van Noesel et al, 2003).

1.4.2.4 Cell cycle regulated genes in neuroblastomas

Altered expression in the positive and negative regulators of the cell cycle is a common event in virtually all types of human tumours. The involvement of factors

that control cell cycle progression in neuroblastomas is discussed in the following sections.

1.4.2.4.1 Expression p53 family in neuroblastomas

p53, the protein product of TP53 gene, is a nuclear phosphoprotein which acts as a check point protein for the cell cycle. It is a ubiquitously expressed protein that monitors DNA damage and upon induction can either cause cell cycle arrest to allow for DNA repair to take place or induce apoptosis if damage is too severe or under unfavourable conditions. The TP53 gene is the most common genetic lesion in human cancers but in neuroblastomas this gene is rarely mutated (Vogan et al, 1993). However, some studies have shown p53 to be inactivated in undifferentiated neuroblastomas by cytoplasmic sequestration, which results in loss of normal G1 checkpoint following DNA damage (Moll et al, 1995).

More recently, two p53 related proteins, p73 and p63, have been identified. This family has also been shown to be important for normal neuronal development (for review see Jacobs et al, 2004). p73 and p63 proteins have high homology to p53 in some domains and share some effects on cell cycle arrest or apoptosis. For instance, the transactivator form of p73 (TAP73) behaves in a manner similar to p53 with respect to inducing cell cycle arrest or apoptosis depending on cellular conditions. Interestingly, the gene encoding p73 protein is located at 1p36.3, which is frequently deleted in neuroblastomas, therefore making it a possible candidate tumour suppressor gene lost in these tumours (Kaghad et al, 1997). However, no mutations in this gene have been reported in neuroblastomas.

A truncated anti-apoptotic isoform, ΔNp73, that lacks the transactivation domain in the amino-terminus of the protein has been identified and shown to be able to antagonize both p53 and p73 protein. In neuroblastomas, this ΔNp73 isoform has been detected as the predominant form of p73 and may provide a mechanism of inactivating wild-type p53 or p73. The clinical evaluation of a representative set of patients showed that the expression (or the lack of expression) of Tap73 is independent of clinical stage, age and survival (Romani et al, 1999). However,

further investigations in larger cohorts need to be carried out before excluding a role for ΔNp73 in neuroblastoma tumourigenesis.

1.4.2.4.2 Cyclin D1 expression in neuroblastoma

The cyclin D1 gene (also referred to as CCND1, BCL1/PRAD1) was originally identified as an oncogene that was activated by chromosomal translocations in patients diagnosed with parathyroid adenoma (Motokura et al, 1991). It has since been found to be one of the most frequently over-expressed genes in a broad range of human neoplasms, including mantle cell lymphoma, head and neck squamous cell carcinoma, oesophageal, lung and breast cancer (Donnellan and Chetty, 1998; Diehl, 2002). This is not surprising when taking into account the functional role of cyclin D1 as a positive regulator of cell cycle progression through the G1 phase of the cell cycle (for review see Sherr, 1994). In most cancer types, cyclin D1 over-expression results from induction by oncogenic signals, rather than a clonal somatic mutation or rearrangement in the cyclin D1 gene (Hosokawa and Arnold, 1998).

Molenaar and colleagues (2003) have also shown very high expression of cyclin D1 at both the mRNA and protein levels in approximately two-third of neuroblastoma cell lines and primary tumours. However, this study showed no correlation between cyclin D1 expression and other prognostic factors such as stage of the tumour, age or MYCN status. More recent studies have shown that cyclin D1 is over expressed in up to 65% of neuroblastoma tumours. This seems to be associated with upstream regulation of expression as the gene itself does not appear to be amplified (personal communication, Dr. Huib N Caron, Dept. of Nuclear Medicine, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands).

The control of mammalian cell proliferation occurs largely during the G1 phase of the cell cycle and involves many regulatory proteins working in concert with each other to direct a timely and orderly progression through the cell cycle. To fully understand the role played by cyclin D1 in the cell cycle regulation, other proteins

are required for its normal function. Thus, the progression from G1 to S phase of the cell cycle in mammalian cells is regulated mainly by the association of D-type cyclins (D1, D2, D3). Cyclin-dependent kinases 4/6 (CDKs) are involved in the early G1-late G1 phase transition in all proliferating cell types (Matsushime at al, 1992; Meyerson et al, 1992), while the late G1-S phase is regulated through the interaction of cyclin E with CDK2 (Dulic et al, 1992). The importance of normal cell cycle progression can further be appreciated by the superimposition of another group of regulatory proteins, the cyclin-dependent kinase inhibitors (CDKIs), which compete with the cyclins to bind their CDKs and hence inhibit their activity.

The coordinated expression and/or association between the positive regulators, CDKs and D-type cyclins, and inhibitors such as CDKIs, and the tumour suppressor protein, pRB, although relevant in the context of normal and malignant cells, has nonetheless received relatively little attention in neuroblastoma cells. In normal non-dividing cells, the presence of anti-mitogenic factors causes the cells to become quiescent as the cell cycle is halted in G0/G1 stage. During this phase the pRB protein is present in a hypo-phosphorylated state and bound to the general transcription factor, E2F. By sequestering E2F, pRB prevents transcription of genes whose products are essential for S phase entry (Chellappan et al, 1991). In addition to this, two other events ensure the maintenance of the quiescent state of a cell. First, the levels of cyclins in the cell are very low and secondly, CDKIs are associated with CDKs, hence preventing them from phosphorylating pRB, which would then become dissociated from E2F and allow entry into S phase. However, upon extracellular mitogenic stimulus, there is a dramatic increase in the levels of specific D-cyclins, depending on cell type, which interact with their respective CDK4/6 partners. This cyclin/CDK complex drives the phosphorylation and subsequent dissociation of pRB from E2F, thus allowing E2F to activate transcription of its target genes, for example, cyclin E (Figure 1.4.2.4.2) (Matsushime et al, 1991; 1994; Meyerson and Harlow, 1994).

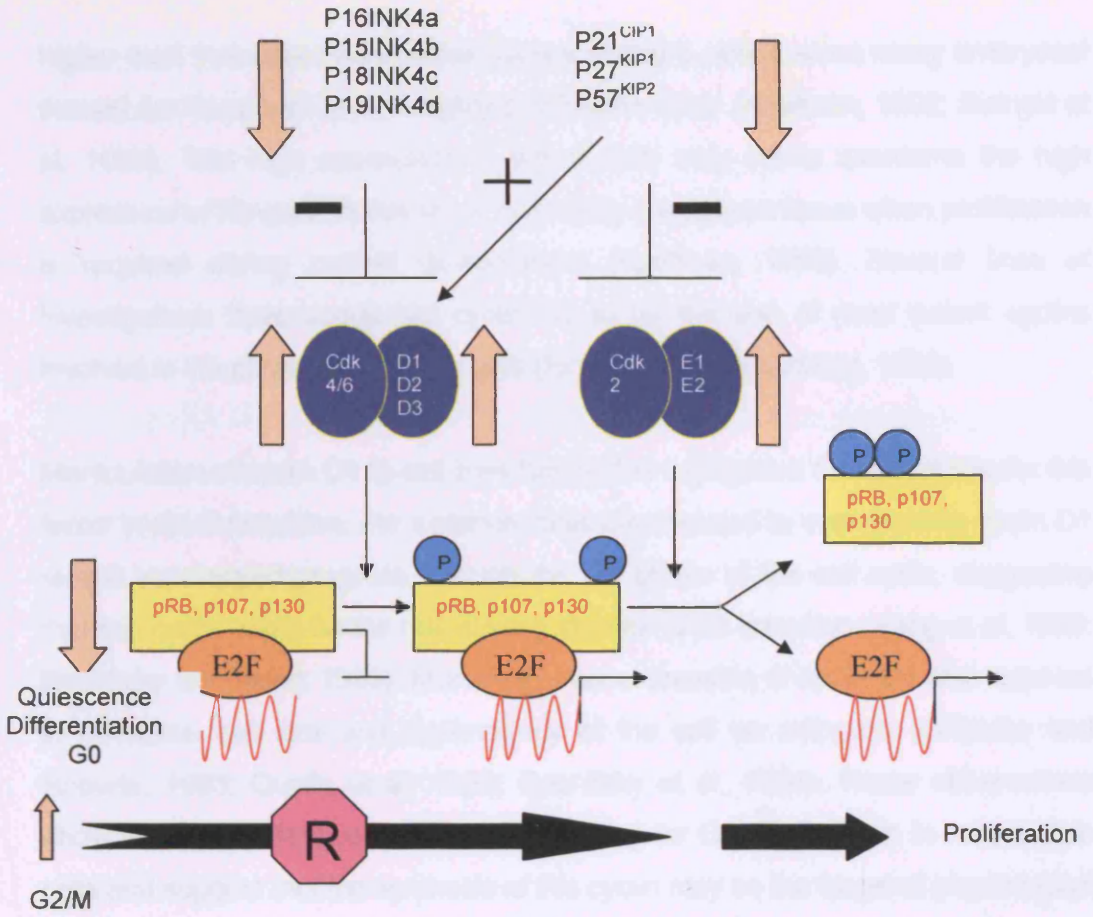


Figure 1.4.2.4.2: Schematic representation of constituents of the cell cycle. Transition from resting phase, G_0 to G_1 requires a coordinated expression of members of cyclin D (D1, D2 and D3) family, and their interactions with specific kinases (CDK4/6). Phosphorylation of retinoblastoma (pRb), p107 and p130, and their dissociation with E2F leads to transcriptional activation of a number of genes, allowing the progression past the restriction point (R) in G_1. During later phases of the cell cycle, E-type cyclins (E1 and E2) and CDKs are expressed with concomitant release of E2F and pRB. Cell cycle inhibitory proteins (p16, 15, 18, 19) of the INK family (INK 4a, b, c, d) serve to inhibit the continued entry of cells in early phases of the cell cycle. Proteins of CIP/ KIP family (p21, 27, 57) also affect the cell cycle in both early and late phases.

The cyclin D1 gene is detected at very high levels in the developing CNS and PNS with significant elevations observed particularly in the developing sympathetic trunk, suggesting an important role for cyclin D1 in the nervous system (Sicinski et al, 1995). Moreover, the levels of cyclin D1 mRNA in the developing retina are

higher than that observed in other tissues during a period when many embryonal tissues are known to be in a highly proliferative state (Kaufman, 1992; Sicinski et al, 1995). This high expression in retinal cells may act to overcome the high expression of Rb gene in this small, but highly specialized tissue when proliferation is required during normal development (Kaufman, 1992). Several lines of investigations have suggested cyclin D1 to be the one of most potent cyclins involved in the phosphorylation of pRb (for review see, Weinberg, 1995).

Manipulation of cyclin D1 in cell lines has further highlighted the critical role for this factor in G1/S transition. For instance, cells manipulated to over-express cyclin D1 exhibit accelerated progress through the G1 phase of the cell cycle, suggesting that this cyclin might be the rate-limiting step for G1/S transition (Jiang et al, 1993; Resnitzky and Reed, 1995). Moreover, over-expression of cyclin D1 also appears to decrease cell size and dependency of the cell on mitogens (Ohtsubo and Roberts, 1993; Quelle et al, 1993; Resnitzky et al, 1994). These observations show that cyclin D1 levels can be rate-limiting for G1 progression in mammalian cells and suggest that the synthesis of this cyclin may be the target of physiological signals that control cell proliferation.

Homologous null mutants of cyclin D1 (cyclin D1-/-) generated have further highlighted the pivotal role of this gene in cellular proliferation (Sicinski et al, 1995; Fantl et al 1995). Mutant mice lacking cyclin D1 are viable but grow more slowly and majority die within the first month post-partum. The most obvious aberrant phenotype involves the nervous system as the mice exhibit retraction of their limbs towards the trunk as well as abnormal clasping of the limbs (Sicinski et al, 1995). This type of clasping of the limbs has been reported in several mutants generated by targeted disruption of genes that are known to be critical for the normal development and functioning of neurons (Urbanek et al, 1994).

Histopathological examination of cyclin D1-/- mice has shown changes particularly in retinal development with reduction in the number of cells in all the layers of the neural retina. This is thought to be due to the reduced ability of the cyclin D1-/- retinal cell precursors to proliferate during embryonic development (Sicinski et al,

1995; Fantl et al, 1995). This retinopathy in cyclin D1 -/- mice is similar to that observed in Brn-3b null mutants (see 1.3.6.2.1).

Another phenotype observed in cyclin D1-/- mice is only apparent in female homozygous mutants after they give birth to litters. In normal mammary epithelium development, there are two phases of intense proliferation during two well-defined periods of development. The first phase occurs during the ductal elongation at puberty and is directly stimulated by oestrogen, whilst the second phase involves the ductal side branching and lobuloalveolar formation during pregnancy in response to both oestrogen and progesterone (Haslam, 1988; Wang et al, 1990). It appears that mammary epithelium of cyclin D1-/- females develop normally during sexual maturation compared with wild-type mice. However, there is a clear impairment of pregnancy induced proliferation in cyclin D1 -/- females as the mammary epithelia fail to undergo the normal proliferation and expansion, resulting in lack of side branching and lubuloalveolar formation (Sicinski et al, 1995; Fantl et al, 1995). Since these changes are a prerequisite for lactation, these mutants fail to nurse their young. These results are further strengthened by the observation that cyclin D1 is the most abundantly expressed D-cyclin in normal mammary glands compared to the low levels of cyclin D2 and cyclin D3 (Yu et al, 2001). Interestingly, mammary glands of cyclin D1-/- mice have modestly elevated levels of cyclin D2 and D3, which might allow normal mammary development during puberty. As ovarian steroid hormones and their receptors are critical in stimulating mammary epithelium proliferation (Haslam, 1988; Wang et al, 1990), the failure of proliferation in mammary epithelium of cyclin D1-/- mice cannot be attributed to these factors as there is no significant difference observed in the mutant mice compared with the wild-type mice. Therefore, the lack of a proliferative response of the mutant mammary epithelial cells can be attributed directly to the loss of cyclin D1 (Sicinski et al, 1995).

As both cyclin D1 and oestrogens have an essential role in regulating proliferation of breast epithelial cells, it is not surprising that several studies have established a relationship between these two proliferative factors. Cyclin D1 has been shown to have a novel function in regulating growth of estrogen-responsive tissues by potentiating transcription of estrogen receptor (ER)-regulated genes (Zwijsen et al,

1997; 1998; Lamb et al, 2000). Cyclin D1 mediates this activation independent of complex formation to a CDK partner. Cyclin D1 activates the ER-mediated transcription through increased binding of both liganded and unliganded receptor to ERE-responsive gene sequences (Zwijsen et al, 1997; 1998; Lamb et al, 2000). Cyclin D1 has been shown to bind directly to the hormone binding domain of the estrogen receptor, causing increased binding of the receptor to ERE, and thereby upregulating ER-mediated transcription (Zwijsen et al, 1997). Cyclin D1 gene is also amplified in approximately 20% of human breast cancers (Dickson et al, 1995) and protein over-expression has been detected in over 50% of human mammary carcinomas, and is associated with poor prognosis and lymph node metastases (Bartkova et al, 1994; Gillett et al, 1994, 1996). Over-expression of cyclin D1 seems to have a causative role in breast cancer formation, as transgenic mice engineered to over-express cyclin D1 in mammary glands develop breast cancers (Wang et al, 1994).

Recent studies undertaken to elucidate the molecular pathways involved in cell cycle arrest and differentiation in neuroblastoma cells, have further highlighted the central role of cyclin D1 in the proliferation of these cells. For example, a significant decrease in cyclin D1 transcript levels is observed in neuroblastoma cells upon treatment with a differentiating agent, dibutyryl cyclic adenosine monophosphate (cAMP), which induces differentiation of these cells and correlates with growth retardation (Munoz et al, 2003). Similarly, treatment of neuroblastoma cells with retinoic acid induces differentiation, and results in a decrease in cyclin D1 level (Wainwright et al, 2001). Hence, these studies have suggested that the regulation of the cyclin D1 in neuroblastoma cells affect proliferative potential of neuroblastoma cells. In the work described in chapter 5 of this thesis, I have examined the regulation of cyclin D1 in human neuroblastoma cells.

Aims

Brn-3a and Brn-3b are potent transcriptional regulators that are expressed in specific sub-set of neurons of the developing and adult nervous system. Studies carried out in mouse neuroblstoma cell lines, ND7, have shown that Brn-3a is highly expressed when these cells are induced to differentiate by serum withdrawal or cAMP treatment while Brn-3b levels dramatically decrease under these growth inhibitory conditions. Conversely, in actively proliferating mouse neuroblastoma cells, Brn-3a levels are detected at low levels while Brn-3b levels increase. Subsequent studies in our laboratory have shown these two transcription factors to have antagonistic effects on several neuronally expressed gene promoters, such as SNAP-25 and α-internexin. Based on these early studies, the aims of this thesis were to study the roles of these proteins in the growth and behaviour of human neuroblastoma cells and are summarized below:

(1) To manipulate the levels of Brn-3b transcription factor in human neuroblastoma cells, IMR-32, and study the effects on cellular events such as growth, proliferation, apoptosis, invasiveness as well as response to growth inhibitory signals (e.g. all-trans-retinoic acid).

(2) To over-express Brn-3a protein in IMR-32, and test whether increasing this factor reduces the growth and proliferation of these cells, whilst enhancing differentiation and cell survival.

(3) To identify and analyse target genes for Brn-3a and Brn-3b in human neuroblastoma cells, IMR-32, in order to provide some mechanistic understanding of how these transcription factors exert their effects on cellular proliferation and differentiation.

CHAPTER 2

Materials & Methods

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Suppliers

Analytical grade laboratory chemicals were obtained from Boehringer Maaheim (Lewes, East Sussex, U.K.), Merck Ltd., (Poole, Dorset, U.K.), or Sigma Chemical Company Ltd. (Poole, Dorset, U.K.). Disposal plasticware was supplied by Greiner (Stonehouse, Gloucester, U.K.) or Sterilin (Stone, Staffordshire, U.K.). Additional laboratory materials were obtained from the suppliers mentioned below (unless stated otherwise):

Amersham International plc. (Little Chalfont, Bucks., U.K.)
[Methyl-^{3}H]Thymidine aqueous solution (70-95 Ci/mmol), Adenosine 5'-[γ-^{32}P] triphosphate triethylammonium salt (3000Ci/mmol), Rainbow™ protein weight marker, Hybond™-C membranes

Amersham Biosciences (Little Chalfont, Bucks., U.K.)
ECL Western blotting detection reagents and analysis system

BD Biosciences Discovery Labware (Bedford, MA, U.S.A.)
BD Biocoat™ Matrigel™ Invasion Chamber

Bio-Rad (Hemel Hempstead, Herts., U.K.)
Ammonium persulphate, N,N'-methylene-bis-acrylamide, N,N,N'N'-tetramethylethylene-diamine (TEMED)

Bio-Rad (Hercules, C.A., U.S.A.)
GS-250 Molecular Imager – densitometer and phosphor imager, SmartSpec™ 3000 spectrophotometer

Dako Ltd. (High Wycombe, Bucks., U.K.)

All peroxidase conjugated secondary antibodies

Difco Laboratories (Basingstoke, Hants., U.K.)
Bacto-agar, Bacto-tryptone, Yeast extract

Gelman Life Sciences (Ann Arbor, Michigan, U.S.A.)
Disposable 0.2 and 0.45 µm sterile filters

Gibco-BRL Life Technologies Ltd. (Renfrewshire, Scotland, U.K.)
1kb DNA ladder, Lipofectin® reagent, all tissue culture solutions, media and supplements

Gibco, Invitrogen Corporation (Paisley, Scotland, U.K.)
Geneticin G-418 Sulphate, PBS tablets

Merck (Darmstadt, Germany)
TLC Aluminium sheets

Nunc (Roskilde, Denmark)
All tissue culture plasticware

Pierce Biotechnology, Inc., (Rockford, IL, U.S.A.)
BCA protein assay kit

Pharmacia
Turner TD-20e luminometer

Promega Corporation (Madison, Wisconsin, U.S.A)
All restriction and modifying enzymes and buffers
Dual-Luciferase® Reporter Assay System

Qiagen (Crawley, West Sussex, U.K.)
Qiagen 'midi/maxi-prep' plasmid DNA extraction kits

Roche (Mannheim, Germany)
Annexin-V-Fluos Staining Kit

Sigma (Saint Louis, Missouri, U.S.A)
Colchicine, 5-Fluorouracil, Hydroxyurea, MTT (Thiazolyl blue), all-trans retinoic acid

Sigma Chemical Company Ltd. (Poole, Dorset, U.K.)
Kodak X-OMAT imaging photographic film, Kodak Professional 64T colour film

Whatman International Ltd. (Maidstone, Kent, U.K.)
3MM chromatography paper

2.1.2. Standard buffers and solutions

The standard buffers and solutions used throughout are given below (all concentrations are expressed at 1X:

Luria Bertani (LB) media:
1% (w/v) Bacto®-tryptone
1% (w/v) NaCl
0.5% Bacto®-yeast extract
LB media was immediately autoclaved after preparation at 120^0C for 20 minutes at 10lb/square inch (psi).

Chloroform/isoamylalcohol:
96% (v/v) chloroform
4% (v/v) isoamyl alcohol

Tris equilibrated phenol:
Liquefied phenol equilibrated twice with excess 0.1 M Tris-HCl pH 8.0

TE:

10 mM Tris-HCl pH 8.0

1 mM EDTA pH 8.0

Tris-Acetate Buffer (TAE):

400 mM Tris base

200 mM Sodium acetate

20 mM EDTA, pH 8.3

Tris Borate Buffer (TBE):

90mM Tris-HCl

90mM Boric acid

1mM EDTA pH 8.0

2.1.3 Description and source of E.coli strain

XL1-blue

Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB Lacl^q ZΔM15 Tn 10 (Tet)^r]*

Source: Stratagene Ltd., Cambridge U.K.

2.1.4 Description and sources of plasmids

pLTR poly

Description: Eukaryotic expression vector in which the MoMuLV promoter drives the expression of the target cDNA cloned into it

Resistance: Ampicillin

Source/Reference: Dr.T.Moroy, Phillips University, Marburg, Germany.

pLTR Brn-3a long

Description: Eukaryotic expression vector under the control of the MoMuLV promoter, expressing the long form of Brn-3a. 3.5kb of the Brn-3a long cDNA is cloned into the SalI/NruI site of the pLTR poly vector.
Resistance: Ampicillin
Source/Reference: Dr.T.Moroy, Phillips University, Marburg, Germany.

pLTR Brn-3b short
Description: Eukaryotic expression vector under the control of the MoMuLV promoter, expressing the short form of Brn-3b. 1.6kb fragment of the Brn-3b short cDNA is cloned into the SalI/HindIII site of the pLTR poly vector.
Resistance: Ampicillin
Source/Reference: Dr.T.Moroy, Phillips University, Marburg, Germany.

pJ4 Brn-3b antisense
Description: Eukaryotic expression vector under the control of the MoMuLV promoter, expressing the first 300 antisense bases of Brn-3b. These first 300 antisense bases of the Brn-3b short cDNA are cloned in a reverse orientation into the Sal1/BamHI site of the pJ4 vector.
Resistance: Ampicillin
Source/Reference: Dr.M.Smith, University College London, U.K.

pCi Neo
Description: Eukaryotic expression vector containing the gene for the neomycin phosphotransferase, allowing selection of stably transfected cells.
Resistance: Ampicillin, Neomycin
Source/Reference: Promega Corporation, Madison, Wisconsin, U.S.A.

pCAT Basic
Description: Lacks eukaryotic promoter and enhancer sequences. It has a chimeric intron located 5' of the chloramphenicol acetyltransferase (CAT) gene and a different polyadenylation site located 3' of the gene. It provides a basis for the quantitative analysis of factors regulating mammalian gene expression.
Resistance: Ampicillin
Source/Reference: Promega Corporation, Madison, Wisconsin, U.S.A.

pCycD1-CAT

Description: Cyclin D1 promoter-driven chloramphenicol acetyltransferase (CAT) vector, which has a 1.9 kb Pvu II (-1652 to +231) fragment from the 5' flanking sequence of the human Cyclin D1 gene subcloned into a pCAT Basic background.

Resistance: Ampicillin

Source/Reference: Dr. J. Nevins, Duke University Medical Centre, Durham, North Carolina, U.S.A.; Ohtani et al, 1995.

pGL3

Description: Provides a quantitative analysis of factors that may potentially regulate mammalian gene expression. It has a coding region for firefly (Photinus pyralis) luciferase, which allows detection of transcriptional activity in transfected eukaryotic cells.

Resistance: Ampicillin

Source/Reference: Promega Corporation, Madison, Wisconsin, U.S.A.

pRL-TK

Description: Renilla Luciferase reporter vector, under a HSV-thymidine kinase promoter. pRL-TK contains cDNA encoding renilla luciferase, acts a control vector for transfection efficiency.

Resistance: Ampicillin

Source/Reference: Promega Corporation, Madison, Wisconsin, U.S.A

2.1.5 Description and sources of cell lines used

IMR32

Description: Human neuroblastoma cells derived from an abdominal mass removed from a 13-month-old Caucasian male. The cell line was submitted to the American Type Culture Collection in the 36th passage.

Culture medium:

1X DMEM

10% (v/v) Foetal calf serum

1% (v/v) Non-essential amino acids

1% (v/v) Penicillin/streptomycin antibiotic

Freezing medium: Culture medium + 10% DMSO

Source/Reference: ATCC, The Global Bioresource Centre™; Tumilowicz et al, 1970.

2.1.6 Description and sources of antibodies used

Actin (I-19)

Immunogen: human actin carboxy-terminus

Specificity: polyclonal

Concentration/dilution: affinity purified goat antiserum used at 1:2000

Secondary: 1: 2000 HRP-conjugated anti-goat Ig

Source: Santa Cruz Biotechnology, Inc., CA, U.S.A.

Brn-3a

Immunogen: mouse Brn-3a carboxyl-terminal domain

Specificity: monoclonal

Concentration/dilution: mouse antiserum used at 1:1500

Secondary: 1: 2000 HRP-conjugated anti-mouse Ig

Source: Chemicon International, Temecula, CA, U.S.A.

Brn-3b

Immunogen: human amino acids 184-252

Specificity: polyclonal

Concentration/dilution: crude rabbit antiserum used at 1:2000

Secondary: 1: 2000 HRP-conjugated anti-rabbit Ig

Source: BAbCo, Berkeley, CA, U.S.A.

Cleaved Caspase-3

Immunogen: peptide corresponding to residues surrounding the cleavage site of human caspase-3

Specificity: polyclonal

Concentration/dilution: crude rabbit antiserum used at 1:1000

Secondary: 1: 2000 HRP-conjugated anti-rabbit Ig

Source: Cell Signaling Technology™ (Cummings Center, Beverly, MA 01915)

Cyclin D1 (DCS-6)

Specificity: mouse monoclonal; recognizes mouse, rat and human Cyclin D1

Concentration/dilution: mouse antiserum used at 1:600

Secondary: 1: 1200 HRP-conjugated anti-mouse Ig

Source: Santa Cruz, Biotechnology, Inc., U.S.A.

2.2. Methods

2.2.1. Bacterial Culturing Methods

2.2.1.1 Propagation of bacteria

Bacteria were grown either on plates prepared from LB media containing 2% Bacto®-agar or in liquid media. Both media contained appropriate antibiotic for selection. Ampicillin was used at a final concentration of 100 μg/ml. Tetracycline was used at a final concentration of 12.5 μg/ml in plates and 10 μg/ml in liquid cultures. Bacterial plates were incubated overnight at 37^0 C in an incubator. Liquid cultures were grown overnight at 37^0 C in a rotary shaker.

2.2.1.2 Long term storage of bacterial cultures

E.coli cells were grown as mentioned above to early log phase indicated by OD^{580} of approximately 0.5. The bacteria were then pelleted by centrifugation in a Sorvall GS3 rotor (or its equivalent) at 3000 rpm for 15 minutes. The LB medium was removed and the bacteria were resuspended in fresh medium with the appropriate antibiotic containing 30% (v/v) sterile glycerol. The culture was then aliquoted at 500 μl in autoclaved vials. These glycerol cell stocks were slowly cooled to -70^0 C and stored at this temperature until ready for use.

2.2.1.3 Preparation of competent cells

Competent XL1-Blue cells were prepared using the calcium chloride technique (Sambrook et al, 1989). LB agar plates containing tetracycline were streaked from a frozen stock of XL1-Blue cells and incubated at 37^0 C overnight. A single fresh bacterial colony was picked and used to inoculate in 20 mls of LB. The cells were grown at 37^0 C overnight, shaking in a 500 ml sterile baffle flask. The following day cells were sub-cultured at a ratio of 1:50 into a litre of LB without tetracycline and grown to an OD^{580} of approximately 0.4. The culture was then chilled for 5 minutes on ice and spun in a Sorvall GS3 rotor at 4000 rpm for 10 minutes at 4^0 C. The

cells were resuspended in 500 ml of 100mM $CaCl_2$, pipetting up and down with a 25 ml pipette. The cells were left on ice for a further 30 minutes, then spun at 5000 rpm for 10 minutes at 4^0 C. The cell pellet was resuspended in 20 ml of $CaCl_2$ and 15% glycerol. 200 µl aliquots were made and left on ice for 1 hour, followed by storage at -70^0 C.

2.2.1.4 Transformation of bacteria

Competent cells were transformed using the heat shock protocol. Competent XL1-B cells were thawed at room temperature and immediately placed on ice for 10 minutes. Plasmid DNA of up to 2/5 volume of cells was added to the competent cells and incubated on ice for further 30 minutes. The cells were then heat shocked by incubating them at 42^0 C for 90 seconds and then returned to ice for 3 minutes. 2-3 volumes of LB was added to each sample. The cell suspension was then incubated for 1 hour at 37^0 C in an orbital shaker. The cells were then plated out on appropriate antibiotic plates. The plates were incubated at 37^0 C overnight. The plate was stored at 4^0 C.

2.2.2 Preparation of plasmid DNA

2.2.2.1 Small scale plasmid DNA extraction from transformed bacteria

The DNA 'mini-prep' extraction method used was based on a standard alkaline lysis protocol (Birnboim & Doly, 1979). Single bacterial colonies were used to inoculate 3 mls of LB containing the appropriate antibiotic selection. Cultures were incubated overnight at 37^0 C with vigorous shaking in an orbital shaker. 1.5 ml of culture was pellet by centrifugation at 12,000g for 1 minute in a bench top microcentrifuge. The cell pellet was then resuspended in 100 µl of resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8, 100 µg/ml Rnase-A). Bacterial lysis was carried out in 200 µl of lysis buffer (200 mM NaOH, 1% (v/v) Triton X-100) and neutralised by the addition of 150 µl of neutralization buffer (3M sodium acetate pH 5.5). The cell lysate was centrifuged at 12,000g for 3 minutes at 4^0 C

and the supernatant transferred to a fresh tube containing 500 μl of isopropanol. To pellet the DNA this supernatant was vortexed and centrifuged for 5 minutes at 12,000g. The subsequent supernatant obtained during this step was discarded. The DNA pellet was washed with 500 μl of 70% ethanol, air dried and then resuspended in 100 μl of double distilled (ddH_2O) containing 20 μg/ml RNase A. The plasmid DNA was stored at -20^0 C.

2.2.2.2 Large scale plasmid DNA extraction from transformed bacteria

400 mls of LB containing the appropriate antibiotic selection was inoculated with a single colony from a bacterial plate or with approximately 50 μl of liquid bacterial culture. This was incubated overnight at 37^0 C with vigorous shaking (300 cycles/minutes on a rotary shaker). The overnight culture was then centrifuged at 4^0 C at 3000 rpm in a Sorvall GS3 rotor for 10 minutes. Plasmid DNA was then extracted from the bacterial pellet using the Qiagen maxi-prep kit following the manufacturer's instructions. Typical yield of DNA obtained via this method was 100 μg, which was resuspended at a final concentration of 1μg/μl in ddH_20.

2.2.2.3 Phenol/Chloroform extraction and precipitation of DNA

To purify DNA, 400 μl of ddH_2O and an equal volume of tris-equilibrated phenol were mixed. The mixture was vortexed and then centrifuged at 12,000g for 2 minutes in a microfuge. The aqueous phase was removed and re-extracted with one volume of chloroform/IAA. The DNA in the aqueous phase was precipitated in 0.1 volumes of 3M sodium acetate (pH5.5) and two volumes of ice-cold 100% ethanol. The pellet was washed with 70% ethanol and centrifuged at 12,000g for 5 minutes in a microfuge. Finally, the ethanol was removed, the pellet dried and resuspended in ddH_2O.

2.2.2.4 Restriction enzyme digestion

DNA analysis was carried out by restriction digests. These digests were performed in 20 μl total volume with approximately 1 μg of DNA. A maximum of 0.1 volumes of restriction enzyme(s) were added and the buffer recommended by the manufacturer was used at 1 X concentration. Digests were incubated at the appropriate temperature for 2 hours. The digested DNA was electrophoresed on an agarose gel of appropriate percentage and the products visualized on a UV illuminator. An undigested sample was run in parallel, allowing a reference against which to compare the enzyme cut samples.

2.2.2.5 Agarose gel electrophoresis

Depending on the expected size of DNA fragments, appropriate percentage agarose gels in 1 X TAE were cast. For example, 1% gel was cast for fragments larger than 1kb, 1.5% for general purpose, and 2% to resolve DNA fragments less than 1kb. Ethidium bromide was added to a final concentration of 0.5 μg/ml. Approximately 0.1 volume of 10 X loading buffer (1 X TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading. A DNA ladder corresponding to the sizes of the fragments being analysed was used, usually a Gibco 1 kilobase (kb) ladder. Electrophoresis of the DNA was carried out at 100 mA for 0.5 to 2 hours, allowing for a good resolution of bands. Finally, the bands were visualized on a long wave UV transilluminator and photographed using the Syngene gel doc system.

2.2.3 Protein Methods

2.2.3.1 Standard protein extraction from cultured cells

80% confluent well of a 6-well plate was washed in 1 X PBS and harvested in 100 μl of standard protein sample buffer (5% β-mercaptoethanol, 50 mM Tris-HCl pH 8.0, 6% (v/v) glycerol, 2% (w/v) SDS, and 0.005% (w/v) bromophenol blue). The samples were immediately placed on ice and heated to 95^0C for 5 minutes and

placed on ice, and either loaded immediately onto a SDS-polyacrylamide gel or stored at -20^{0}C.

2.2.3.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Standard SDS-polyacrylamide gels were prepared with the composition for the stacking and resolving gels as described by Sambrook and colleagues (Sambrook et al, 1989). Protein samples were heated to 95^0 C for 5 minutes. 50-100 μg of protein from each sample and 5 μl of Rainbow marker were prepared and run in a vertical gel electrophoresis system (Sambrook et al, 1989). The gels were run at a constant voltage of 150 V in 1 X running buffer (25 mM Tris, 250 mM glycine and 0.15 (w/v) SDS, pH 8.3). Gels were run until the protein of interest was sufficiently resolved as determined by the migration of the coloured molecular weight marker.

2.2.3.3 Equilisation of protein loading

2.2.3.3.1 Coomassie Blue Staining

In initial experiments in order to establish the integrity of proteins as well as check for variation in total protein concentration, SDS-PAGE mini-gels were run to separate the protein samples. The gel was then placed in Coomassie stain solution (2% (w/v) Coomassie brilliant blue R250, 50% (w/v) methanol, 50% (v/v) glacial acetic acid) for 30 minutes at room temperature on a shaker. Any unbound stain was removed by repeated replacement of destain solution (10% (w/v) glacial acetic acid, 30% (v/v) methanol). Any variation in different samples was observed by the intensity of the protein bands in each sample and densitometry was utilized to obtain values for various band intensities.

2.2.3.3.2 Bradford Chemistry

Protein concentration of triplicate samples was determined by Bradford chemistry using BCA Protein Assay Reagent from Pierce (Illinois, U.S. A.) according to the manufacturer's instructions. Briefly, BCA Reagent A and BCA Reagent B were

mixed at a ratio of 50:1. 300 µl of this mixture was added to the appropriate number of wells of a 96-well microtitre plate. 1, 5, or 10 µl of each protein sample of unknown concentration was added to the individual wells of the plate. Additionally, a titration series of known concentrations of bovine serum albumin were also added to individual wells containing the BCA mixture on the plate. This was carried out to generate a concentration standard curve. The plate was incubated at 37^0 C for 30 minutes and cooled to room temperature prior to exposure to the SmartSpec™ 3000 spectrophotometer (Bio-Rad) which was set to read the absorbance at 562 nm. The value of the absorbance of the blank was subtracted from all other values and a standard curve was generated from the absorbance readings obtained from the albumin titration. Finally, protein concentrations of the unknown samples were determined by comparing their values of absorbance to the albumin standard curve.

2.2.3.4 Transfer of proteins to nitrocellulose membranes (Western blotting)

Proteins of different sizes resolved by SDS-PAGE gels were transferred onto Hybond C membrane using a wet-transfer method as described by Sambrook and colleagues (Sambrook et al., 1989). Briefly, the nitrocellulose membrane and the SDS-PAGE gel were sandwiched between two sheets of 3 MM Whatman paper. The sandwiched gel was presoaked in transfer buffer (50 mM Tris, 380 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol) and a Trans-Blot TM Cell (BioRad) was assembled according to the manufacture's instructions. The transfer was carried out overnight at 150 mA at 4^0C.

2.2.3.5 Immunodetection of proteins on western blots

Following the transfer of proteins onto the nitrocellulose membrane, the membrane was then blocked to prevent subsequent non-specific binding by incubating it in 5% (w/v) skimmed milk powder in 1 X PBS for 1 hour at room temperature with constant shaking. All subsequent incubations were also carried out at room temperature on a shaker unless specified otherwise. The membrane was then

incubated with primary antibody diluted in the appropriate buffer stated by the manufacturers for 1 hour. All the antibodies used in this work, including their sources and the dilutions at which they were used, are listed in section II.1.6. Any unbound, non-specific binding of antibody was removed by washing the membrane 3 X in wash buffer (1 X PBS, 0.1% Tween-20) for 15 minutes. The membrane was then incubated in an appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour. The membrane was washed three times for 10 minutes each in wash buffer to remove any unbound antibody. The bound HRP was detected using an enhanced chemiluminescence system (ECL™) according to the manufacturer's instructions. Finally, the membrane was exposed to X-ray film in order to capture the resultant light emissions. Depending on the strength of the signal, the exposure time varied from 1 second to 15 minutes.

2.2.4 Cell culture

2.2.4.1 Culture conditions

For long term storage all cell lines were stored in liquid nitrogen at -180^{0}C. During cultures the cells were maintained at 37^{0}C in a 5% CO_2 incubator. All manipulations of cells were carried out under standard aseptic techniques.

2.2.4.2 Freezing and recovery of cell stocks

Cell stocks were prepared by resuspending the cell pellet from one 175 cm^3 flask in 1.5 ml of 10% (v/v) dimethylsulphoxide (DMSO), 90% foetal calf serum (FCS). The temperature of the vials was gradually decreased to -70^{0}C before being immersed in liquid nitrogen.

For growth from frozen stocks, cells were recovered by rapidly thawing the contents of one vial and transferring the cell suspension to a 25 cm^2 flask containing 8 ml of pre-warmed medium. The medium was changed and cells passaged as required.

2.2.4.3 Routine cell passage

Cells were grown in the appropriate growth medium until the flasks were ~80% confluent when they were passaged as follows. The monolayer of IMR-32 cells was washed with Hank's Balanced Salt Solution (HBSS) free of calcium and magnesium ions. 2-3 ml of trysin/versene (1:10) were added to detach the cells from the plastic ware and fresh flasks were seeded at a ratio of 1:10 in fresh medium.

2.2.4.4 Transient transfections using Lipofectin® reagent/peptide method

Transfections were carried out using the Lipofectin® reagent (GibcoBRL, Life technologies)/peptide (containing an integrin-targeting domain) complex (kind gift from Dr. S. Hart - ICH). IMR-32 cells were plated out at 1 X 10^5 cells per well in six-well plates and left overnight. The following day transfection complexes, sufficient for 6 μg plasmid per well in 6-well plate, were prepared in the following order and concentration: Lipofectin 4.5 μl (4.5 μg), peptide 240 μl (24 μg) and plasmid 600 μl (6 μg), respectively. The relative amount was scaled up as required for the number of wells being transfected. The complexes were allowed to form at room temperature for 30 minutes to 1 hour, and then diluted with serum free DMEM to a concentration of 6 μg DNA per 1 ml in wells of a 6-well plate. The full growth medium from the cells was removed and the cells were washed in 1 X PBS twice. 1 ml of transfection complex was added to each well and the cells were incubated for 4 h at 37^0C. The transfection medium was removed and replaced with the normal full growth medium. For reporter studies, the cells were left for 48 hours and then assayed for appropriate studies.

2.2.4.5 Construction of stable cell lines

IMR-32 cells were plated out at 1x 10^4 in six well dishes (Nunc, GibcoBRL, UK) and transfected using lipofectin transfection reagent as described in section II.2.4.D. Each plate was cotransfected with a 10:1 molar ratio of plasmid containing

the Brn-3 constructs to that containing a neomycin selectable marker. The cells were grown in G418-containing medium to select for cells stably expressing the neomycin gene. It was assumed that cells exhibiting resistance to G418 were also stably expressing either the recombinant Brn-3b short, or Brn-3b anti-sense, or the empty pLTR vector (all under the control of Moloney Murine leukemia virus promoter). After 12 days (two days after 100% of the mock transfected cells had died under the G-418 selection process), individual colonies were picked by sterile pipettes using a Gilson P-1000 pipetteman under a microscope and transferred in volumes of 200 µl of medium to 96-well plates. Once the cells reached 70% confluency, they were transfected into 24-well plates, then into 6-well plates, and finally into 175 cm^3. Cells from each coloney were split into two flasks and one flask grown for storage as a DMSO stock and the other for characterisation by Western immunoblotting.

2.2.4.6 Cellular growth curve studies

Two IMR-32 clonal cell lines representing each of the stably integrated constructs were grown to 80% confluency, trypsinised, counted and plated into 24-well plates using 1 ml of cells at 1 x 10^3 cells per ml. The number of wells seeded was determined by the number needed to allow 25 days of time points to be taken in triplicate at 48h intervals. The medium was changed every three days to supplement the cells with fresh growth medium containing G418 for 25 days.

2.2.4.7 Saturation density limitation growth studies

5 x 10^3 cells were plated in 24 well plates containing a 13 mm cover slip in each well. After 24 hours, the cover slips were transferred to six well plates containing 4 ml of full growth medium. The medium was changed every 48 hours to ensure maximal growth conditions. After 5 days total cells were harvested and counted at 48 hour intervals for ten days.

2.2.4.8 MTT assay for measuring viability/proliferation of cells

MTT assay was carried out to measure live/proliferating cells in culture. IMR-32 cells were grown in 6-well plates. The medium was removed from the cells and cells were washed in 1 X PBS. 500 μl of PBS was added to each well, followed by addition of 50 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5mg/ml stock solution) to each well. Cells were incubated with this solution in a CO_2 incubator for 2 hours at 37^0C. Equal volumes (500 μl) of the solubilization buffer (0.1M HCl in 1 X PBS/ 0.1% Triton-X) was added to each well and incubated at 37^0C incubator for 5 minutes, followed by cooling to room temperature. The absorbance of the converted dye was measured in disposable plastic cuvettes at a wavelength of 570 nm using a SmartSpec™ 3000 spectrophotometer (Bio-Rad).

2.2.4.9 Tritiated thymidine incorporation proliferation studies

Two IMR32 clonal cell lines representing each of the stably integrated constructs were grown to 80% confluence, trypsinised, counted and plated in 96-well plates using 200 μl of medium containing cells at a density of 1 x 10^2 cells per well. Cells were grown in the normal growth medium containing G418. 24h post-plating, cells were pulsed with 10 μl of 50 μCi/ml [^{3}H] thymidine for 1 hour. Cells were then trypsinised and harvested into glass filters, and washed using a Tomtec cell harvester. The glass filters were then treated with scintillant and counted on a Nuclear Enterprises NE10600 scintillation counter.

2.2.4.10 Anchorage independent growth studies

These studies were carried out in soft agar in 6-well plates where a base layer of 2 ml of growth medium containing 0.7% agarose was topped with 1 ml of growth medium containing 0.35% agarose and 1 x 10^4 cells per ml. Finally, a top layer of 2 ml of growth medium containing 0.7% agarose was added. Colonies were allowed to grow for 21 days after which time colonies comprising of at least 8 cells were

counted using the Ziess axiovert microscope and images acquired using the Axiophot camera and AxioVision imaging sysytem.

2.2.4.11 Annexin V labelling for determination of apoptosis

1 x 10^6 IMR-32 were washed twice in 1 X PBS and scraped off the 6-well plates. Cell suspension was centrifuged at 200 X g for 5 minutes. The cell pellet was then resuspended in 100 µl of labeling solution (1000 µl of incubation buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$), 20 µl of fluorescein isothicyante-conjugated Annexin-V (Roche Applied Science) and 20 µl Propidium Iodide (PI) (Roche Applied Science). This solution was incubated for 15 minutes in dark at room temperature. Following this time period, 400 µl of incubation buffer was added to the cells and immediately analysed on Beckman Coulter Epics XL (High Wycombe) flow cytometer. Analysis was carried out using 488 nm argon laser and bandpass filters of 515nm for Annexin V and 626nm for PI using Expo32 software. Annexin V negative and PI negative cells were taken as live; Annexin V positive and PI negative were taken to represent apoptotic cells; Annexin V positive and PI positive were taken as necrotic; and finally, Annexin V negative and PI positive were nuclear debris. All the readings made on the FACS machine were carried out by Jo Buddle (UCL, London).

2.2.4.12 Matrigel invasion assay

For cell invasion assays, BD Biocoat™ matrigel™ Invasion Chambers were used and manufacturer's instructions followed for preparation of the membranes. Sub-confluent IMR-32 cells were split from flasks using 0.02% EDTA solution without trypsin. The cells were resuspended in growth medium containing 0.1% foetal calf serum (FCS). 5 X 10^4 cells in 0.15 FCS were placed into the upper chamber while the lower chamber contained medium with 10% FCS, hence creating a serum concentration gradient. Cells were incubated at 37^0C, 5% CO_2 for 12 hours. Following this time period, non-invading cells from the upper chamber were removed and the cells attached to the lower surface of the membrane were fixed in

serial dilutions of 100% methanol. Cells were then stained with cresyl violet and counted using the Ziess Axiovert 200 microscope.

2.2.4.13 Retinoic acid treatment

IMR-32 cells were plated out at a density of 1 X 10^4 cells/ well in a 6-well plate and grown in full growth medium. 10 μM all trans-retinoic acid (Sigma) dissolved in DMSO was added to the medium the following day and replenished every 48 hours. Morphological analysis was carried out using the Ziess Axiovert 200 microscope and images acquired using the Axophot camera and Axiovision imaging system.

2.2.4.14 Cisplatin treatment

IMR-32 cells were plated out at 1 X 10^6 cells/ well of a 6-well plate. The following day of plating, 33 μM Cisplatin was added to full growth medium. The cells were left for 48 hours under these conditions before harvested for either MTT assay, Annexin V analysis or Western immunoblotting. Simultaneously, controls cells grown in the absence of Cisplatin were grown at 1 X 10^6 cells/well for 48 hours.

2.2.5 Gene Reporter assays

2.2.5.1 CAT assay

The transcriptional activity of transfected promoter constructs was measured by determining the activity of the chloramphenicol acetyltransferase (CAT) enzyme gene product which inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups. This gene is not found in eukaryotes, and therefore eukaryotic cells contain no background of CAT activity. The enzyme activity was assayed using thin layer chromatography as described by Sambrook and colleagues (Sambrook et al., 1989). The cells were transiently co-transfected with the reporter and effector constructs and washed 48h post-transfection with 1 X PBS and harvested in 100 μl of ice-cold 0.25M Tris-Cl, pH 7.5. The cells were then

lysed by five freeze-thaw cycles; centrifuged at 12,000g for 5 minutes at 4^0 C in a microfuge. The supernatant was transferred to a fresh eppendorf and cellular debris discarded. The supernatant was then stored at -20^0C until the time for assay.

For CAT assays, the following mastermix was prepared: 2.0 µl of 200 µCi/ml [^{14}C]-chloramphenicol (35 to 55 µmCi/mmol), 20.0 µl of 4mM acetyl CoA, 32.5 µl of 1M Tris-Cl (pH 7.5) and 65.5 µl of H_2O. These amounts were used for one reaction for a 30 µl of extract. For each reaction 120 µl of the mastermix was aliquoted into a microcentrifuge tube. 30 µl of the extract was added to the tube and mixed gently. The tube was incubated at 37^0C for 1h. 500 µl of ethyl acetate was added to the reaction and the tube was vortexed for 1 minute. The top layer (containing chloramphenicol and acetylated chloramphenicol in ethyl acetate) was transferred to a fresh microcentrifuge tube. The ethyl acetate sample was then dessicated in a Speedvac for 45 minutes and the sample was then resuspended in 15 µl of ethyl acetate. The sample was spotted onto a plastic-baked thin layer chromatography (TLC) plate. Meanwhile, a chromatography tank was equilibrated by putting 200ml of fresh 49:1 chloroform:methanol in the bottom and left for 2 hours. Finally, the TLC plate was placed in the tank taking care not to submerge the origin spot in the solvent. The chromatography was allowed to run for 2 hours or until the solvent front was close to the top. The TLC plate was removed from the tank and dried in a fume cupboard. It was then covered in plastic wrap and placed on film for autoradiography. For the quantification of CAT activity, the film was lined up with the plate and the location of the acetylated and non-acetylated spots determined. The resultant signal was analysed by densitometry. The ability of the expression vector containing the factor of interest to activate or repress the reporter construct was determined as a percentage of the conversion observed with the empty expression vector.

2.2.5.2 Luciferase assay

The promoter activity of transfected promoters in Luciferase vectors was measured by the amount of Firefly luciferase and Renilla luciferase activity. Firefly and Renilla

luciferase are proteins that emit photons when provided with appropriate conditions and substrates. The Renilla luciferase acts as a control reporter gene for transfection efficiency. The pGL3-Basic vector lacks a eukaryotic promoter or any enhancer elements of its own, therefore, any promoter sequence inserted upstream of the luciferase gene could drive it, generate luminescence and hence be detected by this system. This assay was carried out using the commercially available Dual-Luciferase reporter assay system (Promega).

Cells were harvested 48 hours post-transfections, rinsed with PBS, and 200 µl of the 1X Passive Lysis Buffer (Promega) added to a well of a 6-well plate. The plates were left rotating on an oprbital shaker for 15 minutes at room temperature to ensure complete and even coverage of the cells. The cell lysate was then collected into an eppendorf and vortexed briefly. Following this, the samples were centrifuged at 12,000g for 5 minutes in a microfuge to pellet cell debris and supernatant collected into a fresh eppendorf.

All reagents and samples were thawed at room temperature prior to carrying out the assay. The readings were taken on the TD-20/20 Luminometer (Turner Designs). The luminometer was set to take dual luciferase readings (Firefly and Renilla luciferase), with a 5 second delay, followed by a 20 second integration/reading time. 50 µl of the Firefly luciferase reagent was added to 50 µl of the sample lysate, and the reading taken. Following this, 50 µl of the Stop 'N' Glo reagent (with 20 µl/ml of the Renilla luciferase substrate added) was added to the mix and another reading taken. Thus, providing two independent measurements and a final ration of promoter activity based upon the transfection efficiency.

2.2.6. Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a technique employed to study protein-DNA interactions to determine if a particular protein binds to a specific DNA regulatory sequence. This assay is performed by incubating a cellular extract over-expressing the protein or purified *in vitro* translated protein with a radio-labeled DNA fragment or oligonucleotide that

contains the putative binding site. The complex is then analysed on a non-denaturing PAGE gel. Proteins that are bound to the labeled oligonucleotide will be visible by autoradiography.

2.2.6.1 Extraction of proteins under non-denaturing conditions

Cell extracts were prepared using a modified method of Dignam et al (1983). IMR-32 cells were scraped from 6-well plates and pelleted by centrifugation at 1000g for 10 minutes at 4^0C. The cell pellet was resuspended in 100-300 µl extraction buffer (50 mM Tris pH 8.0, 170 mM NaCl, 25 X protease inhibitor and 0.5% igepal). The cells were then lysed by three freeze-thaw cycles (-70^0C/37^0C) and centrifuged at 12,000g for 10 minutes at 4^0C to pellet debris. The supernatant was aliquoted in 50 µl volumes and stored at -70^0C. 4 to 6 µl was used in the bandshift reactions.

2.2.6.2 Annealing single-stranded oligonucleotides

Single-stranded oligonucleotides of specific regions of human Cyclin D1 promoter were reconstituted in dd-H_2O at 1µg/µl. Equimolar amounts of complementary oligonucleotides were annealed by adding commercially available 1 X buffer C (Promega), heating to 80^0C for 5 minutes and allowing the oligonucleotide mix to cool slowly to room temperature.

2.2.6.3 Labelling oligonucleotide probes

End-labelling of the oligonucleotides with T4 kinase was employed to label probes for the EMSA reaction. Typically, 100 ng of the annealed oligonucleotide was incubated with 20 µCi [^{32}P] γ-ATP, 50 mM Tris.Cl (pH 7.6), 10 mM $MgCl_2$, 5mM DTT, 0.1 mM EDTA and 5-10 units of T4 polynucleotide kinase (Promega). The reaction was carried out at 37^0C for 30 minutes. Following this incubation period, 50 µl of TE buffer was added to the reaction and the labeled DNA was purified from the unincorporated label by spin-eluting through a Sephadex G-25 column. The labeling efficiency was measured using a Geiger counter, comparing the eluate reading value to the radioactivity left on the column. The purified probe was

typically at a concentration of 1 ng/μl in a final volume of 100 μl. The labeled probe was stored at –20^{0}C for later use in a radioactivity designated area.

2.2.6.4 Gel Mobility Shift Assay

This procedure was modified from the method described by Theil et al., (1993) using the total cellular extracts obtained from actively proliferating IMR-32 cells. The following mastermix was prepared without the labeled double-stranded oligonucleotide: 3 μl of cellular extract, 2.0 μl 10 X EMSA buffer (10 mM Hepes (pH 7.9), 60 mM KCl, 4% w/v Ficoll, 1 mM EDTA, 1 mM DTT), 2.0 μg poly (dI-dC) (Pharmacia), 50 – 100 ng cold oligonucleotide (50 – 100 fold molar excess specific competitor) or 50 ng non-specific (ERE oligonucleotide) and dd H_2O to give a final volume of 20 μl. 1 ng of kinase labeled oligonucleotide probe was then added, mixed well and centrifuged at 12,000g for 5 seconds. The reaction mix was then incubated on ice for 1 hour.

The protein-DNA complex was resolved from the free probe by electrophoresis carried out on a pre-run 7% non-denaturing polyacrylamide gel run in 0.5% TBE at 40 mA for 2 to 3 hours. Gels were carefully removed from the running apparatus and attached to two sheets of 3 MM paper, covered by cling film and vacuum dried at 80^{0}C for 1hour on a gel drier. Finally, the dried gels were exposed to X-ray film overnight at 80^{0}C.

2.2.7 Statistical Analysis

Data points were compared using Student's T-test analysis and the level of significance of the test is given as *p*-value in the text. Microsoft Excel and Sigma plot 2001 were the softwares used for the analyses. Statistical help was provided by Dr. V. Budhram-Mahadeo and the statistician, Angie Wade (ICH, UCL).

CHAPTER 3

Results 1

3.0 Growth and invasive characteristics of IMR32 cells with altered expression of Brn-3b

3.1 Introduction

The Brn-3 POU family of proteins represent a diverse and potent group of transcription factors. They were originally identified in the cells of the developing sensory nervous system where they regulate proliferation and neuronal differentiation (Lillycrop et al, 1992; He et al, 1989; Gerrero at al, 1993; Turner et al, 1994).

The two closely related members of the Brn-3 family, Brn-3a and Brn-3b, are associated with distinct cellular phenotypes in ND7 neuronal cell line, which was derived by fusing non-dividing rat dorsal root ganglion cells with the N18 mouse neuroblastoma cells (Wood et al, 1990). ND7 can be maintained in a proliferative state when grown in full growth medium, containing 10% foetal calf serum (Lillycrop et al, 1992). Under these proliferating conditions, ND7 cells show high levels of expression of Brn-3b, but not of Brn-3a. However, upon serum withdrawal, when these cells cease to proliferate, withdraw from the cell cycle and undergo differentiation, Brn-3b levels decrease significantly with a concomitant increase in Brn-3a levels (Lillycrop et al, 1992; Smith et al, 1997). This differential expression has also been observed in the human neuroblastoma cell lines, IMR-32 and SHSY5Y (Smith and Latchman, 1996), suggesting that these two transcription regulators are associated with different growth states in neuroblastoma cells. At a molecular level, Brn-3b was shown to repress the promoters of genes associated with neuronal differentiation, such as α-internexin and neurofilaments (Smith et al, 1997; Budhram-Mahadeo et al, 1994)

The association of Brn-3b with cellular proliferation is strengthened by other studies carried out in our laboratory in relation to breast tumourigenesis. Budhram-Mahadeo et al (1999) have shown elevated levels of Brn-3b in a significant number of breast cancer biopsies compared with levels in normal breast tissue. Moreover, modifying the levels of Brn-3b protein can alter the growth of breast

cancer cell lines, MCF 7 (Dennis et al, 2001). It is interesting to note that these changes were achieved with the short isoform of Brn-3b protein (Brn-3b(s)). Furthermore, the proliferative effect of Brn-3b in breast epithelial cells is associated with its ability to suppress the expression of the tumour suppressor gene, BRCA1 (Budhram-Mahadeo et al, 1999). In fact, Brn-3b is the first transcription factor shown to regulate the expression of BRCA1. Brn-3b is also known to functionally interact and enhance the proliferation associated with estrogen receptor in breast cancer cells (Budhram-Mahadeo et al, 1998).

Hence, the findings of elevated Brn-3b in proliferating neuroblastoma cell lines, led us to investigate the effect of altering the levels of this transcription factor in human neuroblastoma cells. Differences in cellular growth *in vivo* and *in vitro*, as well as changes in invasive properties associated with constitutively increasing or decreasing Brn-3b protein levels in human neuroblastoma cells, IMR-32, were analysed.

3.2 Results

3.2.1 Experimental strategy for alterating Brn-3b(s) levels in cells

Altering the expression of a specific protein in a cell line is a powerful tool for understanding its regulatory role in proliferation, differentiation and/or survival. Thus, cell lines with altered levels of a particular gene product have been created via homologous (Capecchi, 1989; Friedmann, 1989) or non-homologous (Scangos & Ruddle, 1981) recombination. In this study we have modified the gene expression of Brn-3b using non-homologous recombination in which constructs expressing short form of Brn-3b were used to generate constitutively over-expressing cells. An anti-sense strategy was used to generate clonal cell lines in which Brn-3b levels were reduced.

3.2.1.1 Human neuroblastoma IMR-32 cell line

The human neuroblastoma cell line, IMR-32, was used to generate stable clones with either elevated or reduced levels of Brn-3b. Similar to mouse neuroblastoma cells, ND7, IMR-32 cells also show changes in the levels of Brn-3a and Brn-3b depending on growth conditions so that high levels of Brn-3b are expressed when the cells are proliferating and low levels of Brn-3a are expressed when cells are induced to differentiate (Smith and Latchman, 1996), making it a good cell line for our studies of human neuroblastoma. This cell line was derived from a neuroblastoma tumour in a 13 months old Caucasian male, and exhibits properties such as antigen presentation, biochemical and tumour markers, neurotransmitter production, and ability to differentiate into neurons in response to stimulus such as retinoic acid (Tumilowicz et al, 1970).

3.2.1.2 Over-expression of Brn-3b short protein in IMR-32

Proliferating IMR-32 cells were transfected with the expression construct containing the cDNA encoding the short form of Brn-3b under the control of the Moloney murine leukemia virus (MoMuLV) promoter using Lipofectin®/peptide tranfection methodology (see section 2.2.4.4 of materials and methods). The empty expression vector pLTR was also transfected to provide a control for all subsequent studies undertaken with these manipulated cell lines. All transfections included pCi Neo at a 10-fold reduced concentration compared to the expression constructs. This allowed for selection of transfected clones exhibiting neomycin resistance in the presence of antibiotic, G418. Twenty independent G418-resistant clones were selected from each transfection, propagated and characterised by Western immunoblotting for Brn-3b levels.

3.2.1.3 Reducing Brn-3b protein levels in IMR-32

In the work described here, we utilised an eukaryotic expression vector encoding antisense cDNA for 300 bases of Brn-3b short. These 300 nucleotides of Brn-3b sequence do not cross-hybridise with the mRNA encoding other POU domain

members, Brn-3a or Brn-3c mRNA. A pJ4 construct expressing the first 300 bases in a reverse orientation under the control of MoMuLV promoter was cotransfected in a 10-fold excess with a pCi Neo construct into IMR-32 cells using the Lipofectin®/peptide tranfection methodology (see section 2.2.4.4 of materials and methods). Selection was carried out in the presence of an antibiotic, Geneticin-G418 sulphate. Antibiotic resistance clones were further propagated and analysed for any decrease in endogenous Brn-3b protein levels in IMR-32.

3.2.2 Brn-3b expression in stable transformants

Stable cell transformants with increased or reduced levels of Brn-3b were established using the human neuroblastoma cell line, IMR-32. To determine changes in the Brn-3b protein expression in the selected clones, Western immunoblotting was carried out using an antibody that recognises both isoforms of this protein. Each of the clonally selected cell line was harvested in cell lysis buffer. The cell lysates were equalised by Bradford Assay and 60 micrograms of total cell protein was run on a SDS/15% polyacrylamide gel and blotted onto a nitrocellulose membrane. These were incubated with Brn-3b specific antibody (see sections 2.1.6 and 2.2.3 of materials and methods). In order to control for differences in protein loading, duplicate immunoblots were also incubated with an antibody specific for the invariant protein, β-actin.

As shown in Figure 3.2.2A, three of the selected Brn-3b over-expressing clones showed an increase in the short form of Brn-3b (33KDa) which was transfected into the cells but interestingly, there was also an increase in the expression of endogenous long form of Brn-3b (43KDa). This suggests an autoregulatory effect by which the short form of Brn-3b protein can induce the expression of the endogenous long form of Brn-3b. This effect has also been seen in studies using the breast cancer cell line, MCF7 (Dennis et al, 2001). Conversely, the anti-sense clones showed a reduction in the endogenous levels of both long and short forms (Fig. 3.2.2B) as expected since the anti-sense construct is derived from a region common to the mRNAs encoding both forms.

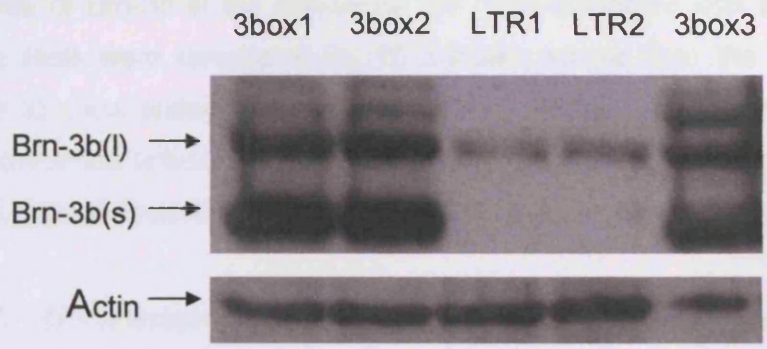


Figure 3.2.2A: Western blot analysis of selected clonal IMR32 cell lines to assess for any increase in Brn-3b protein levels compared to empty vector cells

Significant elevation in both the isoforms of Brn-3b (Brn-3b(l) and Brn-3b(s)) was observed in three over-expressing Brn-3b clones (3box1, 3box2, 3box3) compared with levels in the two empty vector controls (LTR1, LTR2). The blots were developed for only 30 seconds as longer exposure resulted in too strong a signal, making it difficult to discriminate bands in individual lanes. It is important to note that there was detection of Brn-3b(s) in LTR controls at longer exposure of 2 minutes (not shown in the blot here). Therefore, it appears that there is a higher level of endogenous Brn-3b(l) than Brn-3b(s) in LTR controls. The level of the invariant β- Actin protein in each cell line was used to control for variations in protein loading.

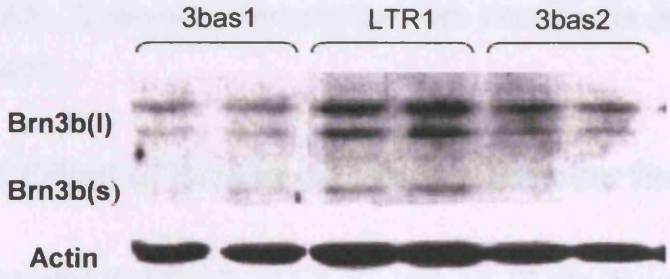


Figure 3.2.2B: Western blot analysis of selected clonal IMR32 cell lines to assess for any decrease in levels of Brn-3b protein compared to empty vector clones

Two anti-sense Brn-3b (3bas1, 3bas2) and one empty pLTR vector controls (LTR1) were tested for any decrease of Brn-3b protein in clonal cells transfected

with the anti-sense construct. There was a reduction of both, long and short, isoforms of Brn-3b in the anti-sense cell lines compared with the LTR control. These blots were developed for 10 minutes, longer than the over-expressing clones to show endogenous levels of Brn-3b in IMR-32 cells and compare any decrease in the anti-sense clones with these basal levels. The level of the invariant β-Actin protein in each cell line was used to control for variations in protein loading.

3.2.3 Characterisation of growth and behaviour under differentiating stimulus of stable transfectants with different levels of Brn-3b

Having successfully obtained clones with variable expression of Brn-3b protein compared to the control cells, the effects of changing Brn-3b levels on cellular proliferation, response to differentiating stimulus and invasive properties were analysed.

3.2.3.1 Effects of manipulating Brn-3b levels on cellular growth in monolayers

Uncontrolled growth of cells is the underlying cause of neoplasia which can be caused due to an aberrant regulation of cellular growth pathways, or defects in differentiation and/or apoptotsis. Since, Brn-3b is associated with proliferation of neuronal cells (see section 1.3.6.2.2. of chapter 1), we investigated the changes in growth of IMR-32 neuroblastoma cell lines with increased or reduced levels of Brn-3b expression.

3.2.3.1.1 Effect of Brn-3b on cell growth over time

Cells were plated at a density of 1 X 10^4 cells per ml into 24 well plates (Nunc) and the number of cells counted using a hemacytometer every 48 hours in triplicates for 26 days. As shown in Fig. 3.2.3.1.1, IMR32 cells which constitutively overexpress Brn-3b showed the most rapid doubling time as well as cellular density at plateau phase compared to the empty vector control cell lines. In contrast, two anti-sense cell lines with decreased Brn-3b showed slower growth

compared to the control LTR clones. Thus, the Brn-3b over-expressing clones 3box1 and 3box2 were the fastest growing, reaching average densities of 2.40 and 2.75 X 10^6 cells per cm^2 respectively, after 26 days (624 hours). In comparison, the control clones LTR1 and LTR2 reached a plateau at an average density of 0.70 and 0.75 X 10^6 cells per cm^2 after the same time period ($p < 0.05$ using Student's *t* test analysis). Moreover, statistically significant differences were observed as early as day 12 (288 hours), between cell numbers in both over-expressing 3box1 and 3box2 cells compared with LTR 1/2 ($p < 0.05$). In contrast, the anti-sense clones with decreased levels of Brn-3b, 3bas1 and 3bas2, reached a plateau at an average density of 0.55 and 0.53 X 10^6 cells per cm^2 after 26 days. The anti-sense clone, 3bas2, showed consistently slower growth compared with 3bas1, with significant differences compared with control cells observed by day 12 ($p = 0.02$ for LTR1 and 0.0001 for LTR2 compared with $p = 0.25$ for 3bas1 for LTR and 0.001 for LTR2). Consistent statistically significant differences in growth were observed for 3bas1 compared with controls by day 18-22 (432-528 hours) ($p = 0.009$ and 0.0007 for LTR1 and 2, respectively). These differences in the growth of the anti-sense clone, 3bas1 and 3bas2, may be due to differences in the resultant loss of Brn-3b protein levels using the anti-sense strategy, with 3bas1 exhibiting lower Brn-3b loss than 3bas2 clone.

Thus, changing the levels of Brn-3b protein significantly modify the growth properties of human neuroblastoma cell lines, with high levels of this protein giving rise to increased growth rate and lower levels having the opposite effects.

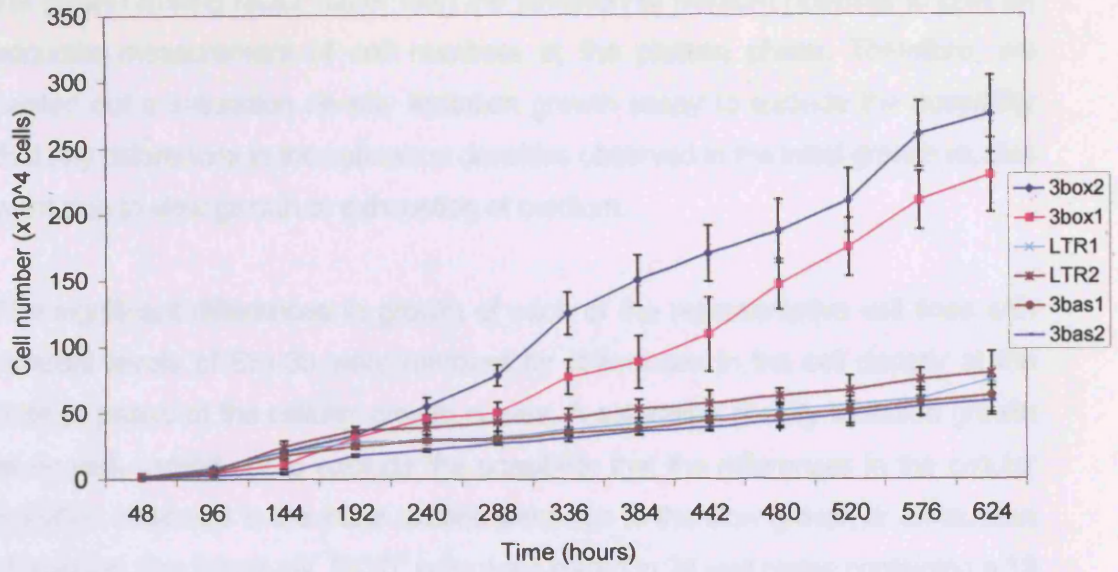


Figure 3.2.3.1.1: Cellular growth curves comparing the rate of growth of stably transfected clonal IMR32 cell lines with different levels of Brn-3b

Cells were plated out at a density of 1 X 10^4 cells per ml in 24-well plates and grown in full growth medium, and counted every 48 hours for a period of 26 days. Both the over-expressing clones (3box1, 3box2) showed significantly higher growth rate than the empty vector controls, LTR1 and LTR2, (p value < 0.05). In contrast, the two anti-sense clones (3bas1 and 3bas2) grew slower than the controls. In particular, the growth rate of 3bas2 was consistently and significantly lower than the two LTR controls (p value < 0.05 from day 12 {288 hours} for 3bas2 and day 18-22 {432-528 hours} for 3bas1). The number of cells shown at each time point represents the mean of three independent experiments (six wells for each clonal cell line per experiment) +/- the standard deviation.

3.2.3.1.2 Saturation density in cells with different levels of Brn-3b

Towards the end of the log phase of cellular growth, the culture becomes confluent as all the available surface area of the substrate is occupied and the cells are in contact with each other. It is a measurement of the cell concentration in the plateau phase that is referred to as the saturation density. Contact inhibition, depletion of growth factors and nutrients in the medium contribute towards diminished growth observed at this stage and hence, it is important to try and promote cell density as

the growth limiting factor rather than the limitation of medium nutrients to give an accurate measurement of cell numbers at the plateau phase. Therefore, we carried out a saturation density limitation growth assay to exclude the possibility that any differences in the saturation densities observed in the initial growth studies were due to slow growth or exhaustion of medium.

The significant differences in growth of each of the representative cell lines with different levels of Brn-3b were mirrored by differences in the cell density at the plateau phase of the cellular growth curves. A saturation density limitation growth study was carried out to exclude the possibility that the differences in the cellular densities observed in the initial studies were due to the slow growth or exhaustion of medium. For this study, $5X10^3$ cells were plated in 24 well plates containing a 13 mm coverslip in each well. After 24 hours, the coverslips were transferred to six well plates containing 4 mls of full growth medium. The medium was changed every 48 hours to ensure maximal growth conditions. After 5 days total cells in each well were harvested and counted at 48h intervals in triplicate for ten days.

As shown in Fig.3.2.3.1.2, similar to the effect seen in the growth curve, Brn-3b over-expressing cells continued to proliferate at higher densities compared with the control LTR cells with statistically significant differences between 3box1/ 2 and LTR1/2 ($p<0.05$). The anti-sense cells with decreased levels of Brn-3b grew at lower densities compared with controls, with 3bas2 showing a significantly greater growth reduction ($p<0.05$). Thus, the saturation densities for each clone were similar to their proliferation rate and at the plateau phase of growth curves. These results support our findings that over-expressing Brn-3b increases the cellular growth rate while decreasing its levels retards the proliferation of neuroblastoma cells.

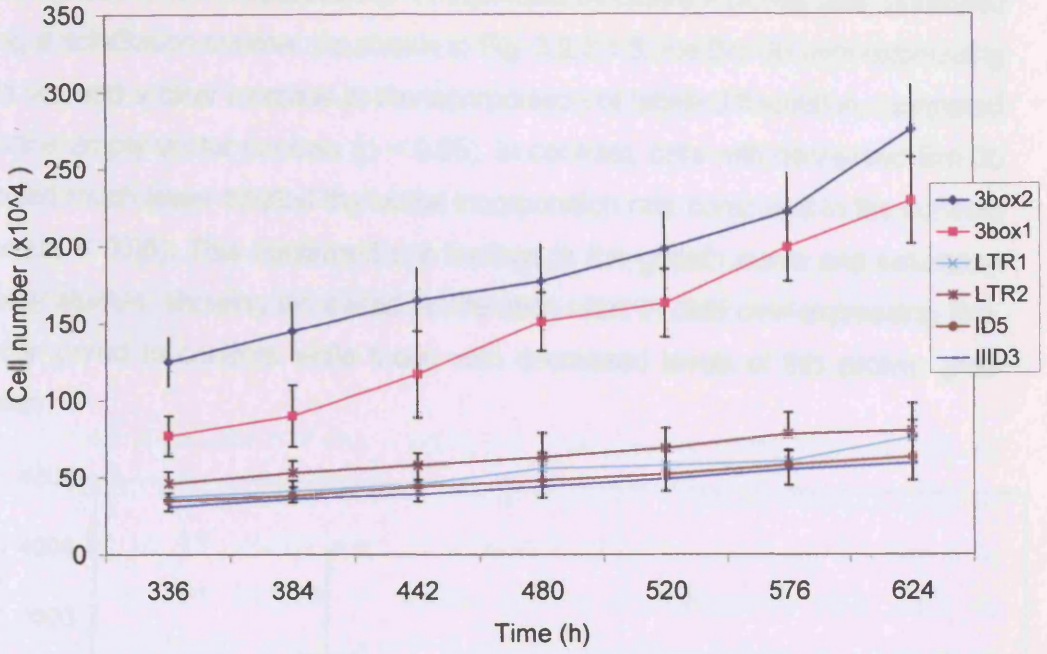


Figure 3.2.3.1.2: Saturation density of IMR32 cell lines with altered levels of Brn-3b

Cells were plated out on a coverslip in 24 well plates and transferred to a 6 well plate containing 4 ml of full growth medium. After 5 days total cells in each well were harvested and counted at 48 hour intervals for 10 days. The two over-expressing clones (3box1, 3box2) showed higher saturation density rate than the empty vector controls (LTR1, LTR2) and the two anti-sense clones, 3bas1 and 3bas2 ($p < 0.05$). The number of cells shown at each time point represents the mean of three independent experiments (six wells for each clonal cell line per experiment) +/- the standard deviation of the mean.

3.2.3.1.3 Tritiated thymidine incorporation of cells with different Brn-3b levels

To further investigate if the differences in the cell numbers of different clonal cell lines were due to changes in their rate of proliferation, we measured differences in the rate of DNA synthesis. For this study the tritium (^3H) labeled thymidine (^3H-thymidine) was incubated with cells and the amount incorporated by replicating cells was used as a measure of proliferative activity. Cells were incubated with

tritiated thymidine for 1h, then harvested and prepared as described in materials and methods. The incorporation of ^{3}H-thymidine by different clones was measured using a scintillation counter. As shown in Fig. 3.2.3.1.3, the Brn-3b over-expressing cells showed a clear increase in the incorporation of labelled thymidine, compared with the empty vector controls ($p < 0.05$). In contrast, cells with decreased Brn-3b showed much lower tritiated thymidine incorporation rate compared to the controls (p value < 0.05). This confirmed our findings in the growth curve and saturation density studies, showing increased proliferation rates in cells over-expressing Brn-3b compared to controls while those with decreased levels of this protein grew slower.

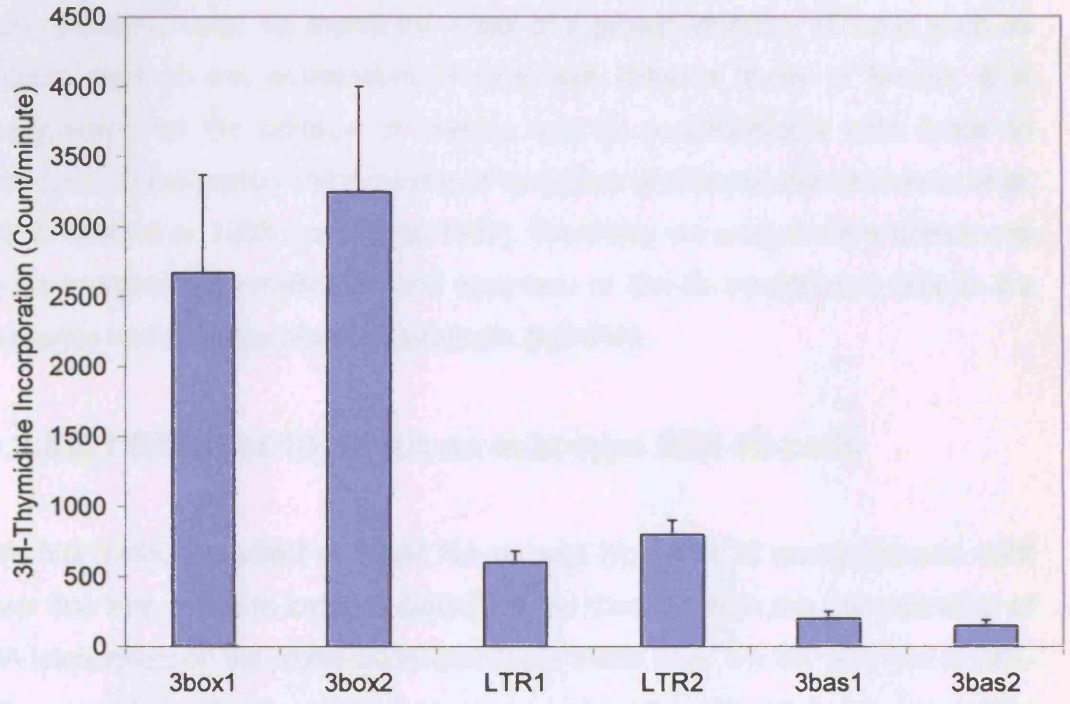


Figure 3.2.3.1.3: Tritiated thymidine incorporation by clonal IMR-32 cell lines with altered levels of Brn-3b

Cells were grown in full growth medium for 24 hours and pulsed with tritiated thymidine for 1 hour prior to harvesting. After the treatment, cells were trypsinised, harvested onto glass filters, and counts per minute from the glass filters were measured using a scintillation counter. The two Brn-3b over-expressing clones (3box1, 3box2) showed increased DNA synthesis, as reflected by the amount of ^{3}H-thymidine incorporation, when compared to the empty vector controls, LTR1 and LTR2 (p value < 0.05). The two anti-sense clones (3bas1 and 3bas2) showed

the slowest incorporation of the labelled thymidine with significant differences compared with the controls (p value < 0.05). The values shown represent the mean of three independent experiments (six wells for each clonal cell line per experiment) +/- the standard deviation. All statistical analyses were carried out using the student T-test.

3.2.3.2 Effect of altering Brn-3b levels on growth, proliferation and apoptosis in response to a growth inhibitor (all-trans-retinoic acid)

Since Brn-3b appeared to have a profound effect on the proliferation of IMR-32 neuroblastoma cells, we tested the effect of a growth inhibitory stimulus such as retinoic acid on the proliferation of cells with different levels of Brn-3b. It is recognised that the addition of retinoic acid to neuroblastoma cells leads to inhibition of proliferation and induction of apoptosis or differentiation (Haussler et al, 1983; Sidell et al, 1983; Lovat et al, 1997). Therefore, we analysed any differences in the morphology, proliferation and apoptosis of Brn-3b manipulated cells in the presence and absence of all-trans-retinoic acid (RA).

3.2.3.2.1 Effect of 10μM RA on wild-type IMR-32 cells

We first tested the effect of 10μM RA on wild type IMR-32 neuroblastoma cells over five time points in order to determine the time at which this concentration of RA takes effect on the proliferation/viability of these cells. 1 x 10^4 cells / ml of IMR-32 were plated out in 6-well Nunc plates and after 24 hours, 10μM RA was added to the full growth medium. Control cells were grown in parallel in the absence of RA. Cells were subsequently harvested at 6h, 12h, 24h, 48h and 72h, in triplicate for each time-point, and MTT assay carried out to measure changes in cell proliferation (see section 2.2.4.8 of chapter 2). In actively proliferating cells, mitochondrial succinic dehydrogenase metabolises the yellow tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide], to yield a purple formazan reaction product. Hence, this reaction can be quantified spectrophotometrically. Conversely, in cells that are undergoing apoptosis, MTT metabolism decreases, reflecting the loss of cell viability. Therefore, the results

arising from this assay can be interpreted as a measure of cellular proliferation, viability and/or cytotoxicity. For the purposes of the study described herein, the results obtained from this assay were used as a quantification of cellular proliferation and viability.

Figure 3.2.3.2.1 shows that the maximal effect of 10μM RA on proliferation and/or viability of IMR-32 cells takes place after 48h of RA addition, when a clear decrease is observed in the percentage of proliferating cells grown in RA compared with cells grown in the absence of RA. At 6, 12 and 24h post-RA treatment, there are no significant differences in the number of proliferating/surviving cells compared to the untreated control cells. Hence, subsequent studies to look at the effect of RA on IMR-32 cells were carried out at a time point after 48h of RA treatment, i.e. 72h, to observe any differences in cellular proliferation/viability of IMR-32 neuroblastoma cell line.

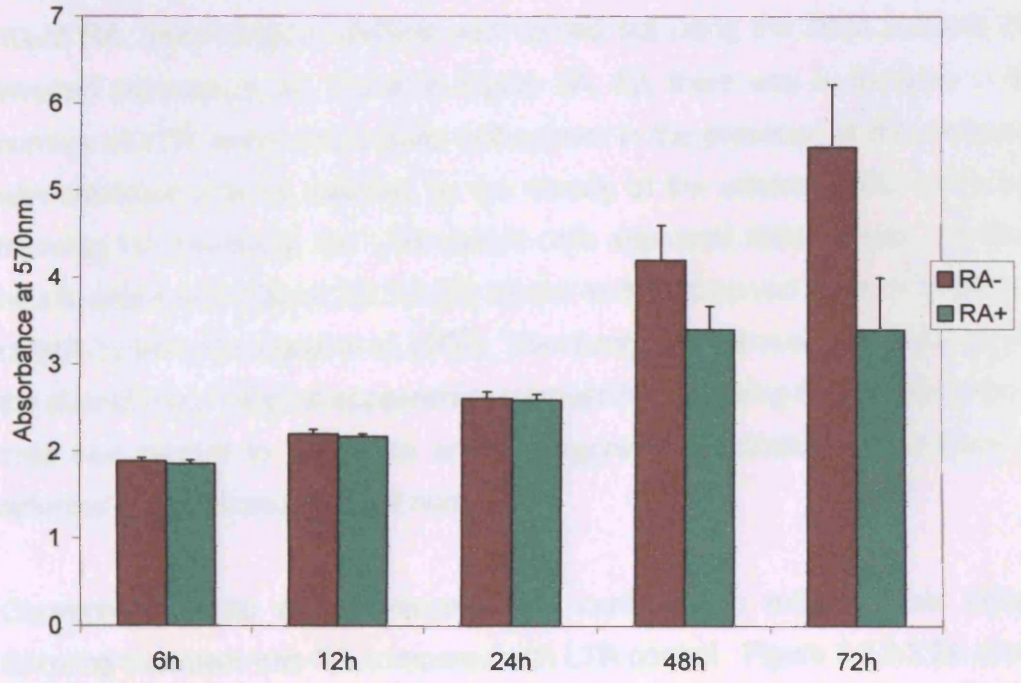


Figure 3.2.3.2.1: Optimal time-point for observing decrease in cell numbers in response to 10μM RA in wild-type IMR32

Wild-type IMR-32 were plated out and 10 μM RA added to the growth medium. Cells were harvested in triplicate at 6h, 12h, 24h, 48h and 72h after addition of RA and MTT assay carried out, with absorbance measured at 570 nm. Untreated

control cells grown in the absence of RA (red bars) were harvested in parallel at each time-point. The absorbance value is representative of the number of proliferating/viable cells. A significant decrease in the number of actively proliferating cells was detected at 48h post retinoic acid treatment. A greater decrease in the number of cells was observed at 72 hours of RA treatment compared to untreated cells. The absorbance at 570 nm shown at each time-point represents the mean of 3 independent experiments (six wells for each clonal cell line per experiment) +/- the standard deviation.

3.2.3.2.2 Morphology and growth in Brn-3b manipulated cells following 10μM RA treatment

To test whether the differentiating factor, retinoic acid (RA), affected the morphology and growth of Brn-3b manipulated IMR32 cells, cells with different levels of Brn-3b were plated and grown in full growth medium either with or without 10μM RA. Morphological analysis was carried out using the Ziess Axiovert 200 inverted microscope. As shown in Figure 8A, 8B, there was a decrease in the number of LTR vector only control cells grown in the presence of RA compared with untreated cells as reflected by the density of the adherent cells. Moreover, following RA treatment, the LTR control cells appeared flattened with extensive neurite processes (Figure 3.2.3.2.2B) similar to that observed upon differentiation of IMR-32 cells (Poongodi et al, 2002). The changes in cell numbers, together with the distinct morphological appearance, suggest that, following RA treatment, these cells had ceased to proliferate and undergone differentiation or apoptosis as reflected by the decrease in cell numbers.

Conversely, Brn-3b over-expressing cells continued to exhibit higher density following treatment with RA compared with LTR control. Figure 3.2.3.2.2A shows that Brn-3b over-expressing clones, 3box1 and 3box2, failed to show decrease in cell numbers upon RA treatment, with the numbers remaining similar to the untreated cells at 48 and 72 hours. Moreover, morphological analysis revealed that the RA treated Brn-3b over-expressing cells did not exhibit the flattened morphology and neurite processes, observed in the control cells, and remained similar to the untreated Brn-3b over-expressing cells. This suggests Brn-3b over-

expressing IMR-32 cells overcome the growth inhibition secondary to RA treatment.

As shown in Figure 3.2.3.2.2C, the antisense clones, 3bas1 and 3bas2, showed a decrease in cell number, as well as an extensive neurite outgrowth, in the presence of RA compared to the untreated control antisense clones. Interestingly, the morphological appearance of these antisense clones is distinct from the LTR control or over-expressing cells as they appear to have smaller cell body with distinctive branching patterns.

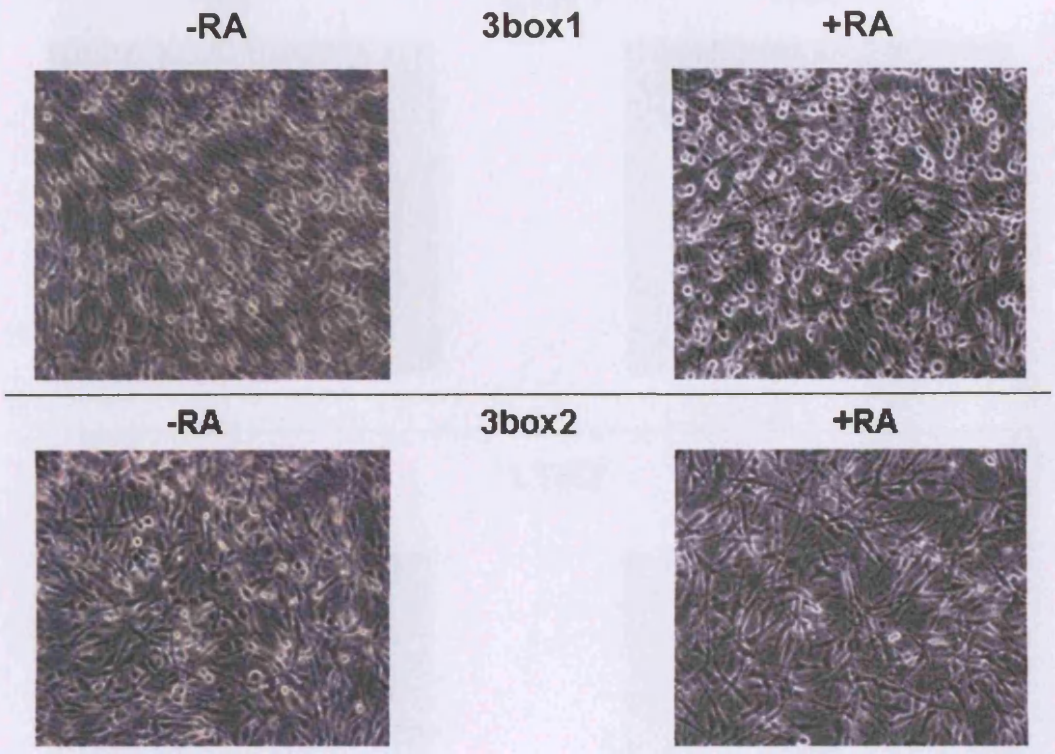


Figure 3.2.3.2.2A: Photomicrographs showing the effect of 10 μM retinoic acid on the growth and morphology of Brn-3b over-expressing clones

The Brn-3b over-expressing cells, 3box1 and 3box2, were plated in six-well plates in full growth medium either with or without RA for 72 hours. Morphological analysis was carried out 72 h post-treatment using a Ziess Axiovert 200 inverted microscope and photomicrographs taken at 10 X magnification. The left-hand panels represent untreated cells (-RA) while the right-hand panels are cells treated with 10μM RA (+RA). There appeared to be no significant difference in cell numbers or morphology between the treated and untreated cells of each clone. These photomicrographs represent one set of three independent experiments carried out.

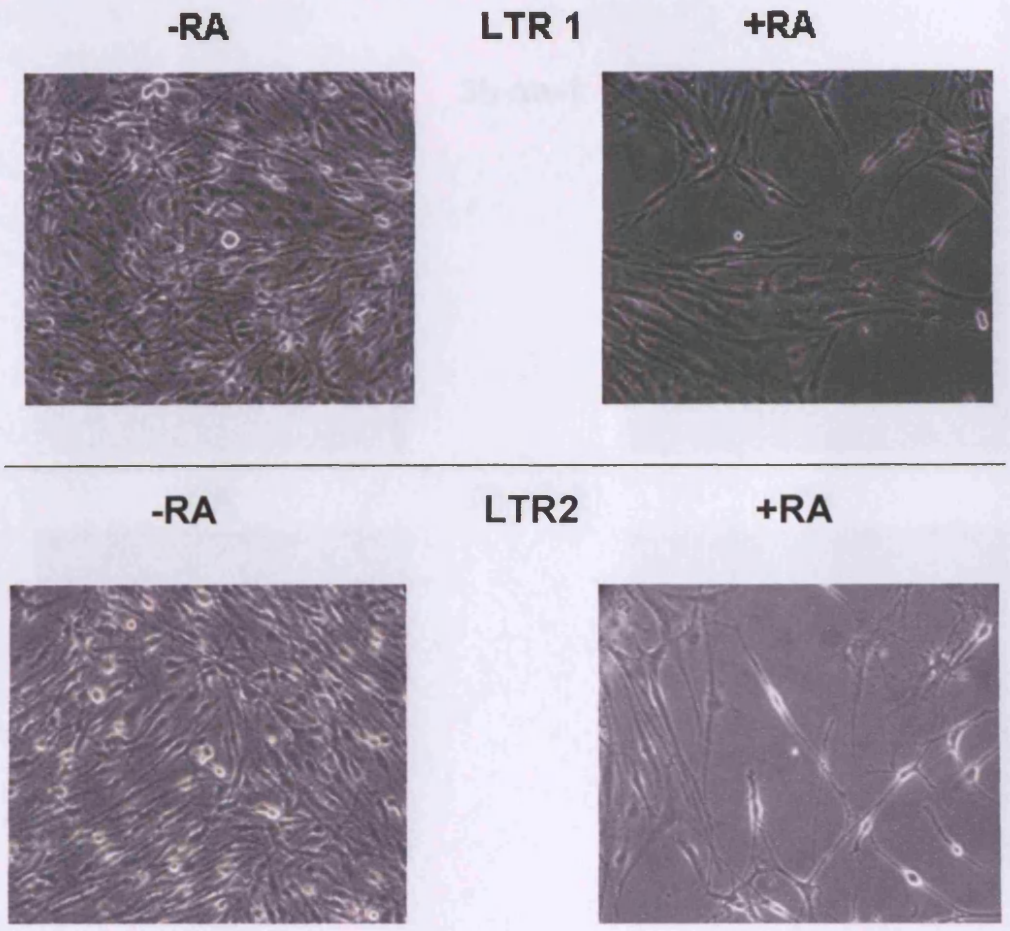


Figure 3.2.3.2.2B Photomicrographs showing the effect of retinoic acid on LTR control IMR-32 cells

The LTR vector only control (LTR1 and LTR2) was grown in full growth medium either in the presence or absence of RA. All left hand panels represent cells grown in the absence of RA (-RA) and all the right hand panels are photomicrographs of cells grown in the presence of 10μM RA (+RA) for 72h time-points. There are significant differences in the number of cells in the treated compared to the untreated cells of each clone at each time point. RA treated cells were lower in numbers and exhibited flattened morphology with neurite processing. These photomicrographs represent one set of three independent experiments carried out.

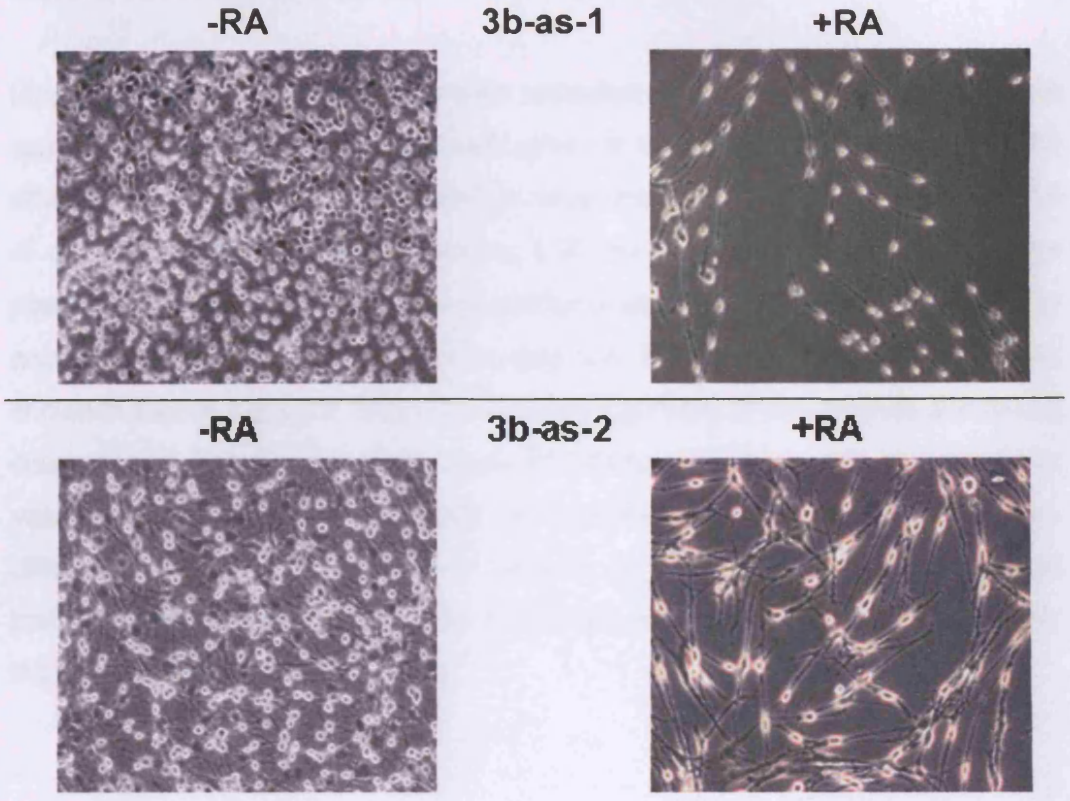


Figure 3.2.3.2.2C: Photomicrographs showing the effect of retinoic acid on anti-sense IMR-32 cells

The Brn-3b anti-sense cells, 3bas1 and 3bas2, were grown in full growth medium either in the presence or absence of RA. The cells grown in the absence of RA (-RA) or presence of 10μM RA (+RA) were photomicrographed at 10X magnification, 72 hours post-treatment. Cells treated with RA showed significant decrease in cell numbers and exhibited neurite processing which was absent in their untreated counterparts. These photomicrographs represent one set of three independent experiments carried out.

3.2.3.2.3 Effect of RA on growth and viability of cells with different levels of Brn-3b

Since RA did not result in the growth reduction of Brn-3b over-expressing cells compared to the untreated control cells grown in the absence of RA, we tested the effect of RA on cell survival/proliferation using the MTT assay (see section 2.2.4.8 of chapter 2). Brn-3b over-expressing, LTR controls and anti-sense cells were plated in triplicate and grown in the presence or absence of 10 μM of RA. The cells were harvested after 48 hours of plating and MTT metabolism measured. As shown in Figure 3.2.3.2.3, a significantly larger number of Brn-3b over-expressing cells were proliferating/viable, following RA treatment compared to control LTR (p value < 0.05 for 3box1and 3box2), while anti-sense cells did not exhibit any differences compared to controls (p value = 0.73 to 0.8). These results suggest that cells with high levels of Brn-3b continue to proliferate and/or survive even in the presence of RA.

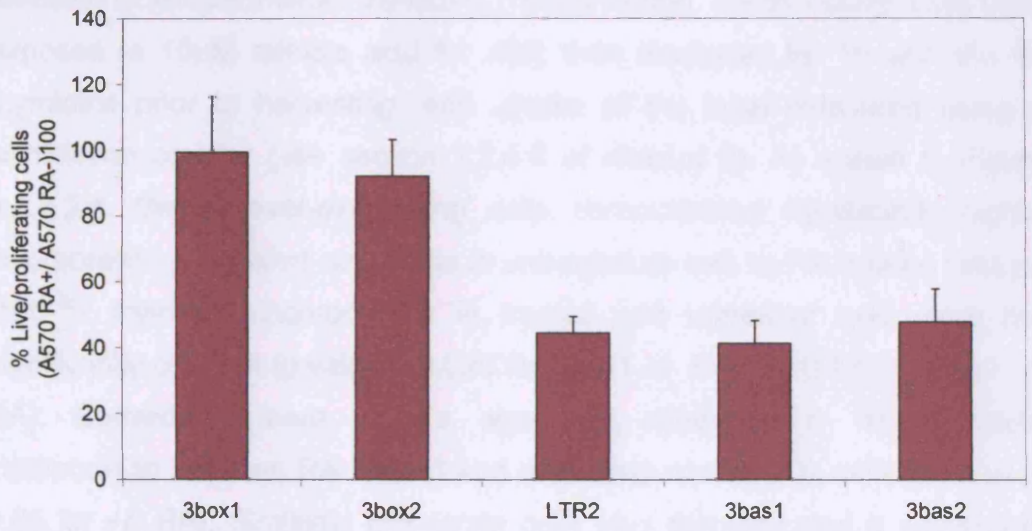


Figure 3.2.3.2.3: MTT assay to observe cell survival/proliferation of Brn-3b manipulated cells following treatment with 10μM RA

Cells were grown in the presence or absence of 10μM RA for 48h and subsequently assayed for conversion of yellow MTT salt to purple formazan crystals, which was measured spectrophotometrically at 570 nm wavelength. The data represents the ratio of conversion of MTT in treated (RA+) compared with corresponding untreated (RA-) controls for each clonal cell lines. Both Brn-3b over-

expressing clones, 3box1 and 3box2, had a higher percentage of proliferating/surviving cells expressed as a percentage of untreated cells compared with the LTR control (p value < 0.05). In contrast, the anti-sense clones, 3bas1 and 3bas2, showed no significant difference compared with control cells (p value = 0.73 and 0.8 for 3bas1 and 3bas2, respectively).

3.2.3.2.4 Effect of RA on Brn-3b mediated proliferation/ survival

The sustained increase in cell numbers seen in the MTT assay in the Brn-3b over-expressing cells in the presence of RA, compared to the control cells may either result from continued proliferation and/or reduction in apoptosis. To elucidate whether these differences seen in cells with higher levels of Brn-3b were due to increase in the rates of proliferation, we directly measured the rate of DNA synthesis in replication cells by quantifying the incorporation of labelled nucleotide tritiated thymidine (^{3}H-thymidine). For this study, cells were exposed to 10μM retinoic acid for 48h, then incubated for 1h with the ^{3}H thymidine prior to harvesting, and uptake of the label measured using a scintillation counter (see section 2.2.4.8 of chapter 2). As shown in Figure 3.2.3.2.4, Brn-3b over-expressing cells demonstrated significantly higher incorporation of labelled nucleotide in untreated as well as RA treated cells so that ^{3}H thymidine incorporation in treated and untreated cells were not significantly different (p value = 0.025 for 3box1 +/- RA and 0.28 for 3box2 +/- RA). Conversely, there was a significant difference in ^{3}H thymidine incorporation between RA treated and untreated control LTR cells (p value < 0.05 for +/- RA). Similarly, anti-sense cells also demonstrated a statistically significant difference in the incorporation of ^{3}H-thymidine between RA treated and untreated controls (p value < 0.05 for both 3bas1 and 3bas2 +/- RA). Thus, in agreement with the previous morphological and growth/viability analysis, these results suggest that both LTR control and anti-sense cells fail to proliferate in the presence of RA, while Brn-3b over-expressing cells continue to proliferate.

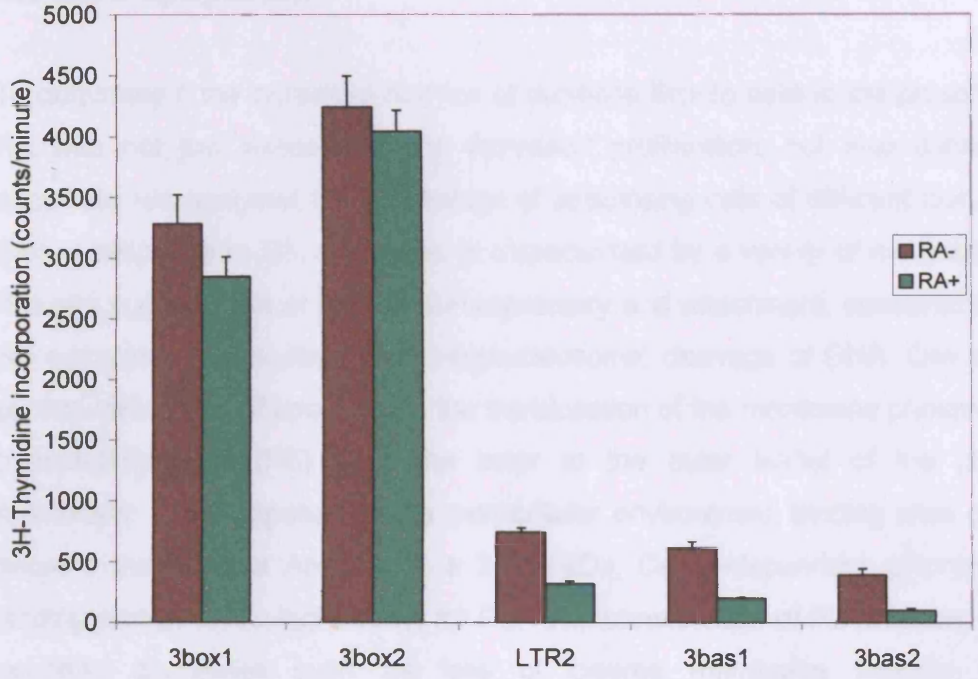


Figure 3.2.3.2.4: Effect of manipulating Brn-3b levels on the proliferation of neuroblastoma cells in response to retinoic acid of neuroblastoma cells

Each clonal cell line was grown in full growth medium with and without 10μM RA for 48 hours. Cells were treated with tritiated thymidine for 1 hour, trypsinised, harvested onto glass filters, and counts per minute from the glass filters were recorded by a scintillation counter. Cells from the two over-expressing clones (3box1 and-2) demonstrated continued proliferation even in the presence of RA (red boxes), as reflected by the amount of ^{3}H–thymidine incorporation. Proliferation rates between RA treated and untreated 3box1 and -2 cells did not differ (p value = 0.025 for 3box1 +/- RA and 0.28 for 3box2 +/- RA). In contrast, the control cells and anti-sense cells showed a significant decrease in proliferation in the presence of RA compared to their corresponding untreated control cells (p value < 0.05 for LTR2 +/- RA and for both 3bas1 and 3bas2 +/- RA). The values shown represent the mean of three independent experiments (twelve wells for each clonal cell line per experiment) +/- the standard deviation.

3.2.3.2.5 Effect of changing Brn-3b levels on retinoic acid induced apoptosis

To determine if the increased number of surviving Brn-3b cells in the presence of RA was not just associated with increased proliferation, but also decreased apoptosis, we analysed the percentage of apoptosing cells of different clonal cell lines in response to RA. Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, a 35-36 kDa, Ca 2+-dependent, phospholipid binding protein with a high affinity for PS. The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. As such, Annexin V conjugated to biotin or a fluorochrome such as FITC, PE, APC, Cy5, or Cy5.5, can be used for identification of cells in the early stages of apoptosis using flow cytometry.

Because PS translocation also occurs during secondary necrosis, Annexin V is not an absolute marker of apoptosis. Therefore, it is often used in conjunction with vital dyes such as 7-amino-actinomysin (7-AAD) or propidium iodide (PI), which bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis (Koopman et al, 1994). Hence, together Annexin V and PI staining can measure for early apoptosis, late apoptosis and necrosis in cells.

Cells from each clonal cell line with different levels of Brn-3b were plated in 6 well plates at a density of 1 X 10^6 cells / well and grown either in RA^+ or RA^- medium, and harvested 48 hours later for FITC-Conjugated Annexin V antibody and PI staining (described in section 2.2.4.11 of chapter 2). As shown in Fig 3.2.3.2.5, similar levels of apoptosis was observed in Brn-3b over-expressing cells compared with control or anti-sense cells. These results suggest that Brn-3b does not affect the survival of these cells and hence any increase in cell numbers observed in RA

treated Brn-3b over-expressing cell lines compared to controls were not necessarily due to increased survival in these cells. Together with the results discussed in section 3.2.2.1.D4, the increase in cell numbers in Brn-3b over-expressing cells is likely to be due to an increase in cellular proliferation and not due to decreased apoptosis. Similarly, the lower cell numbers observed in LTR controls and anti-sense clones in the presence of RA compared to their respective untreated counterparts, is likely to be due to a decrease in proliferation rather than increased apoptosis.

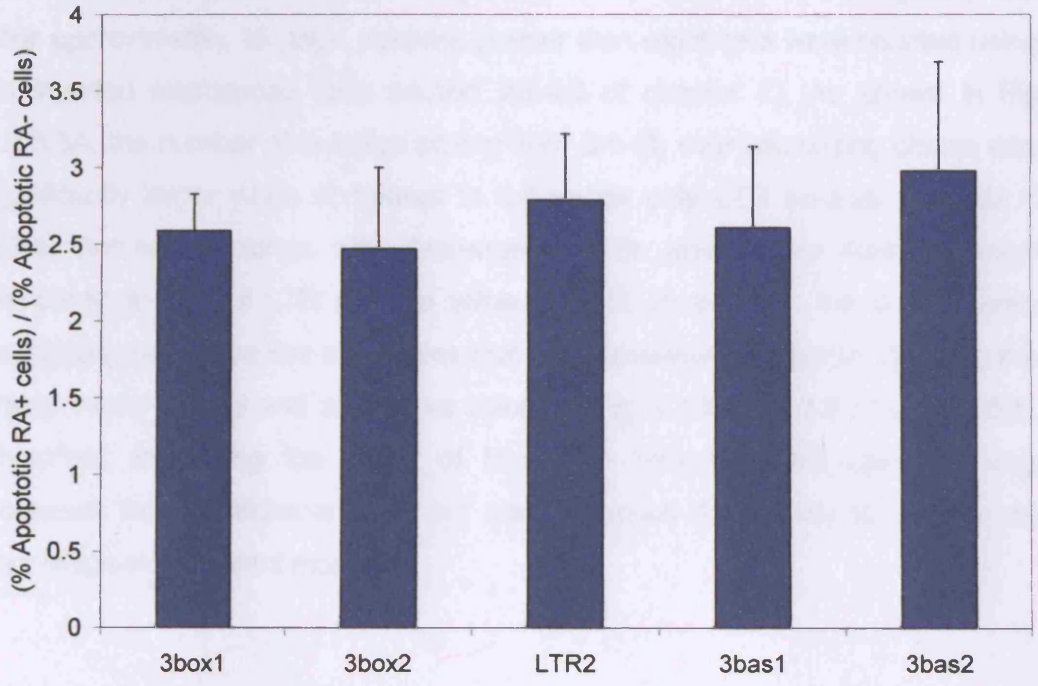


Figure 3.2.3.2.5: Effect of RA on apoptosis of Brn-3b manipulated cells

Clonal cell lines with different levels of Brn-3b were grown in full growth medium with and without 10μM RA for 48 hours. Cells were harvested and incubated with FITC-conjugated Annexin V antibody and stained with propidium iodide (PI), and analysed on a flow cytometer. The cells were dual labelled with FITC-Annexin V and PI to discriminate between apoptotic and necrotic cell populations. The data represents the percentage of Annexin V positive RA treated cells divided by the percentage of Annexin V positive untreated cells. There is no significant difference between the Brn-3b over-expressing (3box1, 3box2), empty vector control (LTR2) and anti-sense IMR-32 cells (3bas1, 3bas2). The data represents the mean of three independent experiments, +/- the standard deviation.

3.2.3.3 Effect of manipulating Brn-3b levels on cellular growth under anchorage independent conditions

The ability of cells to grow independently of anchorage is a hallmark of transformed cells and is an important property that allows tumour growth *in vivo*. We tested whether manipulating levels of Brn-3b could modify the ability to these neuroblastoma cells to grow in an anchorage independent manner, by forming colonies in soft agar. Thus, cells with different levels of Brn-3b were grown in full growth medium containing 0.35% agarose. These were observed regularly and after approximately 15 days, colonies greater than eight cells were counted using an inverted microscope (see section 2.2.4.9 of chapter 2). As shown in Fig 3.2.3.3A, the number of colonies arising from Brn-3b over-expressing clones was significantly larger when compared to the vector only LTR controls (p value < 0.05). Anti-sense clones with decreased Brn-3b levels grew fewer colonies compared to control LTR cells (p value < 0.05). In addition, the Brn-3b over-expressing cells gave rise to colonies that were consistently larger in size than the empty vector control and anti-sense colonies (Fig 3.2.3.3B, 3.2.3.3.C, 3.2.3.3D). Therefore, increasing the levels of Brn-3b in neuroblastoma cells not only increases their proliferative rates but also enhances their ability to grow in an anchorage-independent manner.

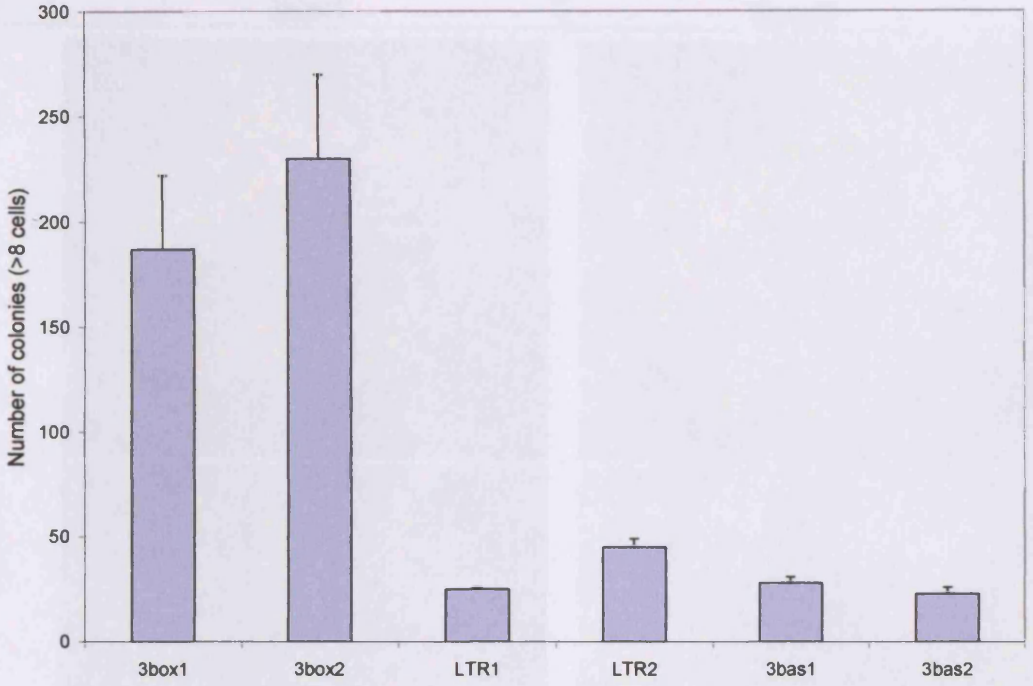
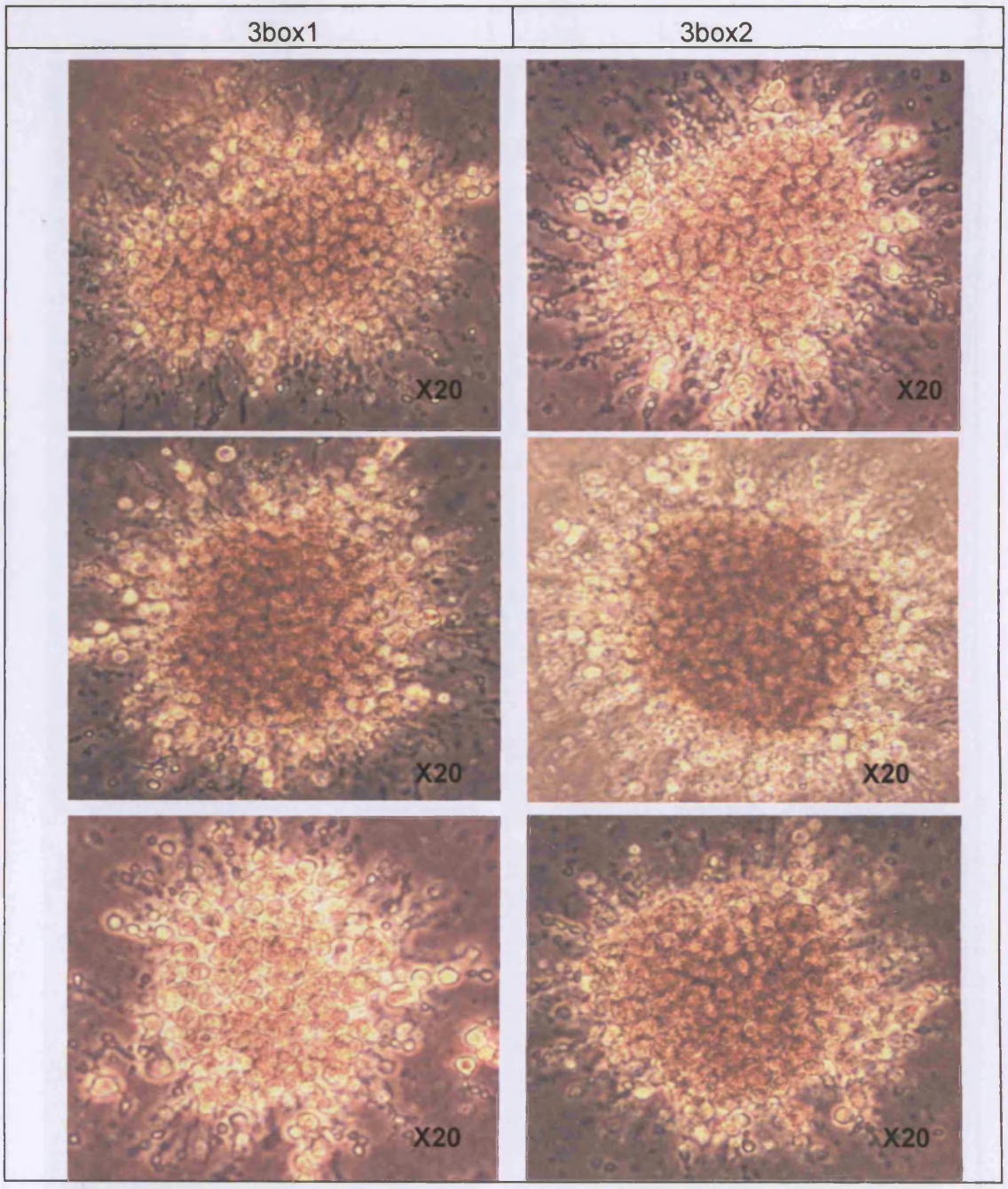


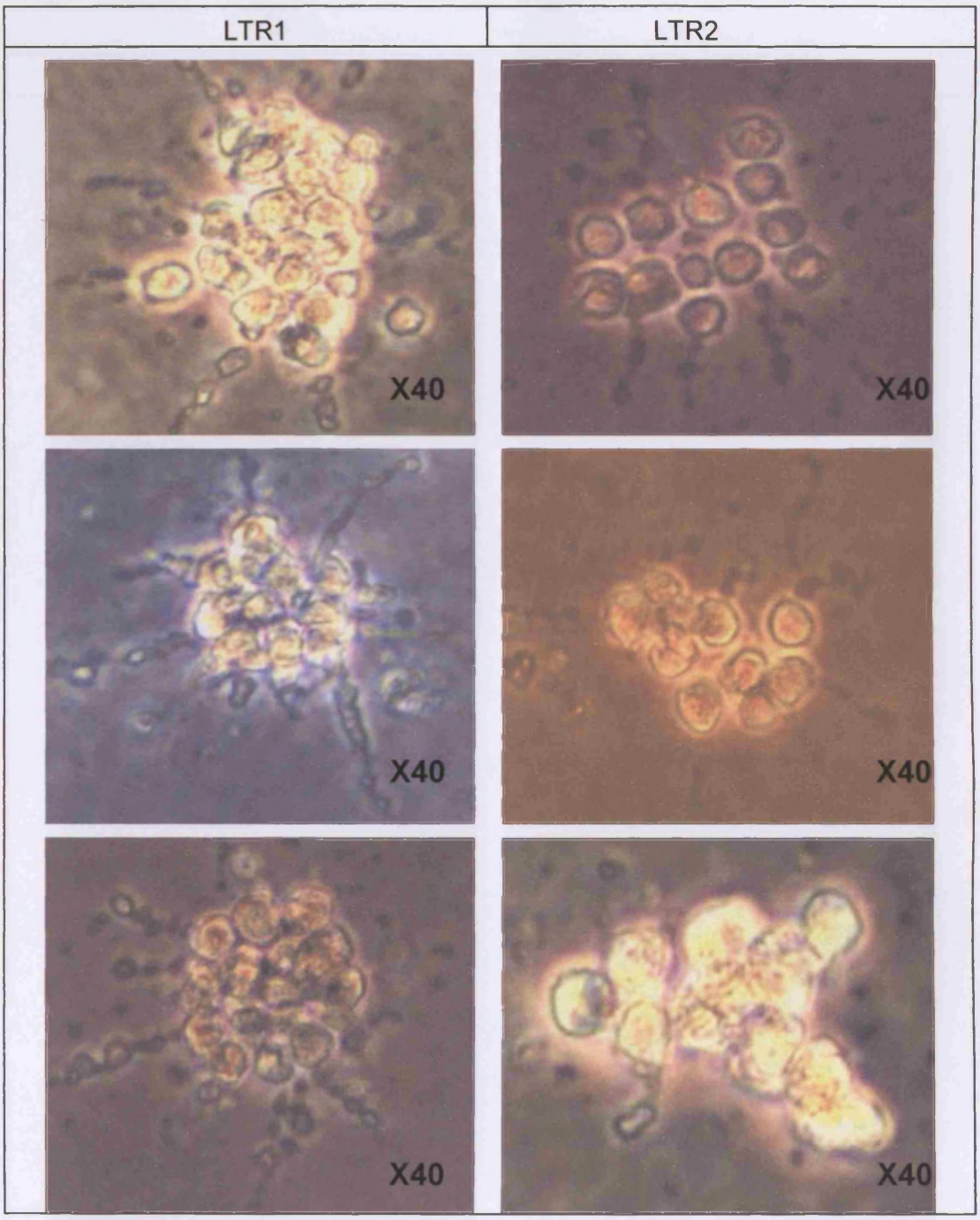
Figure 3.2.3.3A: Effect of manipulating Brn-3b levels on the ability of neuroblastoma cells to grow in soft agar under anchorage independent conditions

Cells were plated in full growth medium containing 0.3% low-melting-point agarose. After 15 days, colonies comprising of greater than 8 cells were counted. Brn-3b over-expressing clones 3box1 and 3box2 gave rise to greater number of colonies on soft agar compared with the vector only controls LTR1, LTR2 (p value < 0.05). In contrast, cells with decreased levels of Brn-3b, 3bas1 and 3bas2 grew fewer colonies compared with control cells (p value < 0.05). The number of colonies counted for each Brn-3b manipulated cell line represents the mean of three independent experiments (three wells for each clonal cell line per experiment) +/- the standard deviation.



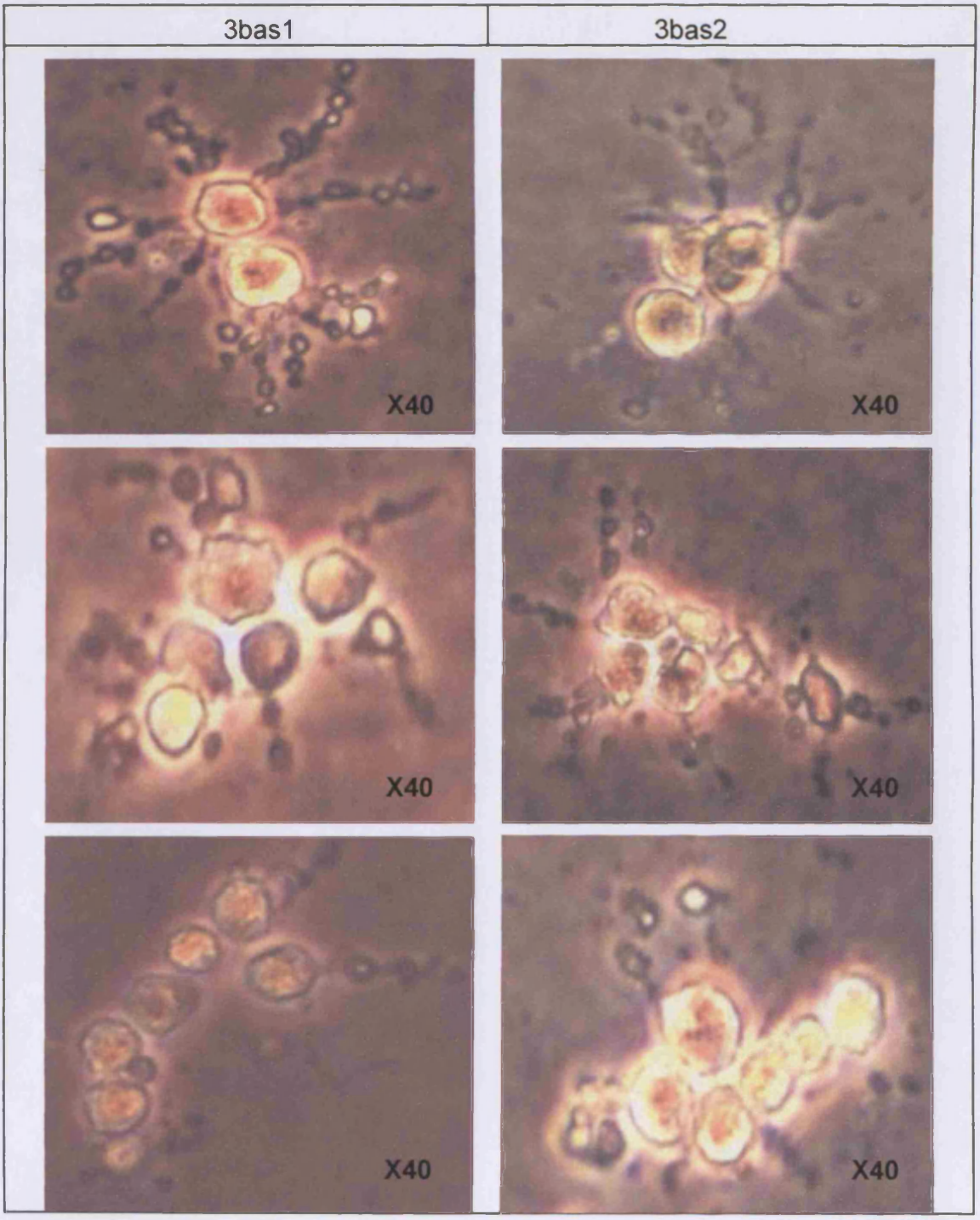
3.2.3.3B: Anchorage independent colonies on soft agar arising from cells with elevated levels of Brn-3b (3box1 and 3box2)

Single cell suspensions from each clonal cell line were grown in full growth medium containing 0.3% low melting point agarose. Cells were allowed to form colonies for 15 days. These representative photomicrographs were taken on day 15 post-plating using a Ziess Axiovert 200 inverted microscope at 20X magnification. Brn-3b over-expressing clones, 3box1 and 3box2, exhibited a greater ability to grow colonies in soft agar, forming much larger and denser colonies compared with the LTR controls or the anti-sense cells (see facing pages for Figure 13C and 13D).



3.2.3.3C: Anchorage independent colonies on soft agar arising from empty vector LTR control (LTR1 and 2)

Single cells were grown in full growth 0.3% agarose medium for 15 days. These representative photomicrographs were taken using a Ziess Axiovert 200 inverted microscope at 40X magnification. LTR cells gave rise to significantly fewer and smaller colonies compared with the Brn-3b over-expressing cells.



3.2.3.3D: Anchorage independent colonies on soft agar arising from IMR-32 cells with decreased Brn-3b levels (3bas1 and -2)

Single cells from each clonal cell line were grown in full growth 0.3% agarose medium for 15 days. These representative photomicrographs were taken using a Ziess Axiovert 200 inverted microscope at 40X magnification. The anti-sense clones, 3bas1 and 3bas2, gave rise to the smallest and the least number of colonies on soft agar compared with Brn-3b over-expressing cells and the LTR control cell lines.

3.2.3.4 Effect of Brn-3b on invasive potential of neuroblastoma cells

Invasion and metastatic growth can profoundly affect the course and outcome of neuroblastoma tumours. Preliminary results of studies using breast cancer cells in our laboratory suggested a role for Brn-3b in regulating expression of genes associated with invasion and / or metastasis (Dennis J et al, unpublished data). We, therefore, investigated whether changing Brn-3b expression could modify the invasive potential of IMR-32 neuroblastoma cells. Wildtype IMR-32 cells have been shown to be relatively invasive (Zaizen et al, 1998). We further compared the ability of cells with elevated (3box1 and 3box2) or lower Brn-3b (3bas1 and 3bas2) levels to migrate through an artificial Matrigel Basement Membrane (BD Biocoat Matrigel^{TM}) in the presence of a serum gradient with wildtype or control LTR vector cells.

We analysed cells migrating due to a serum gradient through the matrix of a BD Biocoat Matrigel^{TM} to the lower surface of the membrane after 12 hours. Following plating of 5 X 10^4 cells of each clonal cell line in 0.1% serum in the upper chamber, the cells that had migrated to the lower chamber containing medium with 10% serum were stained and counted (see section 2.2.4.12 of chapter 2). As shown in figure 3.2.3.4, there was a clear and significant increase in the number of migrating cells from both Brn-3b over-expressing clones, 3box1 and 3box2, compared with the wild-type IMR-32 control cells or the vector LTR control clones (p value < 0.05). Interestingly, the number of anti-sense cells, particularly 3b-as2, migrating in this assay was slightly lower than the wild-type IMR-32 or LTR control cells (p value for 3bas1 = 0.02 and 0.01 and p value for 3bas2 = 0.001 and 0.01 with LTR and wild-type IMR-32, respectively), indicating that the loss of Brn-3b may decrease the invasiveness of these cells. These results suggest that Brn-3b affects the ability of neuroblastoma cells to migrate through a matrix, hence affecting the invasive properties of these cells, implicating a role for this transcription factor in pathways associated with tumour progression.

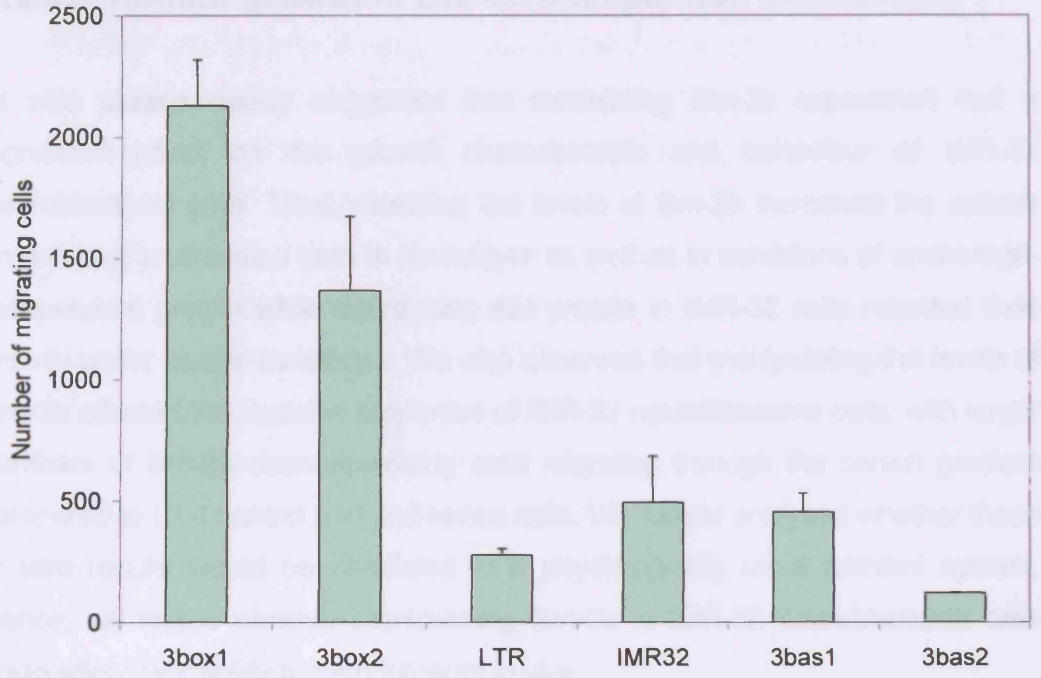


Figure 3.2.3.4: Invasion assay using the BD Bioquote Matrigel™ chamber to test the effect of manipulating Brn-3b expression on migration of neuroblastoma cells
Cells were plated in medium containing 0.1% foetal calf serum (FCS) in the upper chamber of a BD Biocoat Matrigel™ plate and allowed to migrate to the lower chamber containing medium with 10% serum for 12 hours. Cells in the lower chamber were stained and counted. Cells in the upper chamber were also counted and used to determine total cell numbers at the end of the 12 hour incubation period, which appeared to remain the same as at the time of plating for each clone (data not shown). Both clones over-expressing Brn-3b (3box1 and 2) showed increased invasiveness with significantly larger number of cells migrating through the membrane compared with the wildtype IMR-32 cells, LTR control (p value<0.05). Lower number of anti-sense cells of clone 3bas2 migrated through the matrix compared with LTR and wild-type IMR-32 controls (p value = 0.02 to 0.001). Anti-sense clone, 3bas1, did not show any significantly different migratory ability compared to the controls ($p > 0.05$). These results were obtained from four independent counts.

3.2.3.5 Tumour growth of Brn-3b manipulated cells *in vivo*

In vitro studies clearly suggested that modulating Brn-3b expression had a significant effect on the growth characteristics and behaviour of IMR-32 neuroblastoma cells. Thus, elevating the levels of Brn-3b increased the cellular growth and proliferation both in monolayer as well as in conditions of anchorage-independent growth while decreasing this protein in IMR-32 cells retarded their growth under similar conditions. We also observed that manipulating the levels of Brn-3b affected the invasive properties of IMR-32 neuroblastoma cells, with larger numbers of Brn-3b over-expressing cells migrating through the serum gradient compared to LTR control and anti-sense cells. We further analysed whether these *in vitro* results would be paralleled in a physiologically more relevant system. Hence, we tested whether manipulating Brn-3b in IMR-32 neuroblastoma cells could affect their ability to form tumours *in vivo*.

3.2.3.5.1 Tumour growth in athymic nude mice

For initial studies, we utilised xenograft mice models, in which 1 X 10^6 cells of each clone were injected subcutaneously into the flanks of athymic nude mice. Sets of six mice were used for each clone. The mice were observed everyday for signs of ill health as well as changes in the volume of the tumour. When the tumours reached a maximum size of 1 cm^3, the experiment was terminated, keeping in compliance with UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia. The injection and monitoring of mice was kindly carried out by Dr. Barbara Pedley.

As shown in Fig. 3.2.3.5.1, mice injected with cells over-expressing Brn-3b had detectable tumour mass at earlier time points of days 7 and 11 respectively, compared with the LTR control clones, in which tumours were detected by days 15 and 17 respectively. The Brn-3b over-expressing clones grew faster than control cells with significant differences seen by day 8 after injection ($p<0.05$). Tumours arising from 3box2 clones reached volumes of 0.9-1 cm^3 by 10 days and by 14 days for 3box1 cells. The rate of growth of the Brn-3b over-expressing cells, once

established, was rapid in both sets of experiments. Interestingly, in this study all mice injected with Brn-3b over-expressing clones showed that the rapid increase in tumour volume was associated with evidence of invasion into the skin and surrounding tissue at the site of the tumour upon reaching a volume of 0.9-1 cm^3, so these groups had to be terminated by days 10 - 14. In comparison, mice injected with the LTR control cells had tumours reaching similar sizes by 18 to 24 days for LTR1 and 2 respectively. Moreover, unlike the tissue invasion seen with tumours resulting from the over-expressing cells, the control cells and anti-sense cells resulted in more discrete tumours with less tissue invasion. Tumour arising from the anti-sense cells, in particular 3bas2, grew more slowly than tumours arising from control cells and were much smaller after 24 days with an average volume of 0.5 cm^3 for 3bas2 and 0.8 cm^3 for 3bas1. As with the *in vitro* studies on growth, LTR2 cells grew more slowly and were the only other surviving control group remaining with sets of animals injected with anti-sense cells at 24 days. At this stage, while the tumour arising from the anti-sense cells remained small, this LTR2 control group resulted in tumours with mean volume of 1.2 cm^3, which was statistically significant compared with the anti-sense cells ($p<0.05$).

These results paralleled the findings of *in vitro* studies, suggesting that manipulating the levels of Brn-3b in IMR-32 neuroblastoma cells affected their growth rate with cells expressing elevated levels of Brn-3b promoting tumour formation *in vivo* while reduction of Brn-3b retarded tumour growth.

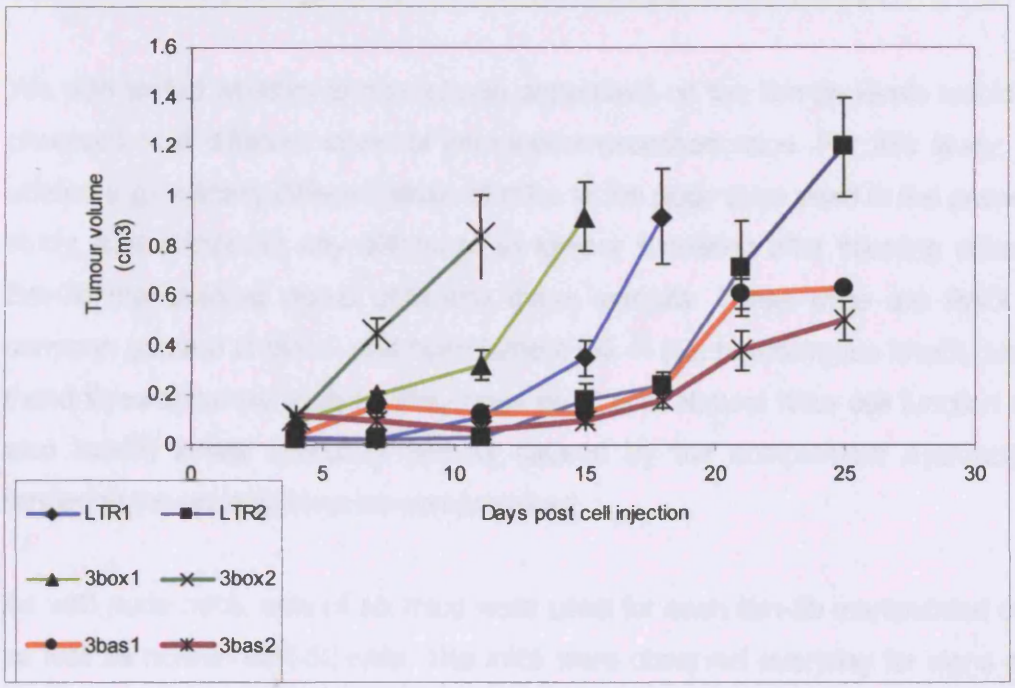


Fig. 3.2.3.5.1 Tumour growth in athymic nude mice injected with Brn-3b manipulated IMR-32 cells

Comparison of in vivo tumour growth resulting from cells with different levels of Brn-3b used as xenograft in nude mice models. Groups consisted of six nude mice for each clonal cell line. 1 X 10^6 cells from each clone were injected subcutaneously into the flank of the animal. Mice were observed daily for signs of ill-health and tumour volume measured following the appearance of a tumour mass. Cells with elevated Brn-3b (3box1 and 2) grew tumours more rapidly compared with control cells, LTR1 and 2 so that significant differences were observed by day 8 post injection (p value = 0.04). The Brn-3b over-expressing tumours resulted in tissue necrosis around the site of tumour growth so that these groups of mice had to be sacrificed at much earlier time points. In contrast, tumours arising from the anti-sense cell lines with lower Brn-3b, 3bas1 and 3bas2, resulted in smaller, slower growing tumours compared with LTR controls. As seen in *in vitro* studies, anti-sense clone, 3bas2, grew slower than 3bas1, reflecting differences in the levels of Brn-3b in these different clones (*p* value for 3bas2 = 0.009 by day 15 compared with the *p* value for 3bas1, day 21 = 0.005). This study was carried out in three independent experiments.

3.2.3.5.2 Tumour growth in immunosuppressed mice

We also tested whether similar effects dependent on the Brn-3b levels would be observed in a different strain of immunocompromised mice. For this study, we utilised a genetically different strain of mice to the nude mice used in the previous study, and compared any difference in tumour formation after injecting different Brn-3b manipulated clonal cells into these animals. These mice are RAG -/-, common gamma chain -/- and complement C5 -/- (i.e. homozygous knock-out for these three separate alleles). They have no T, B or Natural Killer cell function and also exhibit innate immunity defects caused by the complement dysfunction, rendering the animals immuno-compromised.

As with nude mice, sets of six mice were used for each Brn-3b manipulated cells as well as normal IMR-32 cells. The mice were observed everyday for signs of ill health as well as changes in the volume of the tumour. In this experiment, the tumours were allowed to grow to a maximum of 2 cm^3 size before terminating the experiment, keeping in compliance with UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia. The injection and monitoring of mice was kindly carried out by Dr. John Anderson.

As shown in Fig. 3.2.3.5.2, mice injected with cells over-expressing Brn-3b (3box1 and 3box2) showed the fastest tumour growth compared to the controls ($p<0.05$), with 3box2 reaching 20 mm^3 within 18 days post-injection and 3box1 reaching a maximum of 15 mm^3 during the same period. In sharp contrast, mice injected with the empty vector control cells and normal IMR-32 cells had barely detectable tumour size after 18 days, and only reached a maximum size of 12.5 mm^3 and 6 mm^3 after 21 days of injection. Moreover, there was a complete failure of the anti-sense cells, 3bas2, to grow over a period of 21 days while 3bas1 cells also showed retarded growth, reaching a maximum tumour size of only 2.5 mm^3 at 21 days. The difference in the growth of antisense cells was statistically significant compared with the control cells ($p<0.05$). Hence, these results suggest that over-expression of Brn-3b enhances tumour growth *in vivo*, while reducing the levels of Brn-3b retards tumour growth.

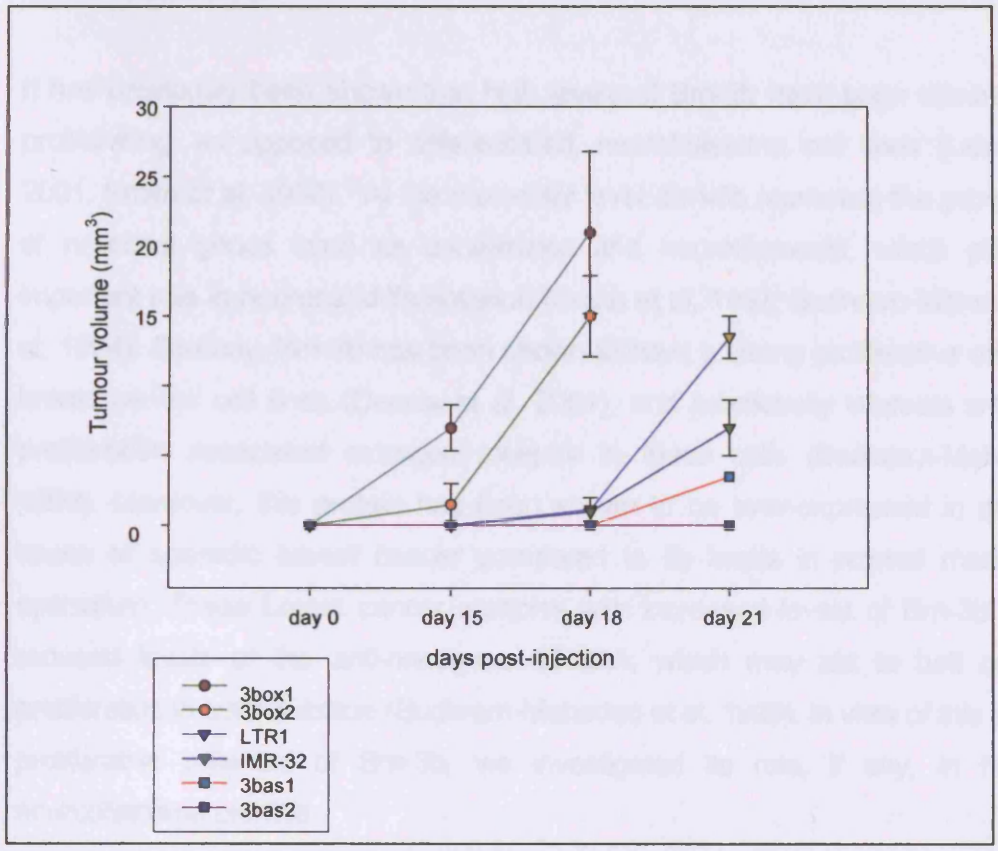


Fig. 3.2.3.5.2: Tumour growth in immunocompromised mice injected with IMR-32 cells with altered levels of Brn-3b

1 X 10^6 cells of each clone with different levels of Brn-3b were injected subcutaneously into the flank of immunocompromised mice. Groups consisted of six immunocompromised mice for each clonal cell line. Mice were observed daily for signs of ill-health and following the appearance of a tumour mass, tumour volumes were measured. Cells with elevated levels of Brn-3b (3b1 and 3b2) gave rise to tumours more rapidly compared with the control cells, LTR1 and 2 (p value < 0.05). In contrast, antisense cells either failed to give rise to tumours (3bas2) or resulted in smaller, slower growing tumours compared to the LTR controls (p value < 0.05).

3.3 Discussion

It has previously been shown that high levels of Brn-3b have been observed in proliferating, as opposed to differentiated, neuroblastoma cell lines (Latchman 2001, Smith et al, 1998). At the molecular level, Brn-3b represses the promoters of neuronal genes such as α-internexin and neurofilaments, which play an important role in neuronal differentiation (Smith et al, 1997; Budhram-Mahadeo et al, 1994). Similarly, Brn-3b has been shown to have a strong proliferative effect in breast cancer cell lines (Dennis et al, 2001), and functionally interacts with the proliferation associated estrogen receptor in these cells (Budhram-Mahadeo, 1998). Moreover, this protein has been shown to be over-expressed in several cases of sporadic breast cancer compared to its levels in normal mammary epithelium. These breast cancer samples with increased levels of Brn-3b show reduced levels of the anti-oncogene BRCA1, which may act to halt cellular proliferation in normal tissue (Budhram-Mahadeo et al, 1999). In view of this strong proliferative potential of Brn-3b, we investigated its role, if any, in human neuroblastoma cell line.

In this chapter the cellular growth, proliferation and invasive characteristics of human neuroblastoma cells with altered levels of Brn-3b is reported. The establishment and confirmation of cell lines which either stably over-expressed the exogenous Brn-3b short or decrease the endogenous levels of both long and short forms of this protein has been confirmed by Western immunoblotting. Over-expression of the short form of Brn-3b in IMR-32 cells was accompanied by an increase in the endogenous longer isoform of this protein. This is suggestive of a possible auto-regulatory mechanism where the short form of the protein causes an increase in the long isoform.

Transfection of the anti-sense construct caused a decrease but not total loss of Brn-3b protein. Antisense strategies are valuable tools to inhibit the expression of a target gene in a sequence-specific manner. The general principle of antisense methodology is based on DNA or RNA oligonucleotides binding a complementary

sequence within a specific mRNA and thereby preventing translation into protein. At present three types of anti-sense mRNA strategies are in use.
(1) Introduction of a DNA-antisense oligonucleotide into the cell, where it binds to its complementary target mRNA and either creates a substrate for endogenous RNase H, leading to directed cleavage of the mRNA (Dash et al, 1987, Dagle et al, 1991) or possibly the oligonucleotide:RNA duplex inhibits translation by steric blockade of the ribosome (Fire 1999). However, the effect of use of oligonucleotides in preventing protein production is only transient.

(2) Use of mRNA-cleaving ribozymes strategy is based on mutating the 5' four – base nucleotide sequence in order to bind complementary sequences on the target mRNA to be cleaved (Doudna and Cech, 2002). The disadvantages of this method is because the specificity is based on only four bases, mismatches are not uncommon (Colman, 1990) and the catalytic activity of ribozymes varies on different target sequences (Cantor et al, 1996).

(3) In the last two years, after the onset of the study described in this thesis, the discovery of RNA interference (RNAi) has further improved the antisense field of research. RNA interference is initiated by long double stranded- RNA molecules, which are processed into 21-23 nucleotide long RNAs, which act as small interfering RNAs (siRNA). They are then incorporated into the RNA-induced silencing complex, a protein RNA complex, and guide a nuclease, which degrades the target RNA (Yu et al, 2002).

In order to achieve efficient methods for gene silencing that are less transient than oligonucleotides treatments, more specific than ribozymes, expression vectors encoding antisense DNA which acts as a template for the synthesis of antisense-mRNA in vivo are used (Colman, 1990). In the work described here, we utilised an eukaryotic expression vector encoding antisense cDNA for 300 bases of Brn-3b short. These 300 nucleotides of Brn-3b short do not cross-hybridise with the other POU domain members, Brn-3a or Brn-3c mRNA. A pJ4 construct construct expressing the first 300 bases in a reverse orientation under the control of MoMuLV promoter was cotransfected in a 10-fold excess with a pCi Neo construct into IMR-32 cells using the Lipofectin®/peptide method of transfection as

described in chapter 2. Selection was carried out in the presence of an antibiotic, Geneticin-G418 sulphate. Antibiotic resistance clones were further propagated and analysed for any decrease in endogenous Brn-3b protein levels in IMR-32.

The results in this chapter have clearly shown that manipulating the levels of Brn-3b in IMR-32 cells had profound effects on the cellular growth, proliferation and invasive characteristics of these cells. Over-expression of Brn-3b levels resulted in increased growth rate in monolayer of neuroblastoma cells, reflected in increased proliferation, saturation densities, and tritiated thymidine incorporation compared to the empty vector control cells. This finding was further strengthened by the decrease in growth rates observed in cells with reduced levels of this protein. Additionally, elevating levels of Brn-3b protein in neuroblastoma cells also significantly enhanced growth under anchorage independent conditions, an essential requirement for tumour cells to gain autonomy and progress. As expected, cells with lower levels of Brn-3b protein, inhibited growth of colonies on soft agar under anchorage independent conditions.

Altering the levels of Brn-3b also affects the invasive ability of these human neuroblastoma cells. A significantly higher number of invading cells are observed from clones with elevated Brn-3b levels while decreasing Brn-3b reduces the invasiveness of IMR-32 cells. This suggests that high levels of Brn-3b may profoundly affect not just the growth but also invasion and progression of neuroblastoma cells.

Another hallmark of tumour cells is their ability to override growth inhibitory or differentiating signals. Our results described herein have shown that cells with enhanced Brn-3b levels exhibited continued proliferation even in the presence of growth inhibitory all-trans retinoic acid (RA). Moreover, IMR-32 cells with decreased levels of Brn-3b proteins as well as the empty vector control cells showed significantly reduced proliferation when grown in the presence of RA. The anti-sense cells with lower levels of Brn-3b exhibited flattened morphology with distinct branching patterns. These morphological differences in IMR-32 cells with altered levels of Brn-3b, and previous studies showing its ability to negate the effect of a differentiation associated protein, Brn-3a, on the expression of some

neuronal structural genes (Smith et al, 1997; Budhram-Mahadeo et al, 1994), suggests a possible indirect involvement of Brn-3b in a complex structural mechanism.

In order to ascertain whether the enhanced growth rate of neuroblastoma cells with high levels of Brn-3b observed in *in vitro* experiments could be mirrored at an *in vivo* level, we used two different xenograft models in which cells over-expressing Brn-3b as well as those with decreased levels of this protein and LTR controls were injected. The aim was to observe any differences in tumour growth rate and size. In keeping with the *in vitro* growth and proliferation studies, Brn-3b over-expressing cells grew tumours of significant size in these mice models in a shorter time period than control cells. Further evidence supporting the role of Brn-3b in proliferation and growth of neuroblastoma tumours emerged with the observation that IMR-32 cells with decreased levels of Brn-3b resulted in slower rate of tumour growth and much smaller size in *in vivo* mice models. Moreover, not only was the size of the tumours arising from Brn-3b over-expressing cells significantly larger than the controls but there was tissue invasion, not observed in mice injected with vector only controls or anti-sense cells. Similar results were obtained from immunocompromised mice in which Brn-3b over-expressing cells injected grew larger tumours in a shorter period of time compared to the controls. However, mice injected with Brn-3b antisense cells showed a much lower growth compared to the over-expressing cells and control cells, with one clone failing to grow into a tumour of considerable size. In fact, the anti-sense cells injected into immunocomromised cells grew to a smaller size compared to same cells injected into nude mice. This is possibly due to the difference in the genetic background of the mice models.

It is clear that the effects of manipulating the levels of Brn-3b in IMR-32 cells gives rise to significant effects on proliferation, growth and behaviour of human neuroblastoma cells both *in vivo* and *in vitro*. It is, however, necessary to elucidate the pathways and transcriptional mechanisms affected by altering Brn-3b levels in neuroblastoma tumours. Chapter 5 investigates the role of Brn-3b transcription factor in the progression of the cells through the cell cycle and its effect on an important cell cycle regulatory protein, cyclin D1.

CHAPTER 4

Results 2

4.0 Growth and apoptosis in IMR-32 cells with altered levels of Brn-3a

4.1 Introduction

The POU domain transcription factor, Brn-3a, is expressed in the developing and adult nervous system (Gerrero et al, 1993; Lillycrop et al, 1992; He et al, 1989; Turner et al, 1994; Ninkina et al, 1993). Brn-3a expression defines the earliest post-mitotic neurons to appear in the central nervous system whereas in the peripheral nervous system, Brn-3a expression is initiated just before the neurons exit the cell cycle (Fedtsova et al, 1995; 1996). Brn-3a is also expressed in the neural crest derived cells (Greenwood et al, 1999), which are associated with neuroblastoma formation (Brodeur and Castleberry, 1993).

Brn-3a is associated with differentiation and survival of sensory neurons. The critical role for Brn-3a *in vivo* is paralleled by its ability to activate transcription from various promoters of neuronally expressed genes such as the neurofilaments (Smith et al, 1997), the related neuronal intermediate filament α-internexin (Budhram-Mahadeo et al, 1995), the synaptic vesicle proteins, involved in the process of neurite outgrowth, SNAP-25 and synaptophysin (Lakin et al, 1995; Morris et al, 1997; Smith et al, 1997). It also activates the anti-apoptotic protein Bcl-2 (Smith et al, 1998), while down-regulating the pro-apoptotic Bax (Budhram-Mahadeo et al, 2002). Two isoforms of the Brn-3a, generated from alternative promoter usage, confer two distinct functions of this protein. Whereas the long isoform of Brn-3a (Brn3al) is required for survival, both the long and the short (Brn3as) isoforms are associated with differentiation.

Over-expression of Brn-3a in ND7 mouse neuroblastoma cell line stimulates neurite outgrowth under normal growth conditions, whereas reduction in the levels of Brn-3a via anti-sense strategy results in inhibition of process formation in these cells (Smith et al, 1997c). Moreover, upon differentiation of ND7 cells by serum removal, endogenous Brn-3a levels are elevated compared to actively proliferating cells with low Brn-3a levels (Budhram-Mahadeo et al, 1994). Similar changes in Brn-3a levels, in differentiating versus proliferating cells, are observed in human

neuroblastoma cells such as IMR-32, SKN-MC and SK-N-SH (Smith and Latchman, 1996). Hence, *in vitro* studies using various neuroblastoma cell lines have highlighted a potential role for Brn-3a in the transition from proliferating neuroblasts to quiescent differentiated sensory neurons, thus suggesting Brn-3a to be a factor associated with differentiated neuronal cells.

Brn-3a is well documented to be involved in cell survival in neuronal cell lines and primary sensory culture, providing an explanation for the profound wave of neuronal apoptosis observed in Brn-3a null mutant mice (McEvilly et al, 1996; Xiang et al, 1996; Huang et al, 1999). For example, over-expression of Brn-3a in mouse neuronal cell line, ND7 cells and primary cultures of trigeminal and dorsal root ganglia protects these cells from apoptotic stimuli such as the withdrawal of nerve growth factor from the medium (Smith et al, 1998b; Ensor et al, 2001). In agreement with this survival role for Brn-3a, ND7, trigeminal and dorsal root ganglia with decreased Brn-3a expression, achieved via anti-sense strategies, exhibited enhanced death (Ensor et al, 2001; Smith et al, 1998b). In addition, Brn-3a over-expressing cells have been shown to express high levels of Bcl-2 (Smith et al, 1998a; Ensor et al, 2001).

An important phenomenon observed in lower stage neuroblastoma tumours in infants is the frequent spontaneous regression in which the tumour cells become a non-malignant ganglioneuroma (Brodeur 2003). The molecular mechanisms leading to regression are ill-defined and it is reasonable to postulate that alterations in the expression of genes that regulate apoptosis and/or differentiation may underlie the spontaneous conversion of a neuroblast to a non-malignant ganglioneuroma. High expression of Brn-3a in differentiating neuroblastoma cells compared to proliferating cells has led us to hypothesise that aberrant expression of this gene might result in dysfunctional differentiation pathway, and hence lead to continued proliferation of cells that is observed in neuroblastomas.

4.2 Results

In this study, we tested whether altering the expression of Brn-3a would change the growth characteristics of human neuroblastoma cells, IMR-32. As with Brn-3b (discussed in Chapter 3), stable cell lines were generated in which Brn3a(l) protein was constitutively over-expressed. We utilised the IMR-32 cell line for these studies because, similar to ND7 cells, the levels of Brn-3a(l) are low in these cells when they are actively proliferating but are elevated upon differentiation of cells (Smith et al, 1996).

4.2.1 Establishment and confirmation of stable cell lines over-expressing longer Brn-3a(l) isoform

Proliferating IMR-32 cells were transfected with the expression construct containing the cDNA encoding the mouse Brn3a(l), or antisense construct to decrease Brn-3a expression, under the control of Moloney Murine Leukemia Virus (MoMuLV) promoter using the Lipofectin/peptide method of transfection (see section 2.2.4.4 of chapter 2). Simultaneously, control cell lines, expressing the empty expression vector pLTR, were also generated in IMR-32 cells. All tranfections include pCiNeo expression constructs, thus allowing selection of neomycin resistant transfected cells in the presence of G418 antibiotic. A number of independent G418-resistant Brn-3a or LTR (vector-only control) transfected clones were selected and grown up for evaluation for any changes in the levels of Brn-3a protein by Western immunoblotting as described in materials and methods. In brief, 25 µg of total cellular protein from each clone was separated on a SDS/10%/Polyacrylamide gel, blotted onto a nitrocellulose membrane, and incubated with Brn-3a specific antibody. The membrane was then stripped in 200mM NaOH and re-incubated with an antibody specific for the invariant protein, β-Actin, thus allowing any differences in protein loading to be detected.

When these stable cells were being generated, the cells that were transfected with Brn-3a(l) gave rise to colonies with different characteristics. While some cells proliferated to give rise to large colonies that could be propagated further, others failed to proliferate and subsequently, died in culture. As such of the twenty Brn-3a

overexpressing clones that were obtained, only four survived and could be grown and three of these were further characterised. Strikingly, the G418-resistant colonies arising from cells transfected with Brn-3a by anti-sense plasmid grew to form small colonies but died soon after and, therefore, could not be propagated for any further studies.

As shown in Figure 4.2, four of the selected Brn-3a(l) over-expressing clones showed expression of the exogenous mouse Brn-3a(l) protein (43KDa) but this band was absent in pLTR control cell lines.

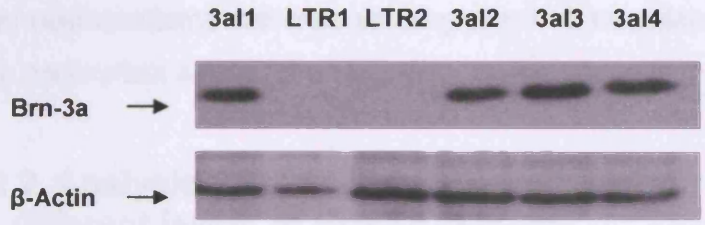


Figure 4.2: Western blot analysis of selected clonal IMR-32 cell lines to determine increase in the levels of Brn-3a over-expressing clones compared to cells transfected with the empty vector

High levels of exogenous mouse Brn-3a(l) protein was observed in four of the selected clones (3al1, 3al2, 3al3, 3al4) compared with no exogenous expression of this protein in two empty vector control cell lines (LTR1 and LTR2). The antibody used is specific for mouse Brn-3a and does not cross react with endogenous human Brn-3a protein. Differences in protein loading were determined by analysing the levels of the invariant β-actin protein in each cell line.

4.2.2 Characterisation of stable transfectants with increased levels of Brn-3a

Having successfully obtained clones that over-expressed Brn-3a protein compared to the control cells, we carried out an analysis of the changes on cellular growth, proliferation and invasive properties of these cells.

4.2.2.1 Effects on cell growth in monolayers

As Brn-3a is involved in events associated with cellular differentiation, we investigated whether increasing Brn-3a changes the growth characteristics of human neuroblastoma cell lines when grown in a monolayer using the standard growth parameters as described below.

4.2.2.2 Analysis of growth and proliferation of IMR-32 cells with different levels of Brn-3a protein

Three clonal cell lines constitutively over-expressing Brn3al and two empty vector LTR controls were studied to determine effects on cellular growth. For the growth curve study, cells were plated at a density of 1 X 10^4 cells per ml into 24 well-plates (Nunc) and the total number of cells counted on a haemocytometer at 48 hour intervals for 26 days. As shown in Fig. 4.2.2.2A, all three over-expressing Brn-3a cells grew at a similar rate to the empty vector control cell lines and exhibited similar cellular densities at the plateau phase. Thus, Brn-3a over-expressing clones 3al1, 3al2 and 3al3, reached average densities of 0.63, 0.69 and 0.65 X 10^6 cells/cm^2 respectively, after 26 days. Similarly, the control clones LTR1, LTR2 and LTR3 reached a plateau at an average density of 0.67, 0.72 and 0.68 X10^6 cells per cm^2 after 26 days of plating. On the basis of these findings it appears that clones with high Brn-3a did not exhibit different growth rates compared to controls.

In order to evaluate whether the similar growth rates observed between Brn-3a over-expressing clones and LTR controls were associated with the actual proliferation rather than any other parameters such as cell death or differentiation, not excluded in the previous analysis of gross cellular growth, we measured the

rate of DNA synthesis in different clones. To this end, the cells were incubated with tritium (^3H) labeled nucleotide thymidine (^3H-thymidine) for 1 hour prior to being harvested. The incorporation rate was taken as a measure of proliferative activity and measured by a scintillation counter as described in materials and methods. As shown in Fig. 4.2.2.2B, the Brn-3a over-expressing cells showed a similar rate of incorporation of ^3H-thymidine as the empty vector controls. This experiment further confirmed our findings in the analysis of growth rate that increasing Brn-3a levels in these cells did not significantly alter cellular proliferation compared to controls. It is important to note that these analyses were carried out in parallel to growth experiments on Brn-3b manipulated clones (discussed in results chapter 3) where significant differences were observed compared to controls, showing that experimental set up was reliable to determine any possible differences.

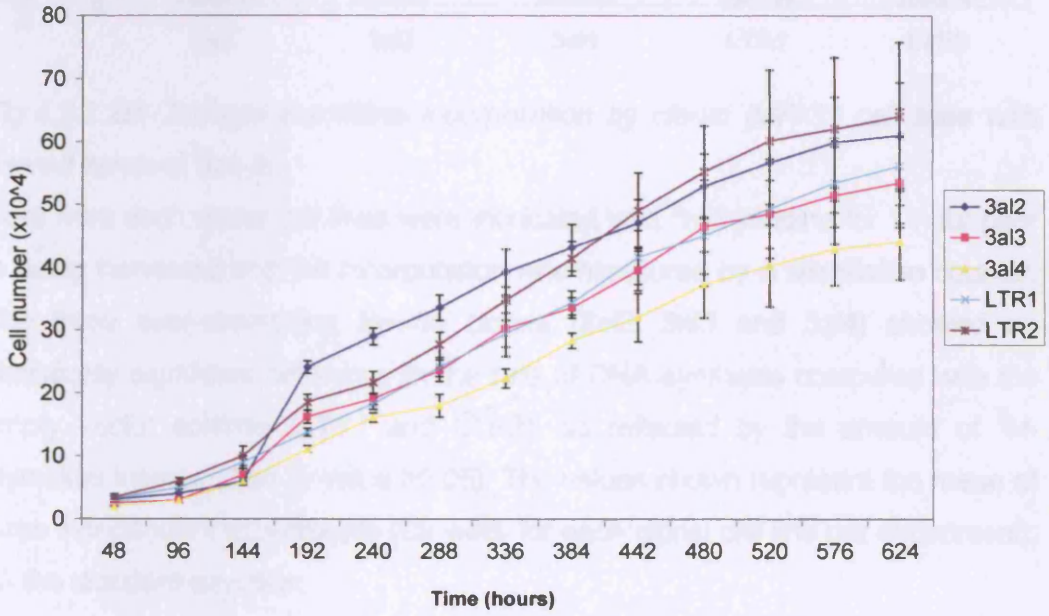


Fig.4.2.2.2A: Growth curves of clonal IMR-32 cell lines with increased levels of Brn-3a protein

Each clonal cell line was grown in full growth medium and counted every 48 hours for 26 days. Analysis of the total number of cells in three Brn-3a over-expressing clones (3al2, 3al3 and 3al4) compared with two empty vector controls, LTR1 and LTR2, showed no statistically significant differences in growth rate (*p* value >0.05). The number of cells at each time point represents the mean of three independent experiments (six wells for each clonal cell line per experiment), +/- the standard deviation.

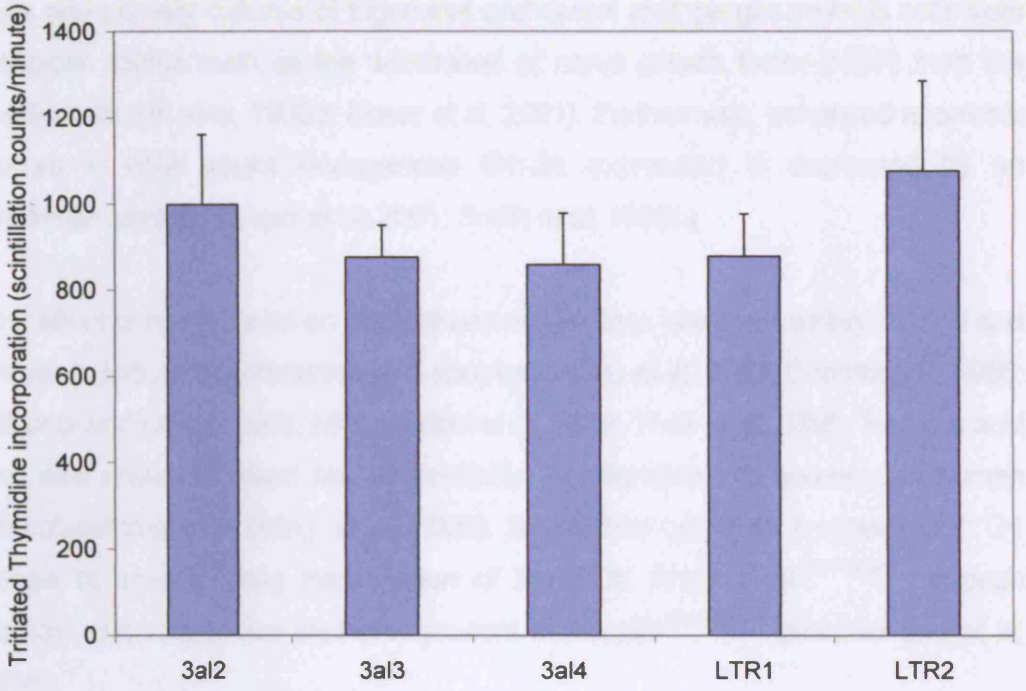


Fig.4.2.2.2B: Tritiated thymidine incorporation by clonal IMR-32 cell lines with altered levels of Brn-3a

Cells from each clonal cell lines were incubated with ^{3}H-thymidine for 1 hour prior to being harvested and the incorporation rate measured by a scintillation counter. The three over-expressing Brn-3a clones (3al2, 3al3 and 3al4) showed no statistically significant difference in the rate of DNA synthesis compared with the empty vector controls (LTR1 and LTR2), as reflected by the amount of ^{3}H-thymidine incorporation (p value >0.05). The values shown represent the mean of three independent experiments (six wells for each clonal cell line per experiment), +/- the standard deviation.

4.2.2.3 Effect on growth, proliferation and apoptosis of neuroblastoma cells in response to a growth inhibitory stimulus, 10 µM all-trans-retinoic acid, RA

Although Brn-3a did not appear to reduce growth of these neuroblastoma cells, we next assessed whether increased Brn3a(l) could modify the survival and differentiation of IMR-32 cells. This is because previous *in vivo* and *in vitro* studies showed that elevated Brn-3a increases survival as well as differentiation of neuronal cells. Artificial over-expression of Brn-3a in mouse neuroblastoma ND7

cells and primary cultures of trigeminal and dorsal root ganglia protects cells from apoptotic stimuli such as the withdrawal of nerve growth factor (NGF) from the medium (Smith et al, 1998b; Ensor et al, 2001). Furthermore, enhanced apoptosis occurs in cells where endogenous Brn-3a expression is decreased by an antisense strategy (Ensor et al, 2001; Smith et al, 1998b).

The effect of retinoic acid on neuroblastoma cell lines has been widely studied and shown to induce differentiation and apoptosis (Chu et al, 2003; Chambon P, 1996; Minucci and Ozato, 1996; Mangelsdorf et al, 1992; Theil et al, 1988). Retinoic acid has also shown to affect the differentiation, proliferation and adhesion of human neuroblastoma cells (Voigt et al, 2000). RA inhibits cell cycle progression in G1 phase by up-regulating transcription of the CDK inhibitor $p21^{CIP1/WAF1}$ through retinoic acid responsive elements present in the $p21^{CIP1/WAF1}$ promoter (Liu et al, 1996).

4.2.2.3.1 Measurement of growth, proliferation and survival on Brn-3a over-expressing cells by MTT assay, ^{3}H-thymidine incorporation and Annexin V labelling

The effect of retinoic acid on cellular growth, viability and survival of different Brn-3a over-expressing cells was analysed using the MTT assay, ^{3}H-thymidine incorporation and Annexin V labelling as described in materials and methods. For these assays, cells were plated in triplicate for each clonal cell line and grown either in the presence or absence of RA (10 μM, the optimal concentration shown to have effect on IMR-32 – discussed in results chapter 3). Similarly, controls were set up in parallel for each clonal cell line.

For MTT assays, the cells were harvested at 24h and 48h intervals after plating and MTT conversion measured by spectophotometry. To determine changes upon treatment with RA the value for cells treated with RA were expressed as a percentage of untreated. As shown in Fig 4.2.2.3.1A, there was no significant difference in the percentage of Brn-3a over-expressing cells proliferating/surviving following ATRA treatment compared to control LTR cells.

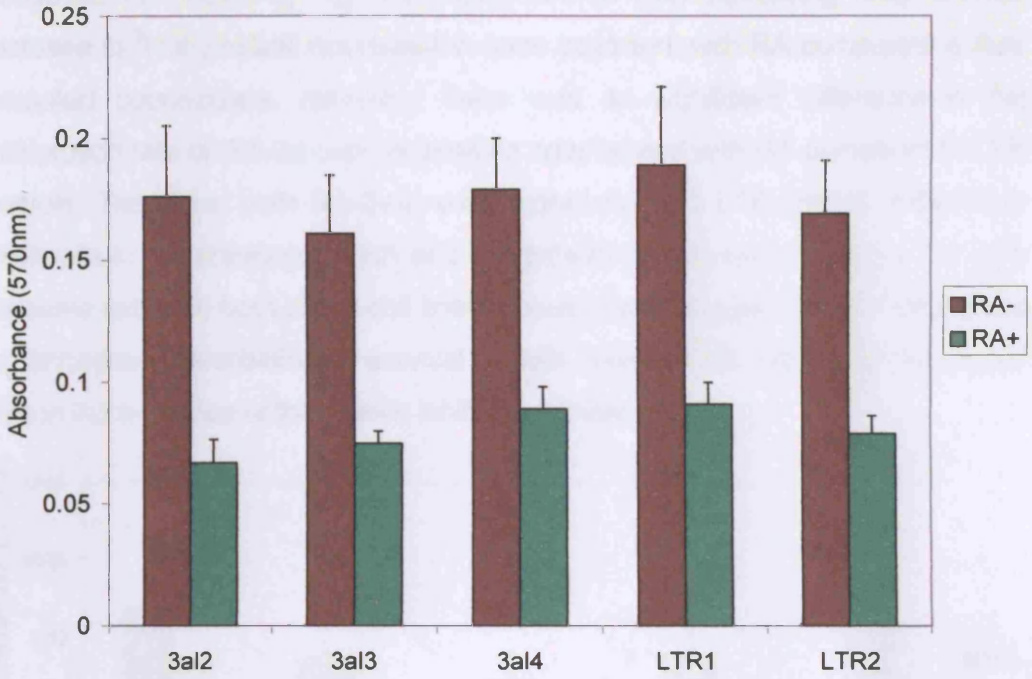


Fig. 4.2.2.3.1A: MTT assay to determine the percentage of surviving/proliferating cells with enhanced levels of Brn-3a(l) in response to treatment with all-trans-retinoic acid

Each clonal cell line was plated out in full growth medium either with or without 10μM RA for 48 hours. Subsequently, a MTT assay was carried out to determine whether increasing Brn-3a could alter survival/proliferation of these cells when grown in the presence of RA compared to untreated controls. The data is represented as absorbance units (at 570 nm) which is taken as a measure of cell number upon treatment with RA compared to untreated cells. The three Brn-3a over-expressing cells (3al2, 3al3 and 3al4) did not exhibit any statistically significant changes in the percentage of surviving/proliferating cells compared to the empty vector controls (LTR1 and LTR2) (p >0.05). The values shown represent the mean of three independent experiments +/- the standard deviation.

This finding was further supported by ^{3}H-thymidine incorporation studies which directly measures DNA synthesis. Cells were grown in medium with or without 10 μM RA for 48h (the optimal time to allow cell arrest and/or differentiation in IMR-32 by RA discussed in described in results chapter 3). The ^{3}H-thymidine was added to the growth medium for 1 hour prior to harvesting and the amount of incorporation

measured. As shown in Fig. 4.2.2.3.1B, Brn-3a over-expressing cells showed decrease in ^{3}H-thymidine incorporation upon treatment with RA compared to their untreated counterparts. However, there was no significant difference in the proliferation rate of Brn-3a over-expressing cells treated with RA compared to LTR controls. Therefore, both Brn-3a(l) over-expressing and LTR control cells fail to proliferate in the presence of RA and the growth retardation induced by RA is to the same extent in both clonal cell lines. These results suggest that Brn-3a(l) does not enhance differentiation or survival of cells compared to the empty vector cell lines in the presence of the growth inhibitor, retinoic acid.

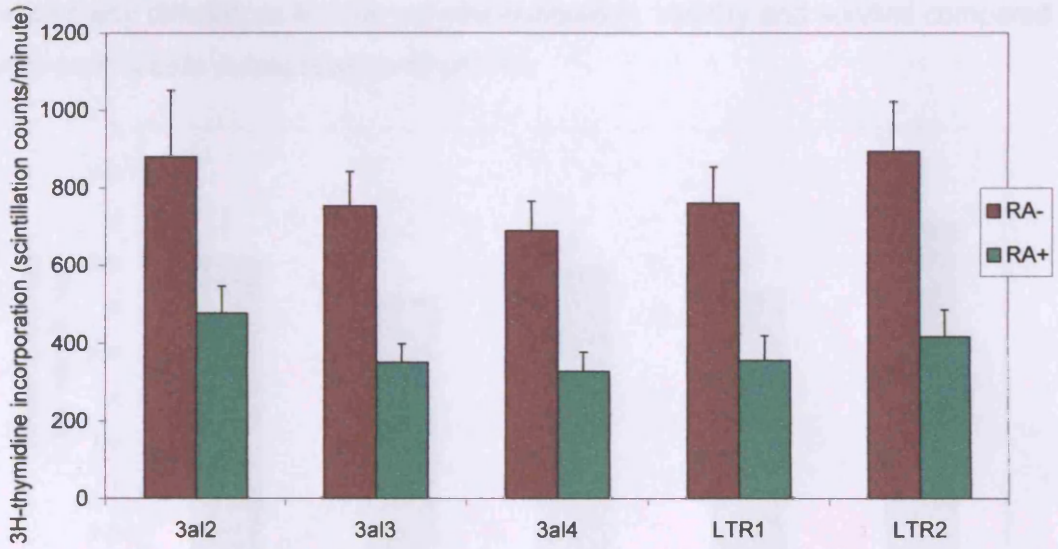


Figure 4.2.2.3.1B: Effect of over-expressing Brn-3a protein levels on the proliferation of human neuroblastoma cells in response to all-trans-retinoic acid

Cells with different levels of Brn-3a protein were grown in full growth medium either with or without 10μM RA. ^{3}H-thymidine incorporation was used as a measure of cell proliferation. The three untreated Brn-3a over-expressing IMR-32 clones (3al2, 3al3 and 3al4) showed similar rates of proliferation as the untreated LTR controls ($p > 0.05$) while addition of RA resulted in a significant decrease in the proliferation of Brn-3a over-expressing cells, similar to that seen in treated controls, LTR1 and LTR2 ($p \leq 0.005$). Therefore, there was no statistical difference in cellular proliferation in response to RA between Brn-3a over-expressing cells and controls. The values shown represent the mean of three independent experiments +/- the standard deviation.

In order to determine whether over-expression of Brn-3a increased the survival of human neuroblastoma cells in the presence of RA, we looked at the changes in the number of cells undergoing apoptosis upon treatment with 10 μM RA compared with untreated cells and LTR controls (treated and untreated). FITC-Conjugated Annexin V antibody staining was carried out as described in materials and methods. As shown in Fig. 4.2.2.3.1C, similar levels of apoptosis were observed in Brn-3a over-expressing cells compared with the control cells. These results suggest that Brn-3a does not affect the survival of these cells in the presence of RA. Hence, IMR-32 cells with manipulated levels of Brn-3a(l) do not exhibit any differences in their growth/proliferation, viability and survival compared with control cells in response to 10 μM RA.

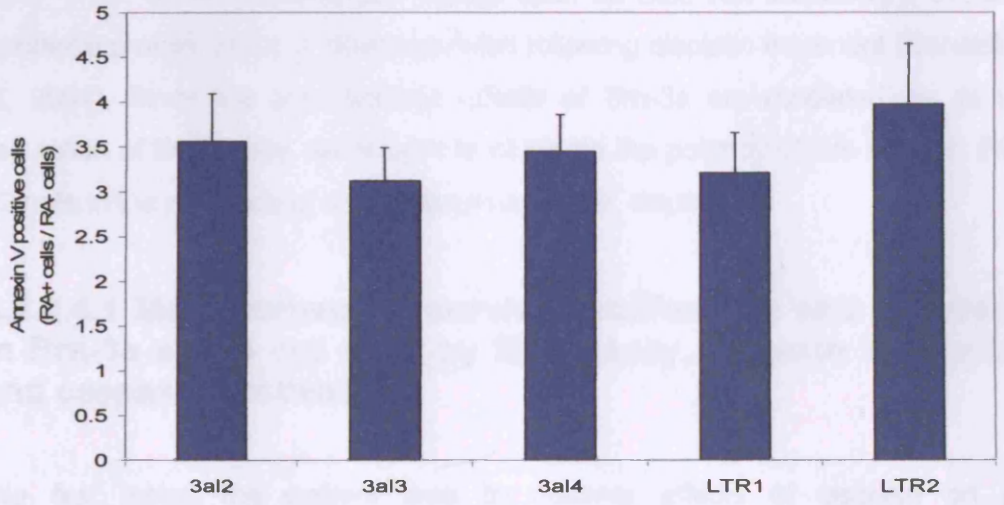


Fig. 4.2.2.3.1C: Effect of RA on apoptosis of Brn-3a over-expressing cells

Each clonal cell line with different levels of Brn-3a was grown in full growth medium either with or without 10μM RA. After 48 hours cells were harvested and incubated with FITC-conjugated Annexin V antibody and stained with PI to determine if RA had any effect on the apoptosis of IMR-32 cells over-expressing Brn-3a. The values represented in the graph above give the percentage of RA treated apoptotic cells (RA+) divided by the percentage of apoptotic cells that were not treated with RA (RA-). The three over-expressing clones (3al2, 3al3, 3al4) showed similar levels of apoptotic cell death as the controls (LTR1, LTR2) ($p > 0.05$). The values shown represent the mean of three independent experiments +/- the standard deviation.

4.2.2.4 Effect of cisplatin on cell survival/apoptosis of neuroblastoma cells

cis-Platinum(II)diamminedichloride (cisplatin), is an important chemotherapeutic agent which is used to treat a variety of human cancer, including neuroblastomas (Yaris et al, 2004). It is generally believed that cisplatin mediates its effect by binding to the DNA, causing structural alterations such that DNA replication is prevented. Subsequently, intracellular transduction pathways are triggered that are involved in regulating the cellular equilibrium between apoptotic death and the cell cycle (for review see Boulikas and Vougiouka, 2003). Genes upregulated following cisplatin treatment of the cells include the pro-apoptotic p53, and its target genes $p21^{CIP1/WAF1}$ or the pro-apoptotic factors such as Bax. Not surprisingly, the anti-apoptotic protein, Bcl-2, is downregulated following cisplatin treatment (Donzelli et al, 2004). Since the anti-apoptotic effects of Brn-3a are mediated via its up-regulation of Bcl-2 gene, we sought to elucidate the potency of this effect in IMR-32 cells in the presence of a Bcl-2 down-regulator, cisplatin.

4.2.2.4.1 Measurement of survival/proliferation and apoptosis in Brn-3a stable cell lines by MTT assay, Annexin V labelling and caspase-3 activation

We first tested the optimal time to observe effects of cisplatin on the viability/proliferation of IMR-32 cells. To this end, wild-type IMR-32 cells were plated and grown in the presence of cisplatin for 6 different time-points (4h, 8h, 12h, 24h, 48h, 55h) and harvested at specific times for MTT assay as described in materials and methods. Untreated cells were harvested at each time-point as controls. We used 33μM cisplatin as other researchers in our laboratory have shown this concentration to be optimal for inducing apoptosis in neuronal cells (personal communication Smith MP; Ensor L; Hudson C). As shown in Fig. 4.2.2.4.1A, there was no difference in spectrophotometric values between cisplatin treated and untreated IMR-32 cells after 4h, 8h, 12h and 24h exposure to the drug. However, a statistically significant difference in the absorbance at 570nm wavelength (indicative of mitochondrial activity in living cells) was observed between treated and untreated cells after 48h of exposure to cisplatin. Hence, for

further analyses to determine the effect of cisplatin on Brn-3a manipulated IMR-32 cells, all assays were carried out after 48 hours of cells being grown in the presence of cisplatin.

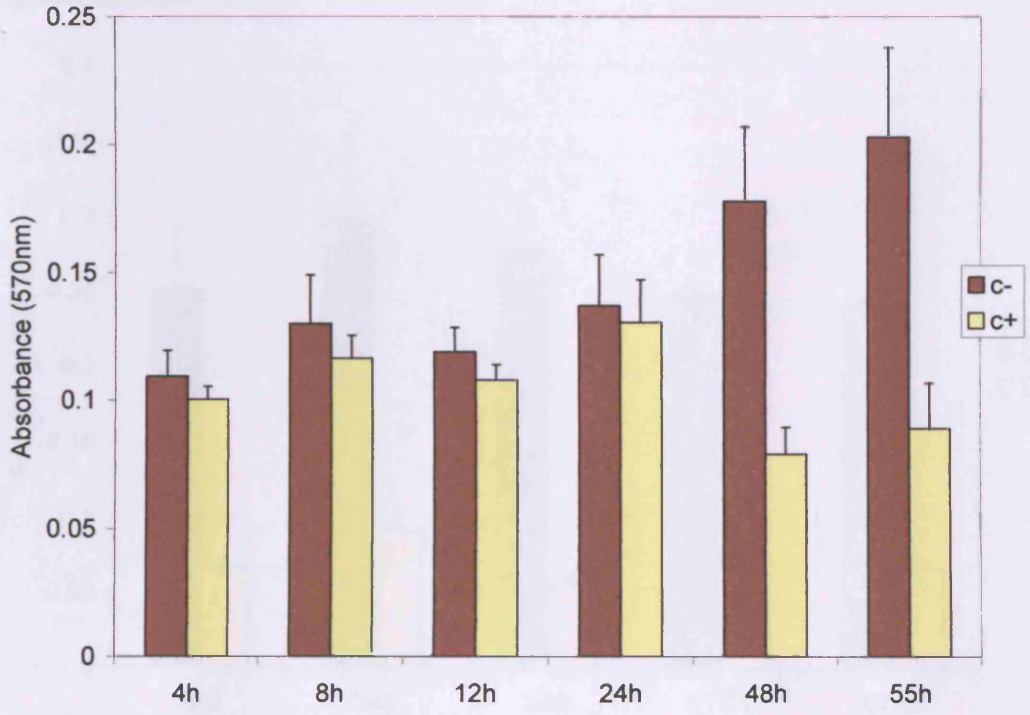


Fig. 4.2.2.4.1A: Effect of cisplatin on IMR-32 cells over time determined by MTT assay

IMR-32 cells were grown in full growth medium supplemented either with (C+) or without (C-) 33μM cisplatin. Cells were harvested over the indicated time periods (4h, 8h, 12h, 24h, 48h and 55h) and determined the mitochondrial dehydrogenase activity in viable cells by spectrophotometrically measuring the cleavage of tetrazolium rings of the yellow MTT to purple formazan crystals. Readings at 570nm wavelength indicated that cisplatin exerts its cytotoxic effect only after 48 hours in IMR-32 cells.

We tested the effect of cisplatin on cell survival/proliferation using the MTT assay. Brn-3a over-expressing and control cells were plated at a density of 1 X 10^6 cells / well in full growth medium. The following day 33μM cisplatin was added to the medium. Untreated cells from each clone were grown in parallel. All cells were harvested after 48 hours of plating and MTT assay carried out as described in materials and methods. As shown in Figure 4.2.2.4.1B, there was no significant

difference in the survival/proliferation of Brn-3a over-expressing cells in the presence of cisplatin compared with empty vector control cells. These results suggest that Brn-3a did not protect IMR-32 human neuroblastoma cells against cisplatin induced death.

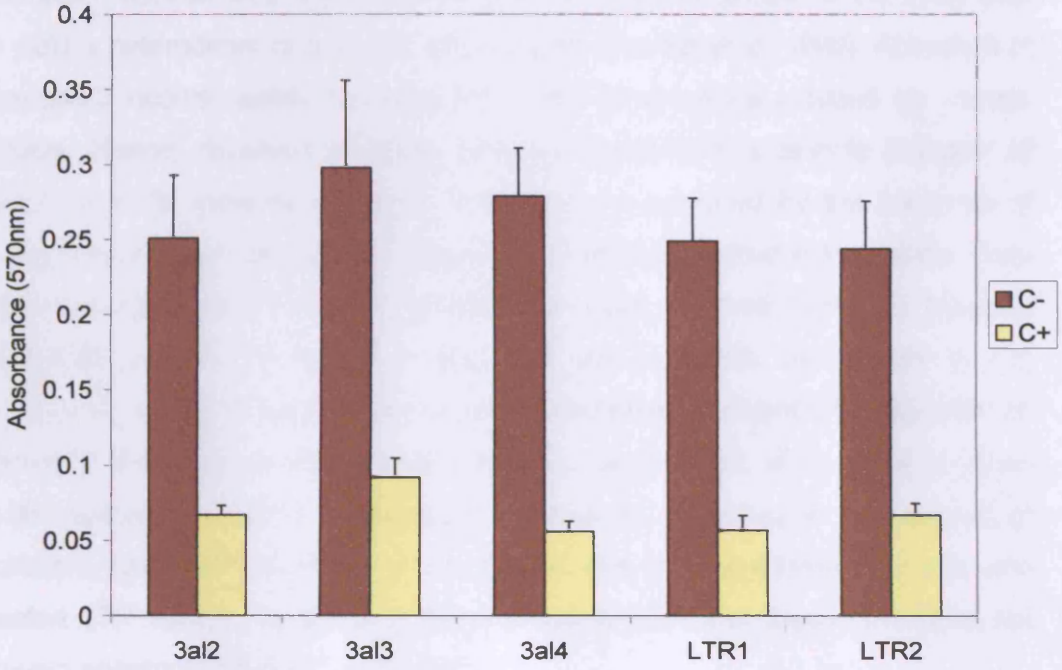


Fig.4.2.2.4.1B: MTT assay on cisplatin treated IMR-32 clonal cell lines

Clonal cell lines with different levels of Brn-3a were grown in full growth medium in the presence or absence of 33μM cisplatin and harvested 48 hours after treatment. MTT assay was carried out to determine whether increasing Brn-3a altered the survival of cells when grown in the presence of cisplatin compared to control cells. All three Brn-3a over-expressing IMR-32 clones (3al2, 3al3 and 3al4) exhibited a significant decrease in survival/proliferation in the presence of cisplatin compared to their corresponding untreated counterparts (p value >0.05). The empty vector control cell lines (LTR1 and LTR2) also exhibited a similar reduction in their survival/proliferative rates in the presence of cisplatin (p value >0.005). However, the extent of cell loss between cisplatin treated Brn-3a over-expressing clones and the two empty vector control cells was not significantly different (p value > 0.05). The values shown represent the mean of three independent experiments +/- the standard deviation.

To directly test the effect of cisplatin on apoptosis of Brn-3a manipulated IMR-32 cells, Western blot analysis was carried out to measure the levels of activated caspase-3 in treated and untreated Brn-3a over-expressing cells and LTR controls. Caspase-3 is a key down-stream protease that is critical for apoptosis and its activation requires proteolytic processing of its precursor protein in the cytoplasm to yield a heterodimer of p17 and p12 subunits (Tewari et al, 1995). Activation of caspase-3 occurs rapidly following the onset of apoptosis induced by various signals. Hence, detection of active caspase-3 protein is a reliable indicator of apoptosis in its irreversible stages. Therefore, we analysed for the presence of active caspase-3 protein in the various clonal cell lines treated with cisplatin. Cells were harvested 48 hours after treatment in whole cell lysis buffer for Western immunoblotting as described in materials and methods. As shown in Fig 4.2.2.4.1C, activated caspase-3 was readily detected in cisplatin treated cells as shown by the presence of two bands, indicated as p17/p12, which were not seen in the untreated controls. However, there was no difference in the amount of activated caspase-3 detected in the treated Brn-3a over-expressing cells and treated LTR controls, suggesting that increasing Brn-3a in these cells does not prevent apoptosis induced by cisplatin.

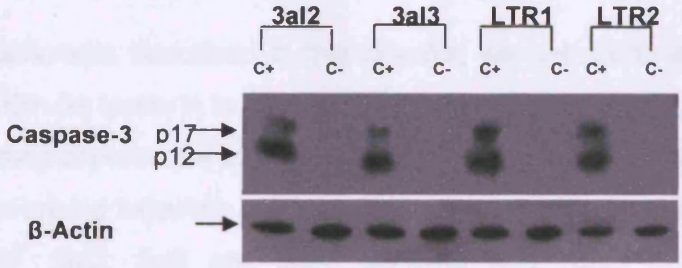


Fig.4.2.2.4.1C Detection of activated caspase-3 protein in cisplatin treated Brn-3a over-expressing IMR-32 cells

Clonal cell lines with different levels of Brn-3a were grown in full growth medium supplemented either with (C+) or without (C-) 33μM cisplatin. Total cellular proteins were harvested from Brn-3a over-expressing cells (3al2, 3al3) and empty vector clones (LTR1, LTR2) and analysed by Western immunoblotting for activated caspase-3 protein following cisplatin treatment. Similar levels of activated caspase-3 were detected in cisplatin treated Brn-3a over-expressing cells and LTR controls. In contrast, activated caspase-3 proteins were not detected in untreated cells of either the Brn-3a over-expressing or LTR control cells.

4.3 Discussion

The role of the POU domain transcription factor, Brn-3a, has been extensively studied in mouse neuroblastoma cell line, ND7. ND7 cells can be induced to differentiate to a non-dividing neuronal phenotype bearing numerous neurite processes by removal of serum from the growth medium or treatment with cyclic AMP (cAMP) (Wood et a, 1990; Suburo et al, 1992). Dramatic increase in the levels of Brn-3a was observed in ND7 cells induced to differentiate to a non-dividing phenotype while levels of the closely related factor, Brn-3b, were virtually undetectable under these conditions (Budhram-Mahadeo et al, 1994). Moreover, over-expression of Brn-3a in ND7 cells resulted in enhancement in the proportion of cells bearing neurites, an event associated with differentiation of cells into mature neurons (Smith et al, 1997). In actively proliferating cells, the ratio of Brn-3a to Brn-3b is reversed. Similar changes are observed during the *in vitro* differentiation of human neuroblastoma cells such as IMR-32, SKN-MC and SK-N-SH, with levels of Brn-3a rising dramatically whilst those of Brn-3b fall (Smith and Latchman, 1996). Hence, these studies have shown Brn-3a to be associated with neuronal differentiation while Brn-3b to be active in proliferating cells.

In the experiments described in this chapter, we set out to study the effects of modifying Brn-3a levels in human IMR-32 neuroblastoma cells. We generated cell lines that over-express the long isoform of Brn-3a protein. However, of the twenty clonal cells arising following the transfections to over-express Brn-3a, only four of these (3al1, 3al2, 3al3 and 3al4) survived and could be propagated. This suggested that in other clones, Brn-3a may be inhibiting proliferation, thereby preventing colony growth.

Three of the constitutively over-expressing Brn-3a clones (3al2, 3al3 and 3al4) were used in to study the effects on growth, proliferation and response to therapeutic drugs such as all-trans-retinoic acid and cisplatin, and compared with empty vector transfected cells (LTR1, LTR2). Results of proliferation assays confirm that over-expressing Brn-3a in IMR-32 cells did not significantly alter the cellular growth or proliferation compared to cells containing the empty vector alone.

Moreover, previous studies in ND7 cells engineered to over-express Brn-3a exhibited striking morphological changes such as neurite outgrowth even in serum-containing medium which would not normally promote differentiation (Smith et al, 1997). However, similar analyses in human neuroblatoma IMR-32 cells over-expressing Brn-3a did not show any enhancement in the proportion of cells bearing processes or in the length of the processes compared to the control cells (data not shown).

We also tested whether Brn-3a affected survival of these cells since in addition to its effect on neuronal differentiation, high levels of Brn-3a has been shown to protect ND7 cells as well as primary cultures of trigeminal and dorsal root ganglia from apoptotic stimuli such as the withdrawal of nerve growth factor (NGF) from the medium (Smith et al, 1998b; Ensor et al, 2001). *In vivo* studies also demonstrated that increasing Brn-3a expression protected dorsal root ganglia following sciatic nerve lesion (Smith et al, 2001). Conversely, increased death has been observed in ND7, TG and DRG neurons in cultures where endogenous Brn-3a expression is decreased using an antisense strategy (Ensor et al, 2001; Smith et al, 1998b). Subsequently, Brn-3a was shown to regulate survival by enhancing expressing of anti-apoptotic genes associated with survival in neuronal cells either directly or indirectly upon association with p53 protein. These include activation of the Bcl-2 P2 promoter (the predominant promoter that is active for this gene in neuronal cells) (Smith et al, 1998a; 1998b; Ensor et al, 2001). Moreover, Brn-3a has been shown to repress p53 mediated activated of pro-apoptotic factors Bax and Noxa (Budhram-Mahadeo et al, 2002; Hudson et al, 2004). Therefore, given this protective role of Brn-3a in neuronal cells and the failure to propagate cells containing the Brn-3a anti-sense plasmid, we tested if IMR-32 cells over-expressing this factor exhibited any protection to apoptosis following all-trans-retinoic acid (RA) or cis-Platinum(II)diamminedichloride (cisplatin) treatment. Both of these agents are known to induce apoptosis in IMR-32 and have been used as chemotherapeutic agents to treat children with neuroblastoma (Nitschke et al, 1981; DeBernadi et al, 1992; Reynolds et al, 2003). However, no statistically significant difference in the number of apoptotic/differentiating cells was observed between Brn-3a over-expressing and control cells. This was determined by

different methods such as MTT assays, Annexin V antibody staining and activated caspase-3 analysis.

The failure of Brn-3a to alter growth or survival of these cells is surprising given that previous studies have shown Brn-3a to promote neuronal differentiation as well as survival against a number of apoptotic stimuli both *in vivo* and *in vitro*. In addition, the significant effects observed in the studies using the Brn-3b manipulated IMR-32 cells (discussed in chapter 3) which employed many of the assays used in the studies with Brn-3a over-expressing cells discussed here, would suggests that the experiments were adequately set up and functional. As such the lack of differences in survival and proliferation in the IMR-32 over-expressing cells compared with controls may have resulted from the limitations of constitutively altering Brn-3a gene expression in these cells. Since previous studies have shown that increasing Brn-3a enhances differentiation and hence prevents cellular proliferation, it is feasible to accept that we might only have propagated cell lines that have growth advantage either due to the site of integration of the Brn-3a plasmid or other cellular changes that could overcome the effects of Brn-3a in these cells. Even though we tested three different Brn-3a cell lines, these were selected out of twenty original colonies, most of which failed to proliferate in culture and were subsequently lost. As such analysis of the parameters that would normally be affected by Brn-3a (survival and differentiation) did not give rise to the expected effects.

Another possible reason to explain the lack of effects seen in IMR-32 over-expressing Brn-3a might be due to the heterogenous population of IMR-32 cells. These human neuroblastoma cell lines consist of two types of cells of neuroblasts and neurofibroblasts. It is possible that while selecting the clonal cells transfected with Brn-3a plasmid, we actually might have selected neurofibroblasts rather than neuroblasts.

Additionally, we also attempted to generate IMR-32 cells with reduced levels of Brn-3a. However, cells transfected with Brn-3a anti-sense construct gave rise to a number of colonies but all of these died early after transfections. This is attributable to the established role of Brn-3a as a surviving factor in neuronal models and

abolishing its expression in knock-out studies results in enhanced cell death of specific neurons (Smith et al, 1998a; 1998b; Ensor et al, 2001; McEvilly et al, 1996; Xiang et al, 1996; Huang et al, 1999). These problems observed in both the constitutively over-expressing and anti-sense IMR-32 Brn-3a clones can be overcome in the future by establishing an inducible expression system to generate Brn-3a stable cell lines such that the gene of interest is expressed only when induced. This could be achieved by, for example, constructing a Brn-3a cDNA construct that is under the control of a tetracyclin inducible promoter so that it is only expressed after the addition of a specific substrate such as tetracycline or doxycyclin.

CHAPTER 5

Results 3

5.0 Functional effect of Brn-3b in neuroblastoma cells

5.1 Introduction

We have shown that over-expressing of Brn-3b in IMR-32 neuroblastoma cells clearly increased proliferation (see chapter 4). This finding lead us to further investigate how this transcription factor mediates its effects by identifying putative target genes regulated by Brn-3b in these cells.

One of the hallmarks of transformed cells is their ability to grow uncontrollably. Cancer cells either do not require mitogenic stimulation to proliferate or their requirements are far less stringent compared to those of normal cells. Similarly, cancer cells do not exit the cell cycle to enter a quiescent state in response to growth inhibitory signals. These acquired properties often result from alterations in growth regulatory pathways, which may either directly or indirectly involve cell cycle regulation. Some of the proteins most often altered in human cancers are those involved in the control of G1/S transition of the cell cycle at a time when a cell becomes committed to a cell division. In particular, the alterations of members of the cascade associated with cyclin-dependent kinase (CDK)-cyclin D/CDK-4/retinoblastoma (pRB)/E2F (described in section 4.4 of introduction) have also been found in more than 80% of human cancers. Such changes arise either because of mutations within the genes or in the regulators that control their expression in specific cells. In this study, we investigated cyclin D1 as a possible candidate gene regulated by Brn-3b.

Cyclin D1 expression is normally increased in early G1 when it complexes with and activates CDK4. Hence, cyclin D1 is critical for cell cycle progression and commitment to cell division. Interestingly, cyclin D1 knock-out mice (Sicinski et al, 1995) share significant phenotypic features with Brn-3b homozygous null mutants (Gan et al, 1996). Thus, similar to Brn-3b homozygous mutants, cyclin D1-/- mice develop to full term but exhibit drastic reduction in the retinal cell numbers due to inability of retinal cell precursors to develop during embryogenesis. In addition, Brn-3b has been implicated in the proliferation of mammary epithelial cells

(Budhram-Mahadeo et al, 1999; Dennis et al, 2001) and it is interestingly to note that cyclin D1-/- females are unable to nurse due to failure of pregnancy-associated mammary proliferation. Additionally, independent studies have demonstrated that cyclin D1, like Brn-3b, is over-expressed in human breast cancers (Buckley et al, 1993; Wang et al, 1994; Budhram-Mahadeo et al, 1998).

A recent study has shown high expression of cyclin D1 at both the mRNA and protein levels in approximately two-third of neuroblastoma cell lines and primary tumours (Molenaar et al, 2003). However, amplification of cyclin D1 gene was found only in one neuroblastoma cell line and four neuroblastoma tumours, suggesting that the increased cyclin D1 result from altered expression of the gene thus implicating upstream mechanisms controlling its expression. Furthermore, a significant decrease in cyclin D1 transcript levels is observed in neuroblastoma cells upon treatment with a differentiating agent, dibutyryl cyclic adenosine monophosphate, which induces differentiation of these cells and correlates with their growth retardation (Munoz et al, 2003). Similarly, treatment of neuroblastoma cells with retinoic acid induces differentiation into neuronal cells, and a decrease in cyclin D1 level (Wainwright et al, 2001).

We have previously shown that the POU-domain transcription factor, Brn-3b, promotes cellular proliferation in human neuroblastoma cell lines (discussed in chapter 4). The changes in levels of this protein in differentiating versus proliferating cells, lead us to investigate whether the *in vivo* and *in vitro* functional overlap between Brn-3b and cyclin D1, reflects some common underlying molecular mechanism that regulate cell proliferation. It is possible that the potent proliferative effect of Brn-3b in IMR-32 is mediated via its direct ability to transcriptionally increase cyclin D1 expression, causing rapid cell cycle progression in neuroblastomas. Therefore, we undertook studies to investigate the possible role of and inter-relationship between Brn-3b, cyclin D1, and proliferative kinetics of IMR-32 cells with altered levels of Brn-3b.

5.2 Results

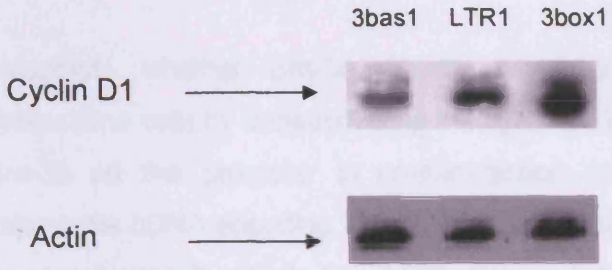
5.2.1 Cyclin D1 protein levels in Brn-3b overexpressing IMR-32 cells

Proteins were extracted from manipulated IMR-32 cells with altered levels of Brn-3b. Immunoblotting was carried out to determine the levels of cyclin D1 in the Brn-3b overexpressing cells, and compared to levels in anti-sense cells with decreased Brn-3b, and vector control cells using densitometry. Total cellular protein from each cell line was harvested at 60% confluency of 6-well plates and loaded onto a 15%/SDS/PAGE gel for resolution of different proteins, which were then transferred onto a nitrocellulose membrane, and probed with an anti-cyclin D1 antibody. Membranes were stripped and reprobed with the invariant protein actin to control for any differences in protein concentrations.

As shown in Fig. 5.2.1A, the levels of cyclin D1 protein were significantly increased in Brn-3b over-expressing cell lines compared to the control cells whilst reduced levels were detected in the Brn-3b anti-sense cells. This correlation of cyclin D1 levels with Brn-3b protein levels in neuroblastoma cells was observed in at least three independent experiments as shown on the graph (Fig. 5.2.1B).

The correlation of Brn-3b protein levels with cyclin D1 protein in Brn-3b manipulated IMR-32 cells could reflect either a direct effect of Brn-3b in transactivating the cyclin D1 gene promoter or an indirect effect associated with altered proliferation rates of these cells.

(A)



(B)

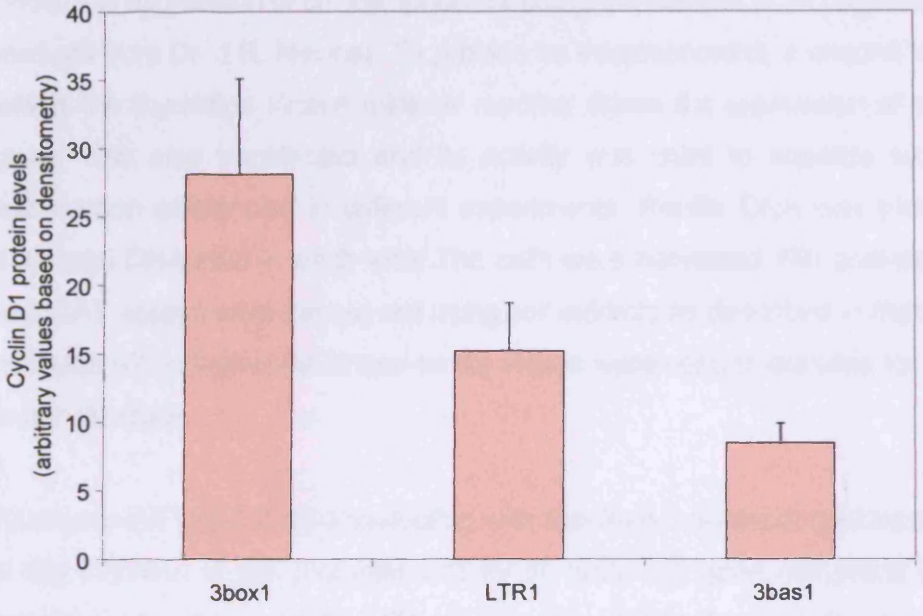


Fig. 5.2.1: Levels of cyclin D1 protein in Brn-3b over-expressing cell line (3box1) compared to the antisense (3bas1) and empty vector control (LTR1)

(A) Proteins were extracted from IMR-32 cells with altered levels of Brn-3b and Western immunoblotting carried out to determine the levels of cyclin D1 protein in the Brn-3b over-expresssing cells (3box1), empty vector control (LTR1), and anti-sense Brn-3b cells (3bas1). Higher levels of cyclin D1 protein were detected in 3box1 compared to LTR1, and even lower levels were found in 3bas1. Invariant β-Actin protein was used as an internal control for differences in protein concentration.

(B) Densitometric analysis on three sets of independent Western immunoblotting experiments (+/- the standard deviation) confirmed the differences in the cyclin D1 protein levels in Brn-3b manipulated cells. Statistical analysis using Student's T-test revealed significant increase in 3box1 compared with LTR ($p \leq 0.05$) and decrease in 3bas1 (p value≤ 0.05). The values have been normalized using β-actin as an internal control on three sets of independent experiments +/- the standard deviation.

5.2.2 Effect of Brn-3b on cyclin D1 gene promoter

To elucidate whether Brn-3b directly regulates cyclin D1 expression in neuroblastoma cells by transactivating the cyclin D1 promoter, we tested the effect of Brn-3b on this promoter in co-transfection assays. An expression vector containing the cDNA encoding Brn-3b was co-transfected into IMR-32 cells with a reporter construct in which the 1.9kb fragment from the 5' regulatory region upstream of the human cyclin D1 gene was cloned into pBLCAT reporter so that it drives the expression of chloramphenicol acetyl transferase (CAT) reporter gene (a kind gift from Dr. J.R. Nevins). To provide an internal control, a second reporter in which the thymidine kinase minimal reporter drives the expression of the renilla gene, was also transfected and its activity was used to equalize for different transfection efficiencies in different experiments. Renilla DNA was tranfected at 1:10 total DNA ratio in each well. The cells were harvested 48h post-transfection and CAT assays were carried out using cell extracts as described in materials and methods whilst thymidine kinase-renilla values were used to equalize for variability in transfections.

As shown in Fig 5.2.2, co-transfection with Brn-3b(l) resulted in approximately 3.5-4 fold increase in the promoter activity of cyclin D1 gene compared to control transfections where empty LTR vector was co-transfected with the promoter construct. Similarly, Brn-3b(s) activated the promoter by 2.2-2.7 fold. Hence, Brn-3b can directly transactivate the cyclin D1 promoter resulting in significant increase in promoter activity ($p<0.05$).

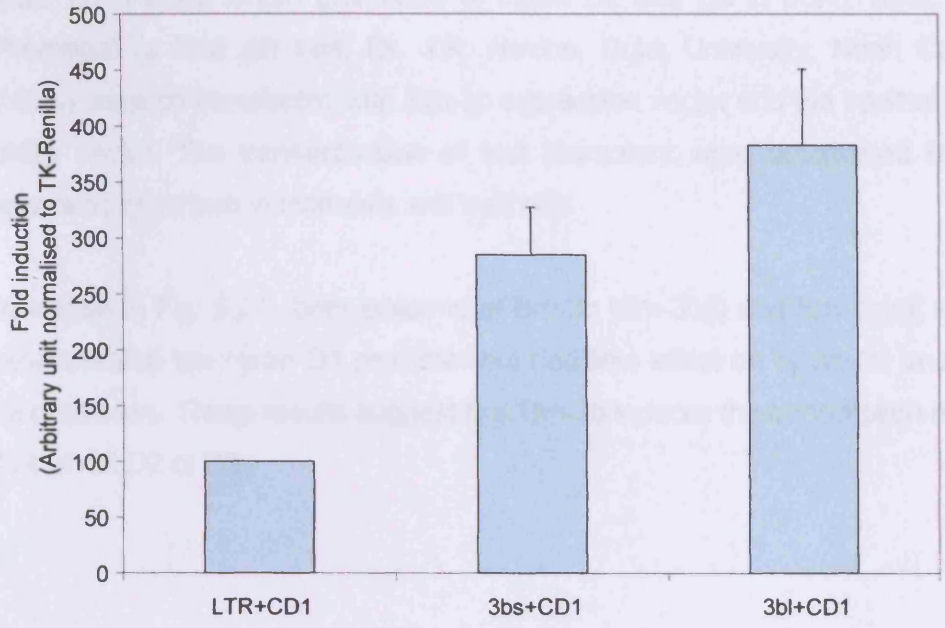


Fig. 5.2.2(ii) Effect of Brn-3b(l) and Brn-3b(s) on full length cyclin D1 promoter determined in a CAT reporter construct

IMR-32 cells were co-transfected with 2.7 µg of Brn-3b(l) or Brn-3b(s) or LTR DNA and 2.7 µg of CAT reporter construct DNA encoding 1.9 kb of the 5' regulatory region of cyclin D1 gene. A control in which minimal thymidine kinase promoter drives the expression of the Renilla gene was also transfected at 1:10 total DNA ratio in each transfection in order to equalize for variability between different transfections. Cells were harvested 48h post-transfections and CAT assays carried out to determine the expression levels of the reporter gene. Brn-3b(l) increases the activity of cyclin D1 promoter by approximately 4 fold ($p<0.05$), whereas the Brn-3b(s) increases it by about 3 fold compared to the empty vector LTR control ($p<0.05$). The data represents the mean of three independent experiments (carried out in triplicate in each experiment), +/- the standard deviation.

5.2.3 Effect of Brn-3b on cyclin D2 and cyclin D3 promoters

To determine whether the ability of Brn-3b proteins to transactivate the promoter of cyclin D1 gene was specific for this gene promoter, we tested its effect upon co-transfection with reporter constructs containing the promoters of other D-type cyclins, namely cyclin D2 and cyclin D3. Using a similar approach described for

cyclin D1, the full length promoters of cyclin D2 and D3 in pCAT Basic vector (Promega) (a kind gift from Dr. J.R. Nevins, Duke University, North Carolina, U.S.A.) were co-transfected with Brn-3b expression vector and the internal control renilla vector. The transactivation of test promoters were determined by CAT assays as described in materials and methods.

As shown in Fig. 5.2.3, both isoforms of Brn-3b [Brn-3b(l) and Brn-3b(s)] strongly transactivated the cyclin D1 promoter but had little effect on cyclin D2 and cyclin D3 promoters. These results suggest that Brn-3b induces the transcription of cyclin D1 but not D2 or D3.

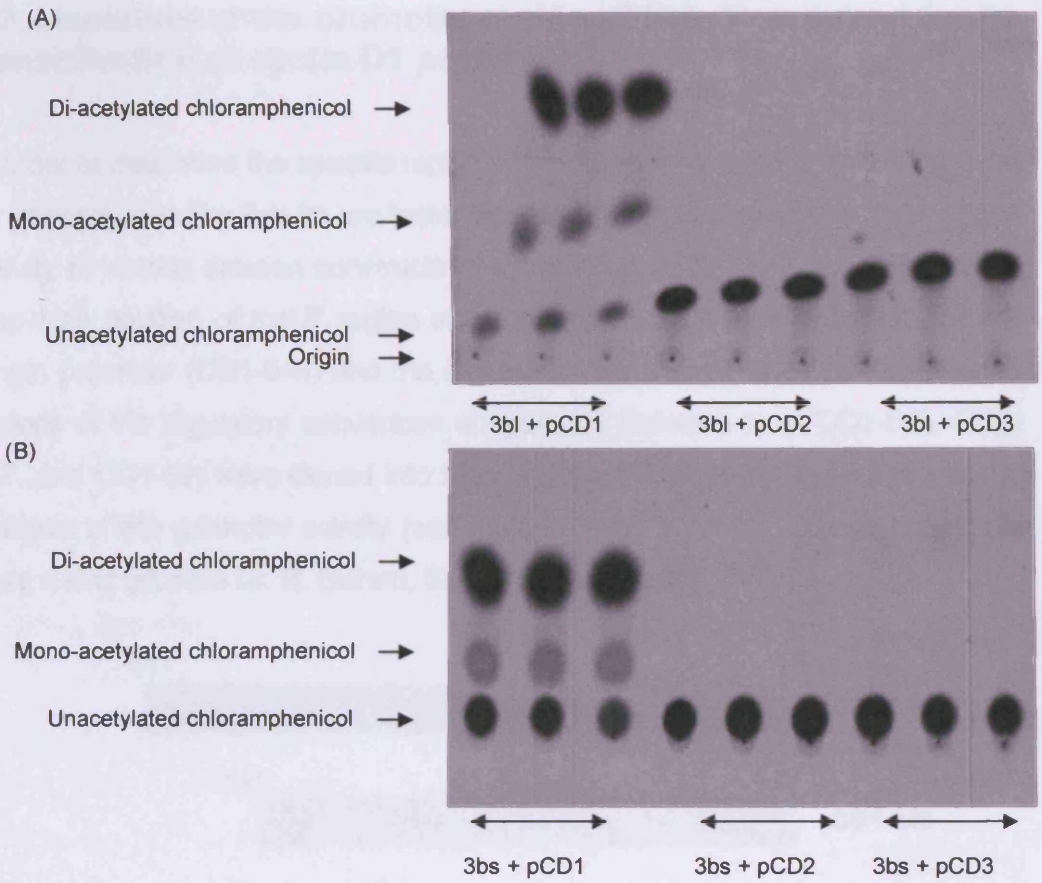


Fig. 5.2.3: CAT assays showing transactivation of cyclin D1 promoter, but not cyclin D2 or cyclin D3, by Brn-3b proteins

IMR-32 cells were co-transfected with 2.7µg of Brn-3b(l) or Brn-3b(s) DNA and 2.7µg CAT reporter constructs containing the promoters of cyclin D1/cyclin D2/cyclin D3 DNA. Thymidine kinase Renilla minimal reporter was also transfected at 1:10 total DNA ratio in order to provide an internal control for differences in transfections efficiencies of different experiments. Cells were harvested 48 hours post-transfection and CAT assays carried out to determine the acetylation at one or both of the hydroxyl groups of chloramphenicol by chloramphenicol acetyltransferase (CAT) gene product resulting from activation of the cyclin D1 promoter. Panel (A) shows the activation of the promoter and consequent high expression of the CAT gene product, resulting in both mono- and di-acetylated chloramphenicol, which reflects a significant transactivation of cyclin D1 promoter upon co-transfection with the long isoform of Brn-3b(l) (3bl + pCD1). However, the absence of acetylated products on cyclin D2 (3bl + pCD2) and cyclin D3 (3bl + pCD3) promoters suggests that Brn-3b(l) does not transactivate these D-type cyclins. Panel (B) shows similar results of with the short isoform of Brn-3b (3bs) with reporter vectors containing the different D-type cyclins. Therefore, both isoforms of Brn-3b (Brn-3b(l) and Brn-3b(s)) are able to transactivate the cyclin D1 promoter but not the cyclin D2 or cyclin D3 promoters.

5.2.4 Isolation of the promoter region of Brn-3b required for its transctivation of cyclin D1 promoter

In order to determine the specific region of the cyclin D1 promoter that is required for transactivation by Brn-3b, we tested the effect of Brn-3b(l) and Brn-3b(s) on the activity of various deletion constructs of cyclin D1 promoter in which there was a step-wise deletion of the 5' region of the promoter, (see figure 5.2.4(i)). The full length promoter (CD1-944) and the deletion constructs which contained different regions of the regulatory sequences of cyclin D1 (referred to as CD1-848, CD1-567, and CD1-69) were cloned into the pGL2 luciferase vector to facilitate easier analysis of the promoter activity (see materials and methods). These constructs were a kind gift from Dr. R. Gerard, Saint-Antoine Hospital, Paris.

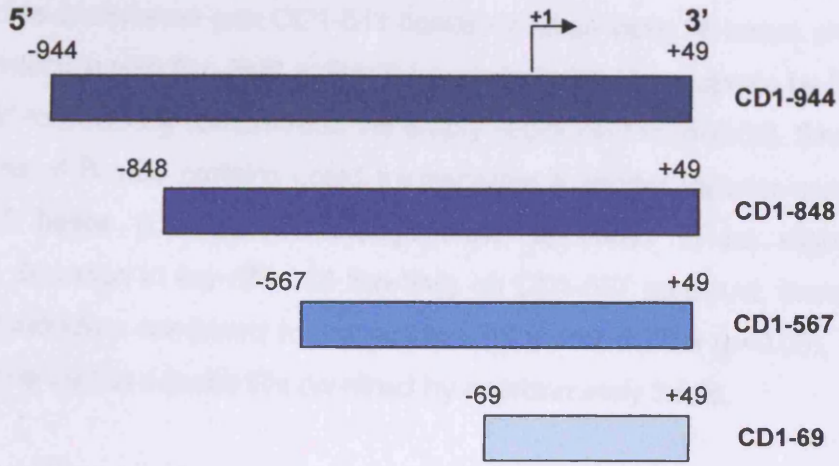


Fig. 5.2.4(i): Diagrammatic representation of cyclin D1 promoter deletion constructs

Step-wise deletions of the 5' end of the cyclin D1 promoter (CD1-944) gave rise to constructs lacking 96 (CD1-848), 377 (CD1-567) and 875 (CD1-69) bases. These deletions were cloned into pGL2 luciferase vector and used in co-transfection studies with Brn-3b expression vectors.

5.2.4.1 Brn-3b requires sequences between -567 and -69 of cyclin D1 for transactivation

As can be seen in Fig. 5.2.4(ii), Brn-3b(l) is able to activate the full length cyclin D1 promoter (CD1-944) by 4-5 fold compared to the empty LTR vector ($p<0.005$). Similarly, Brn-3b(s) activates this CD1-944 promoter by approximately 3 fold ($p<0.005$). Ability of both Brn-3b(l) and Brn-3b(s) to transactivate the full length cyclin D1 promoter cloned into the luciferase reporter construct parallels the effect seen using the cyclin D1 CAT reporter construct, thus further supporting Brn-3b as a regulator of the cyclin D1 promoter activity and validated using these deletion constructs for further analysis.

Similar studies undertaken with CD1-848 construct which lacks 96 bases showed that co-transfection with Brn-3b(l) or Brn3b(s) continued to transactivate by 5- and 2.5 to 3- fold respectively compared to the empty vector control ($p<0.05$). Similarly, both isoforms of Brn-3b proteins could transactivate a shorter reporter construct lacking 377 bases (CD1-567). Although there appeared to be slight but insignificant decrease in the effect of Brn-3b(l) on CD1-567 construct, there was still a 4-fold induction compared to the empty LTR vector control ($p<0.05$), while Brn-3b(s) continued to activate this construct by approximately 3-fold.

Co-transfection of Brn-3b expression vectors with a shorter promoter construct (CD1-69), lacking 875 bases, resulted in the loss of ability of Brn-3b proteins to transactivate this promoter. These results suggest that the regulatory element required for Brn-3b to transactivate the cyclin D1 promoter resides within the region of -567 and -69.

It is important to note that the basal transcriptional activity is still intact in the shortest CD1-69 construct, as determined by the relative luciferase activity of different promoter constructs when transfected alone into IMR-32 cells. The basal activity of CD1-69 was similar to that observed with the full-length (CD1-944) and another deletion construct (CD1-848) (Fig.5.2.4(iii)), suggesting that the loss of this region of the promoter in the sequentially deleted constructs studied does not affect the basal transcription.

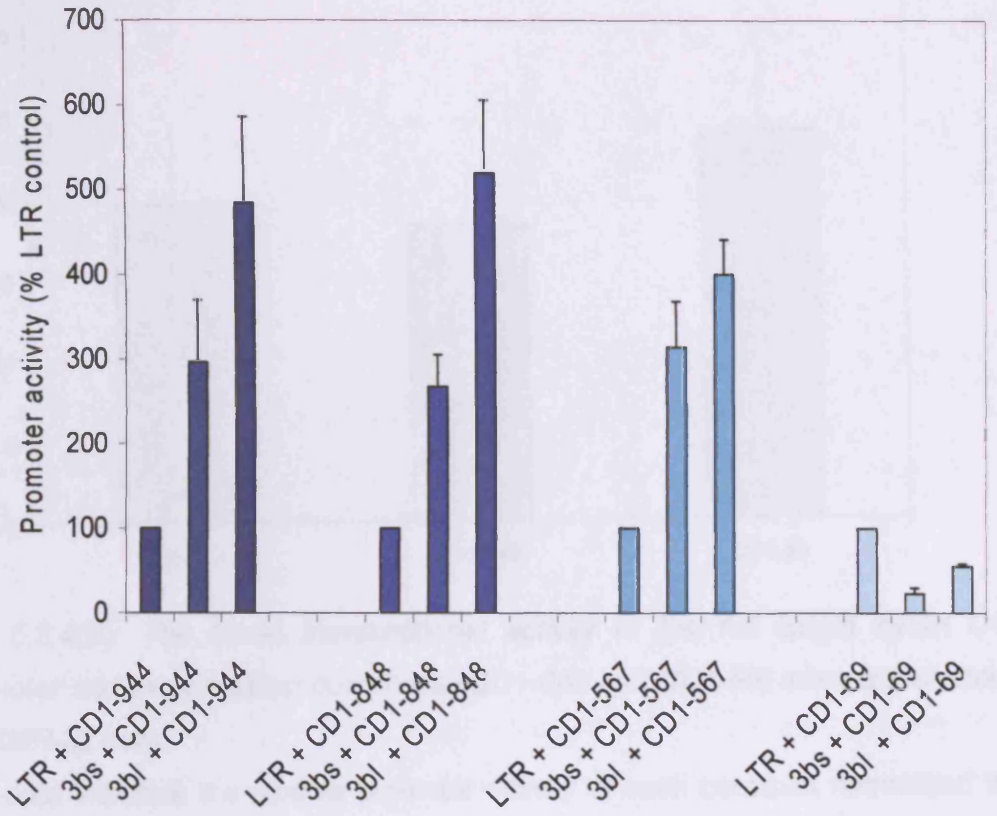


Fig. 5.2.4(ii) Fold induction of cyclin D1 promoter activity by Brn-3b proteins

Co-transfection experiments were undertaken in which the Brn-3b(l) or Brn-3b(s) or LTR control vector were introduced into human neuroblastoma cells, IMR-32, with Firefly luciferase reporter constructs containing the full-length cyclin D1 promoter or different deletions constructs (as indicated on the graph). The values shown were standardized using TK-Renilla. Each experiment was performed in triplicate in three independent experiments. The error bars represent the standard error of the mean. Brn-3b(l) and Brn-3b(s) activates cyclin D1 full-length promoter constructs (CD1-944), as well as deletions CD1-848, and CD1-567 between 3-5 fold. However, neither isoforms of Brn-3b could activate the CD1-69 deletion construct compared to the empty vector LTR control ($p<0.05$).

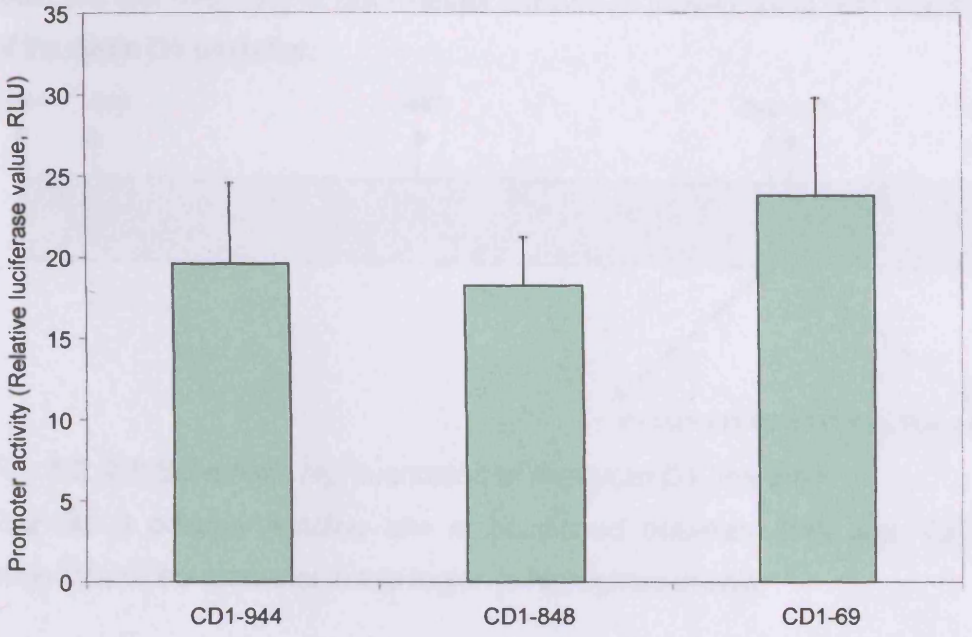


Fig. 5.2.4(iii): The basal transcriptional activity of the full length cyclin D1 promoter and two deletions constructs (CD1-848 and CD1-69) when transfected into IMR-32 cells.

The data indicates the relative promoter activity of each construct normalized to TK-Renilla. The basal promoter activity of the shortest deletion construct (CD1-69) is similar to the full-length promoter (p value >0.05), indicating that basal transcription is still intact on this promoter.

5.2.5 Determining whether Brn-3b regulates cyclin D1 by binding directly to the cyclin D1 promoter by electrophoretic mobility shift assay

We next attempted to elucidate whether Brn-3b transactivated the cyclin D1 promoter by interacting directly with cis-regulatory DNA sequences. Analysis of the cyclin D1 promoter from -567 to -69 for putative Brn-3b consensus binding sequences revealed an A/T rich region with sequence homology to common preferential binding site for POU domain proteins. This region was identified between position -248 to -228 of the promoter. In fact, online MatInspector search results identified an octamer binding motif within this site. Based on this observation, an oligonucleotide was synthesized (5'-CGA TTT GCA TTT CTA TGA AAA-3') and used in electrophoretic mobility shift assay (EMSA) (described in

materials and methods) to test whether the Brn-3b protein could bind to this region of the cyclin D1 promoter.

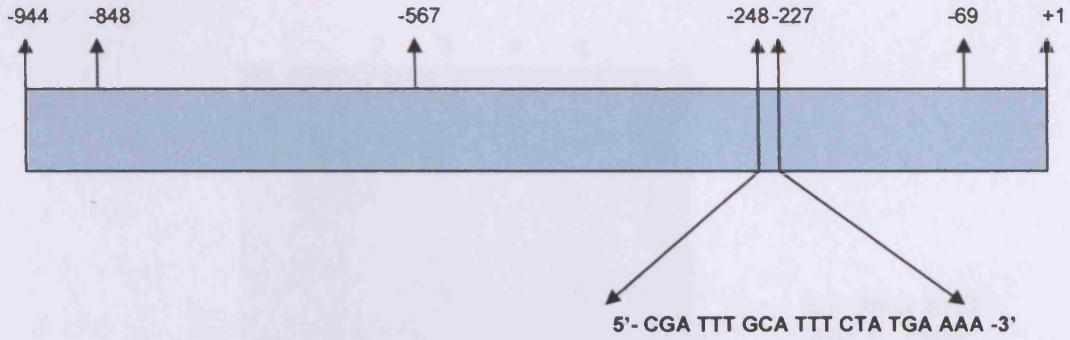


Fig. 5.2.5(i): Schematic representation of the cyclin D1 promoter
The Brn-3 octamer binding site is positioned between -248 and -227. The sequence of the promoter in this region is highlighted in bold.

Fig. 5.2.5(ii) shows the result of an EMSA in which the radiolabelled oligonucleotide, corresponding to the putative Brn-3 binding site, in the cyclin D1 promoter (see above) was incubated with protein extract obtained from IMR-32 cells over-expressing the Brn-3b protein (3box1), giving rise to protein/DNA complexes that form retarded bands that can be separated by electrophoresis. Hence, incubation of cell extract obtained from IMR-32 expressing high levels of Brn-3b with ^{32}P labeled oligonucleotide probe resulted in the formation of a number of protein-DNA complexes (lane 2) but only two of these complexes were competed by addition of increasing molar concentration of unlabelled Brn-3 binding site (lane 3, 4, 5) but not by a non-specific oligonucleotide (lane 6). This suggests the presence of proteins that strongly and specifically bind to the DNA probe so that they are only competed by the addition of the specific oligonucleotides. The presence of two bands with different mobility could suggest that the protein may be bound in different conformation, for example, smaller complex may represent monomers of Brn-3b whilst larger complex may be associated with dimers of Brn-3b. We failed to super-shift any of these complexes with Brn-3b specific antibody (data not shown), perhaps due to the masking of the epitope once the protein is bound to the sequence. Therefore, to confirm that Brn-3b protein was present in these complexes, wild type probe was incubated with SAOS 2 cells that lack Brn-3b (personal communication Perez-Sanchez C). Lane 1 shows that both these complexes were specific to Brn-3b as they were absent in SAOS2 cells. Hence, it

is likely that Brn-3b protein is able to bind to the cyclin D1 at promoter sequence located between -248 and -228 from the transcriptional start site.

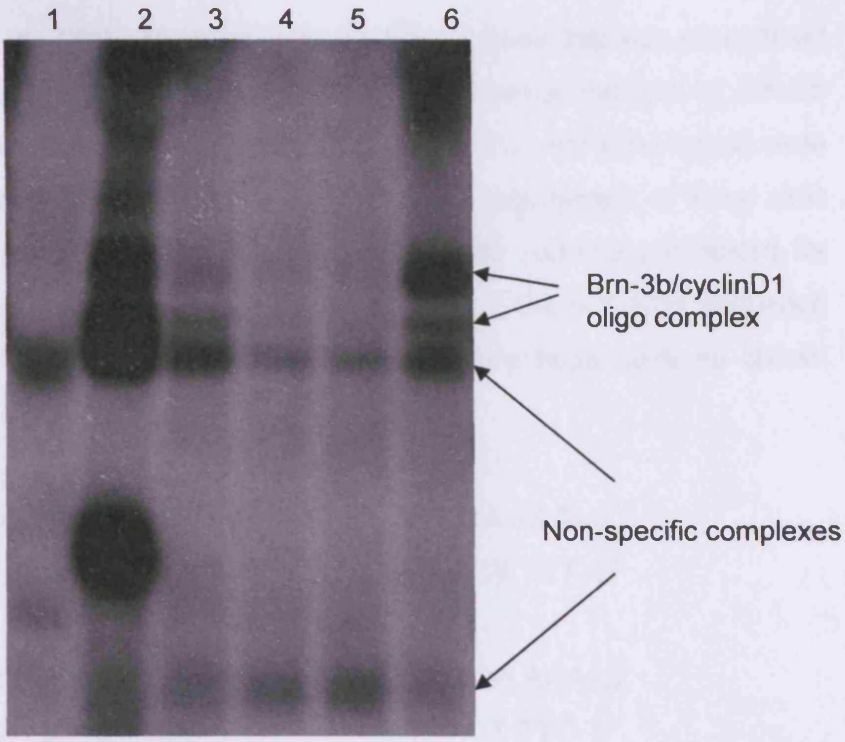


Fig. 5.2.5 (ii) Electrophoretic mobility shift assay (EMSA) using a radioactively labeled oligonucleotide (oligo) probe containing the putative Brn-3b binding sequence (CD1 Brn-3b).

The oligonucleotides were designed to correspond to the putative Brn-3b binding site within the cyclin D1 promoter and labeled with ^{32}P. The labelled oligos were subsequently incubated with cell lysate prepared from SAOS 2 which lacks endogenous Brn-3b or IMR-32 cells over-expressing Brn-3b (3box1). The reaction complex was then analysed on a non-denaturing PAGE gel and visualised by autoradiography.

Lane 1: SOAS 2 cell lysate + labeled oligo

Lane 2: 3box1 cell lysate + labeled oligo

Lane 3: 3box1 cell lysate + unlabeled CD1 Brn-3b (50-fold molar excess) + labeled oligo

Lane 4: 3box1 cell lysate + unlabeled CD1 Brn-3b (100-fold molar excess) + labeled oligo

Lane 5: 3box1 cell lysate + unlabeled CD1 Brn-3b (200-fold molar excess) + labeled oligo

Lane 6: 3box1 cell lysate + Non-specific competitor (ERE) + labeled oligo

5.2.6 Identification of the specific bases required for the binding of Brn-3b to the cyclin D1 promoter

Since we identified the DNA sequence in cyclin D1 promoter that was recognized by Brn-3b, we next attempted to determine the specific bases required by Brn-3b to bind the cyclin D1 promoter sequence tested earlier. Two A/T rich regions were found within the putative binding site so we tested the requirement of these sites for binding of Brn-3b protein. As such these sites were mutated and tested for binding of Brn-3b using EMSA assays. Two variants of the wild-type sequence were synthesized, each containing changes in different base pairs as shown below:

Wild-type (WT/CD1): 5'-CGA TTT GCA TTT CTA TGA AAA- 3'
3'-GCT AAA CGT AAA GAT ACT TTT- 5'

Mutant 1 (M1/CD1): 5'-CGA TTT GCA GGG CTA TGA AAA- 3'
3'-GCA AAA CGT CCC GAT ACT TTT- 5'

Mutant 2 (M2/CD1): 5'-CGA TGG GCA TTT CTA TGA AAA- 3'
3'-GCT ACC CGT AAA GAT ACT TTT- 3'

The wild-type and the mutant oligonucleotides were labeled (see materials and methods) and an EMSA carried out. Fig. 5.2.6 shows the differences in binding by Brn-3b protein on the three oligonucleotides. As also shown previously, incubation of 3box1 cellular extracts with ^{32}P labeled oligonucleotide probe corresponding to the wild-type probe (WT/CD1) resulted in formation of a specific complex (lane 1). Interestingly, incubation of labeled M1/CD1 mutant probe with neuroblastoma cellular extract (3box1) did not give rise to any specific Brn-3b protein/DNA complex (lane 2, 3, 4). However, incubation of 3box1 cellular extract with the second mutant (M2/CD1) resulted in a specific complex formation (lane 5), similar to the WT/CD1. This was further confirmed by the addition of specific competitor (lane 6) and non-specific competitor (lane 7). These results indicate that 5'- TTT -3' in this sequence is critical for binding of the Brn-3b protein to the cyclin D1 promoter and suggests that the sequence 5'- A TTT CTA T -3' is likely to be the

consensus octamer site bound by this protein in the cyclin D1 promoter. However, further studies will need to be undertaken to confirm the significance of these findings by testing the effect of inserting such mutations in the full length promoter using co-transfection studies.

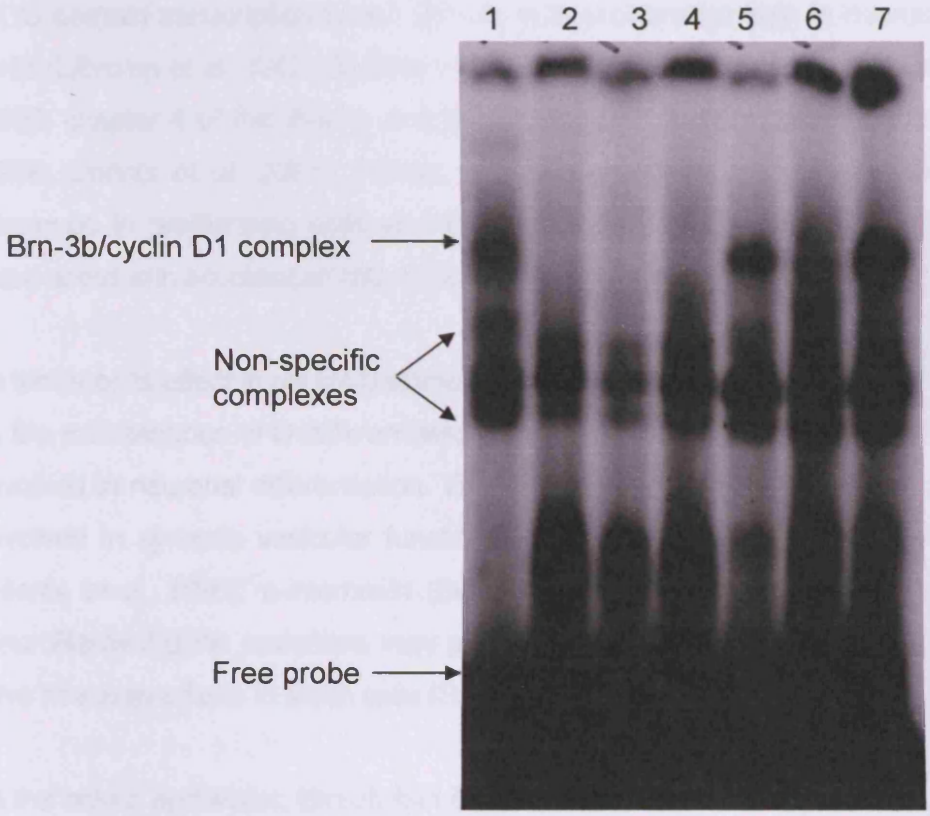


Fig. 5.2.6: EMSA undertaken to test whether Brn-3b binding to cyclin D1 promoter was compromised by mutating two A/T rich sub-sites within the putative Brn-3b site

Wild-type (WT/CD1) and mutant (M1/CD1; M2/CD1) oligonucleotides were labeled with ^{32}P and then incubated with neuroblastoma cellular extract over-expressing Brn-3b (3box1). The complex was then analysed on a non-denaturing PAGE gel and radio-labelled oligonucleotides then visualised by autoradiography.

Lane1: 3box1 cell lysate + labeled WT/CD1

Lane 2: 3box1 cell lysate + labeled M1/CD1

Lane 3: 3box1 cell lysate + labeled M1/CD1 + unlabelled M1/CD1 (150-fold molar excess)

Lane 4: 3box1 cell lysate + labeled M1/CD1 + unlabelled M1/CD1 (200-fold molar excess)

Lane 5: 3box1 cell lysate + labeled M2/CD1

Lane 6: 3box1 cell lysate + labeled M2/CD1 + unlabelled M1/CD1 (100-fold molar excess)

Lane 7: 3box1 cell lysate + labeled M2/CD1 + non-specific competitor ERE

5.3 Discussion

One of the acquired characteristic of tumour cells is their ability to divide uncontrollably. Several lines of evidence have shown a strong correlation of the POU domain transcription factor, Brn-3b, with proliferation both in neuroblastoma cells (Lillycrop et al, 1992; Budhram-Mahadeo et al, 1995a; Smith and Latchman, 1996; chapter 4 of this thesis) and breast cancer cells (Budhram-Mahadeo et al, 1998; Dennis et al, 2001). Hence, in these cell types high Brn-3b levels are observed in proliferating cells and increasing this factor has been shown to be associated with accelerated rate of cell proliferation.

In terms of its effect in neuroblastoma cells, Brn-3b has been shown to be involved in the maintenance of undifferentiated state by repressing the promoters of genes involved in neuronal differentiation. Thus its ability to repress expression of genes involved in synaptic vesicular function and structural genes, namely SNAP-25 (Morris et al, 1996), α-internexin (Budhram-Mahadeo et al, 1995) and all three neurofilament gene promoters may provide one mechanism by which Brn-3b can give rise to its effects in these cells (Smith et al, 1997c).

In the breast epithelium, Brn-3b has been shown to mediate its potent proliferative effect by directly activating the expression of CDK4 in breast cancer cells (Samady et al, 2004) whilst repressing the anti-proliferative BRCA-1 gene (Budhram-Mahadeo et al, 1999). However, Brn-3b can also modify gene expression by increasing the estrogen-receptor mediated transcriptional activity on estrogen-receptor-element containing promoter (Budhram-Mahadeo et al, 1998). Moreover, recently Brn-3b has been shown to repress the expression of plakoglobin, which is known to mediate adhesive functions and suppress tumorigenicity in a number of tumours such as breast and neuroblastoma (Zhurinsky et al, 2000; Amitay et al, 2001). Brn-3b levels are inversely correlated to those of plakoglobin in breast tumours (Samady, personal communication). Whilst genes such as BRCA-1 and ER are more relevant to breast cancers, other factors such as CDK4 may be relevant in other cell types.

As described in chapter 3 of this thesis, altering levels of Brn-3b alone was sufficient to significantly change growth properties of neuroblastoma cells, IMR-32. Hence, increasing Brn-3b levels resulted in increased proliferation while decreasing this factor caused retarded *in vitro* and *in vivo* growth of IMR-32 cells. These results paved the way to examine whether Brn-3b could directly affect genes associated with proliferation in these cells. In particular, the cell cycle protein, cyclin D1, which is required for G1 to S transition of the cell cycle, was an attractive target since it has been showed to be involved in aberrant proliferation of cells in a number of all human neoplasia (for review see, Weinberg 1995). Even though cyclin D1 mRNA and protein levels have been shown to be over-expressed in neuroblastoma primary tumours and cell lines (Molenaar et al, 2003), these are not associated with any mutations or amplifications of the gene in these tumours. This suggests possible dysregulation of pathways that control cyclin D1 expression.

Furthermore, there are some striking similarities in the phenotypic features of Brn-3b homozygous null mutants (Gan et al, 1996) and cyclin D1 knock-out mice (Sicinski et al, 1995). Thus, both mutants are viable but have a marked reduction in the retinal cell numbers. Moreover, the effect of Brn-3b on increased proliferation in breast epithelial cells (Budhram-Mahadeo et al, 1999; Dennis et al, 2001) is mirrored by the effect of increased cyclin D1 in these cells (Zwijsen et al, 1997; 1998; Lamb et al, 2000). Moreover, both Brn-3b (Budhram-Mahadeo et al, 1998) and cyclin D1 have been shown to be over-expressed in human breast cancers (Buckley et al, 1993), further highlighting cyclin D1 as an interesting potential candidate to study with respect to being a possible transcriptional target of Brn-3b.

In this study, we showed that neuroblastoma cells manipulated to express high levels of Brn-3b showed an increase in the levels of endogenous cyclin D1 compared to the control cells. Reassuringly, the levels of endogenous cyclin D1 protein was decreased in cells with reduced levels of Brn-3b(l) and Brn-3b(s) compared to the empty vector control cells, suggesting a correlation of expression of cyclin D1 and Brn-3b. Therefore, in order to elucidate whether this apparent coincidence in the levels of these two proteins is not simply reflecting changes in cellular proliferation caused by increasing Brn-3b(s) or conversely, decreased

proliferation rate in Brn-3b anti-sense cell lines, we next tested the whether Brn-3b(l) and Brn-3b(s) could transactivate the cyclin D1 regulatory region. Results of co-transfection experiments showed that Brn-3b(l) could significantly activate the cyclin D1 promoter with a 4-5 fold increase in the promoter activity compared to the empty LTR vector control, whilst Brn-3b(s) increased this promoter activity by 2-3 fold compared to the basal activity. Hence, this suggests both isoforms of Brn-3b regulate the cyclin D1 promoter and induce it.

The ability of Brn-3b to transactivate cyclin D1 promoter was specific to this gene promoter as regulatory regions of other D-type cyclins, namely cyclin D2 and cyclin D3, were not affected by Brn-3b in similar co-transfection studies. It is interesting that during mouse development and in adult tissue, the three D-cyclins are expressed in a highly orchestrated manner in mutually exclusive cell types. The D-cyclins play similar roles in promoting cell cycle progression but exhibit high tissue specificity (Robkar and Richards, 1998; Wianny et al, 1998). Even though the three D-type cyclins share 50-60% identity in their coding region (Inaba et al, 1992; Xiong et al, 1992), analysis of the regulatory region that control the expression of each of the three D-type cyclins shows very limited homology (Blast search, NCBI). This suggests that different transcription factors may be involved in the regulation of expression of these genes. Hence, our findings (reported here) showing that Brn-3b only affects the transactivation of cyclin D1 further supports this notion.

In order to determine the region of the cyclin D1 promoter that is required for Brn-3b to mediate its transactivation effect, we tested deletion constructs in which specific regions of regulatory regions of cyclin D1 were lost. Loss of up to 377 base pairs of the promoter in the 5' region (-848 and -567 deletion constructs) did not compromise activation of the promoter by Brn-3b as the effects were similar to wild-type. However, loss of 875 bases (-69) resulted in the loss of activation of the cyclin D1 promoter by both the isoforms of Brn-3b proteins. This lead us to conclude that the region between positions -567 and -69 was critical for Brn-3b to exert its effect. Analysis of this region for possible Brn-3b consensus binding sequences was undertaken using sequences described in other studies that were shown to bind Brn-3b. This led to the identification of a closely related sequence

(5'-CGA TTT GCA TTT CTA TGA AAA-3') between -248 and -228 from the transcriptional start site in the cyclin D1 promoter. Electrophoretic mobility shift assay was used to show that Brn-3b could bind strongly and specifically to this oligonucleotide as shown by competition using both specific and non-specific competitors. Attempts to super-shift the Brn-3b/cyclin D1 oligonucleotide with commercially available Brn-3b antibody failed to affect the mobility of the complex. This may possibly be due to the masking of the epitope on the protein required for the antibody recognition during the complex formation. However, to ensure that these complexes were specific to Brn-3b, similar studies using cellular extracts from SAOS2 which do not express any endogenous Brn-3b did not give rise to these complexes. This confirmed that specific complexes were unique to and only present when Brn-3b is in the reaction.

We also identified the specific site required for recognition of this DNA sequence by Brn-3b by mutational analysis. These results showed that mutations of bases at positions -239 and -237 resulted in loss of Brn-3b binding whereas changing bases at positions -244 and -243 did not affect the binding specificity. This suggests that the core sequence of 5'-A TTT CTA TGA-3' is critical for Brn-3b to bind this region. The requirement of this sequence for Brn-3b to stimulate the expression of this promoter will need to be confirmed using site directed mutagenesis with the intact promoter. Furthermore, chromatin immunoprecipitation (ChIP) assay can also be used to confirm the association of Brn-3b protein on the cyclin D1 promoter *in vivo*. However, with the results presented in this chapter, we can suggest that the correlation of cyclin D1 expression with Brn-3b in human neuroblastoma cells may result from the ability of this transcription factor to activate transcription from this gene promoter and hence, increase expression of the cyclin D1 gene.

The ability of Brn-3b to regulate the expression of cyclin D1 promoter may give a possible mechanistic explanation for the altered growth rate observed in these neuroblastoma cells (chapter 4). Cyclin D1 regulates cell cycle, being involved in the processes such as phosphorylation and inactivation of the retinoblastoma (Rb) protein, which in turn causes the release of E2F transcription factors required to drive the cell out of the G1 restriction point and initiation of S phase of the cell cycle. Given the significant alteration in growth and proliferation upon increased

Brn-3b and decrease upon reducing endogenous expression, it is feasible to suggest that the effect on proliferation observed in manipulated Brn-3b cells may be partly due to its ability to transactivate the cyclin D1 promoter. An upregulation in cyclin D1 levels by Brn-3b may account for the increased proliferation rate in Brn-3b over-expressing cells. Furthermore, the positive regulatory effect of Brn-3b on CDK4 expression (Samady et al, 2004), a factor that associates with cyclin D1 to allow cell cycle progression, suggests a combined effect that promotes cellular proliferation.

Hence, Brn-3b might act in a complex manner, regulating tissue specific genes, as exemplified by its regulation of breast specific BRCA-1 and its effects on ER targets in breast cancers, or SNAP-25 and α-internexin in neuronal cells whilst also regulating more general targets such as those associated with the cell cycle, namely CDK4 and cyclin D1. Its activation of the CDK4 gene has been observed in breast adenocarcinoma cell lines while here we have shown its effects on cyclin D1 activation in neuroblastoma cells. It will be interesting to see if CDK4 is also activated by Brn-3b in neuroblastoma cells and whether its effects on cyclinD1 in neuroblastoma cells are paralleled in breast cells. It is also important to determine other neuronal specific targets for Brn-3b that are associated with proliferation in neuroblastoma cells by methods such as Affymetrix™, to further understand how this transcription factor exerts its potent proliferative effect in these cells.

CHAPTER 6

Discussion

6.0 General Discussion

Regulation of gene expression is controlled primarily at the level of transcription and involves an intricate networking between several intra- and extracellular factors in a spatio-temporal manner. Transcription factors are potent proteins that regulate gene expression by either activating or inhibiting production of messenger RNA. There are two types of eukaryotic transcription factors. General transcription factors which are common to all RNA polymerase II transcribed genes, while gene specific transcription factors act on a more restricted number of genes depending on the cell type and precise timing of external stimuli.

The POU domain proteins are a family of gene regulators that have evolved to contain a structurally conserved component shown to be present in organisms as divergent as *C.elegans*, *Drosophila*, *Xenopus*, zebrafish and humans (for review see Andersen and Rosenfeld, 2001). POU proteins exhibit diverse functions such as cellular homeostasis (for example, Oct-1 regulation of the housekeeping histone H2B promoter [Pierani et al, 1990]) as well as specific spatio-temporal developmental processes (for example, Pit-1 control of the prolactin and growth hormone genes in the anterior pituitary gland [Nelson et al, 1988]). This family of transcription factors has been sub-classified into six different classes, based on the degree of homology in the common structural domains. The two proteins, Brn-3a and Brn-3b, which we have studied in the context of neuroblastoma cells, belong to the class IV sub-group of POU family, and are critical for the survival and normal function of specific neuronal cell types (for review Latchman, 1999).

The initial isolation of Brn-3a from brain cDNA (He et al, 1989) and that of Brn-3b from mouse neuroblastoma cells, ND7, (Lillycrop et al, 1992) suggested a role for these factors in neuronal cells. Subsequently, other studies have highlighted the critical roles for these factors in the normal development of particular neuronal cells. For example, using mouse models in which the expression of these genes had been deleted, Brn-3a has been shown to have a distinct developmental role in the maintenance and terminal differentiation of specific neuronal cells of the CNS such as the red nucleus, as well as in the trigeminal ganglia of the PNS (McEvilly

et al, 1996; Xiang et al, 1996; Eng et al, 2001). Similarly, i*n vivo* deletion studies of Brn-3b have shown this factor to be critical in the terminal differentiation of a sub-set of retinal ganglial cells (Erkmann et al, 1996; Gan et al, 1996; 1999).

In parallel with studies determining the expression and role of Brn-3a and Brn-3b *in vivo*, extensive work has been carried out using *in vitro* models to elucidate the precise mechanisms by which these transcription factors exert their functional effects. Studies in mouse neuroblastoma cell line, ND7, have shown that Brn-3a and Brn-3b expression is regulated in opposite directions by common signaling pathways activated by factors such as cAMP or growth factors. Hence, elevated levels of Brn-3a mRNA and protein have been detected upon differentiation of ND7 cells by serum withdrawal and/or addition of cAMP. Moreover, concomitant decrease in the levels of Brn-3b is observed under these differentiating conditions. Conversely, high levels of Brn-3b mRNA and protein are expressed when ND7 cells are actively proliferating, in sharp contrast to the low levels of Brn-3a in these dividing cells (Lillycrop et al, 1992; Budhram-Mahadeo et al, 1995a). Similarly, Brn-3a and Brn-3b also exhibit reciprocal expression in human neuroblastoma cell lines, IMR-32. High levels of Brn-3a are present in differentiated cells while low levels of Brn-3b are expressed in these cells. This expression pattern is reversed in actively proliferating IMR-32 cells (Smith and Latchman, 1996).

Over-expression of Brn-3a in ND7 cells in full growth medium enhances neurite outgrowth even under conditions that would not normally promote process formation (Smith et al 1997c). This is further supported by studies in ND7 cells where anti-sense strategy has been employed to reduce the levels of Brn-3a. Under such conditions loss of Brn-3a results in the failure of extension of neurites even under differentiation promoting conditions such as serum withdrawal (Lakin et al, 1995). Furthermore, there is also a decrease in the levels of some neuronal proteins associated with differentiation, such as SNAP-25, synaptophysin, synaptotagmin I, synapsin I and neurofilament proteins, NFH, NFM and NFL in these cells with reduced levels of Brn-3a while Brn-3a over-expressing cells have a corresponding increase in the levels of these proteins (Smith et al, 1997a; 1997b). Similar studies in ND7 with increased levels of Brn-3b showed a failure of neurite outgrowth even when the cells are grown in differentiation medium (Smith et al

1997a). This contrasts with the effects of Brn-3a in these cells which is associated with mature, differentiated neuronal phenotype.

The opposite expression patterns of Brn-3a and Brn-3b in differentiating versus proliferating ND7 cells is accompanied by antagonistic effects of these factors on the activities of various promoters. While Brn-3a is a strong activator of promoter of genes associated with differentiation such as SNAP-25, synaptotagmin I, synapsin I, NFH, NFM and NFL (Smith et al, 1997a; 1997b), Brn-3b appears to repress genes expressed in differentiated neurons such as SNAP-25, α-internexin, and all three neurofilament proteins (Morris et al, 1996; Budhram-Mahadeo et al, 1995; Smith et al, 1997c). Brn-3b, like Brn-3a, activates the promoter of synapsin 1, suggestive of a certain degree of compensatory function between these two closely related transcription factors (Morris et al, 1996). However, the opposite expression pattern and antagonistic effects on most target promoters observed in ND7 cells might reflect the involvement of Brn-3a and Brn-3b in providing a fine balance between proliferation and differentiation in these cells.

The role of Brn-3b in cells of neuronal origin presents an interesting paradox. While *in vivo* knock out studies have shown Brn-3b to be critical for the survival and terminal differentiation of retinal ganglial cells, it has been shown to be associated with proliferation in neuroblastoma cell lines. The lack of abnormalities in other regions of the CNS and PNS in Brn-3b homozygous null mutants has made it difficult to elucidate the precise role for this factor in other neuronal cell types. It has been speculated that Brn-3a might actually compensate for this factor in regions where its expression precedes that of Brn-3b such as dorsal root ganglia and trigeminal ganglia of the developing PNS (Gerrero et al, 1993; Turner et al, 1994). This would suggest that Brn-3b acts in a similar manner to Brn-3a and is associated with differentiation and maturation of these neurons. While this hypothesis remains to be tested, caution is needed in generalizing the function of a protein as exemplified by other members of the POU family of transcription factors. For example, Pit-1 POU protein acts by stimulating genes associated with terminal differentiation and maturation of the anterior pituitary such as PRL, GH and TSHβ genes (Nelson et al, 1988). Simultaneously, this protein also promotes cellular proliferation in other pituitary specific cells such as somatotrophs (Li et al, 1990).

Similarly, the role of Brn-3b in preventing differentiation of neuroblastoma cells while promoting differentiation in highly specialized retinal ganglial cells suggests that Brn-3b might work in a complex manner, dependent on cell specific factors.

Since their initial identification in neuronal cells, Brn-3a and Brn-3b have also been detected in other cells such as breast, testis, prostate, heart and lungs (Turner et al, 1994; Budhram-Mahadeo et al, 2001). In fact, it was the role of these factors in breast and testis that first highlighted their potential role in tumourogenesis. Brn-3b has been shown to be elevated in several cases of sporadic breast cancers and in mammary tumours with reduced levels of the tumour suppressor gene, BRCA-1 (Budhram-Mahadeo et al, 1998; 1999). Indeed, Brn-3b represses the expression of the BRCA-1 promoter in breast cancer cell lines, suggesting that it is associated with promoting proliferation of these cells. The role of Brn-3b in breast cancers has been extensively studied and other targets for this factor in these cells have been identified and analysed. These provide some of the functional mechanisms by which high levels of Brn-3b may affect the proliferation of cells in breast cancer cells. For example, in breast adenocarcinoma cell lines, MCF7, Brn-3b activates the positive cell cycle regulator, CDK4 (Samady et al, 2004) while repressing the adhesion associated protein, plakoglobin (personal communication, Dr. L. Samady, MMBU, ICH, UCL). Furthermore, Brn-3b has also been shown to functionally interact with the estrogen receptor and hence, potentiates transcription from genes containing the oestrogen-responsive elements which are associated with growth and proliferation in breast tissue (Budhram-Mahadeo et al, 1998). Over-expression of Brn-3b in MCF7 cells results in increased proliferation and enhanced ability to grow under anchorage independent conditions compared to control cells or cells with decreased levels of Brn-3b (Dennis et al, 2001). Hence, there is strong evidence to suggest that Brn-3b promotes proliferation and invasion of breast cancer cells.

In the experiments described in this thesis, we have attempted to analyse the effects of Brn-3a and Brn-3b in human neuroblastoma cell lines, IMR-32, with a view to examine whether altering levels of these potent developmental regulators may have a role in the initiation and/or progression of neuroblastomas. Neuroblastomas are highly malignant, undifferentiated tumours originating in

neuroectodermal cells derived from the neural crest that are destined for the adrenal medulla and the sympathetic nervous system (for review see Schwab et al, 2003). It accounts for 8-10% of all paediatric malignancies, with an annual incidence of 1 in every 7,000 live births. The molecular mechanisms contributing to the onset of this disease are poorly understood and are compounded by the remarkable clinical heterogeneity. This cancer can range from life-threatening aggressive undifferentiated neuroblastoma, to less malignant tumours that differentiate to ganglioneuroblastomas or ganglioneuromas. It is interesting to note that spontaneous regression of tumours is also observed in very young infants at a high frequency.

Hence, we tested the effects of manipulating Brn-3a and Brn-3b levels by either constitutively over-expressing Brn-3a/Brn-3b or reducing their expression using anti-sense strategies in IMR-32 cells as discussed in the chapter 3 and 4. Firstly, IMR-32 cells were stably transfected with plasmids either containing the Brn-3b sense cDNA or anti-sense cDNA sequence. Having established cells with either increased or decreased levels of Brn-3b, we tested for effects on cellular growth in monolayers and under anchorage independent conditions, as well as their invasive properties and response to differentiation/apoptotic stimulus such as all-trans-retinoic acid (RA).

Altering Brn-3b protein levels affects the growth and proliferation of neuroblastoma cells

Most human neoplasias result from abnormalities in the expression of genes that participate in the control of cell proliferation and differentiation. We have shown (chapter 3 of this thesis) that alteration in the levels of Brn-3b has profound effects on the cellular growth characteristics of IMR-32 cells. Upregulation of Brn-3b(s) (and subsequent increase in Brn-3b(l) possibly via an auto-regulatory loop) results in cells which have greater proliferation rates as well as saturation densities when grown in monolayers. Conversely, cells with reduced levels of Brn-3b exhibit slower proliferation and saturation densities compared to controls. We also examined the ability of Brn-3b manipulated cells to grow tumours *in vivo,* the ultimate marker for experimental malignant cell transformation, by injecting them into athymic and immuno-compromised mice models. Mice injected with Brn-3b

over-expressing cells grow larger tumours in a significantly shorter time than controls while Brn-3b anti-sense cells do not promote tumour growth in these xenograft mice models. Therefore, increasing the levels of Brn-3b in neuroblastoma cells increased their growth and proliferation both *in vitro* as well as *in vivo*.

Brn-3b may mediate its effects on cellular proliferation by transactivating the cyclin D1 gene expression

The potent proliferative effect of Brn-3b in IMR-32 cells led us to determine a possible mechanism by which this transcription factor could cause changes in cellular growth and proliferation. In this regard, we showed for the first time that Brn-3b can transactivate cyclin D1 gene, an important regulator of the mammalian cell cycle. Expression of cyclin D1, and subsequent association with its partner CDK4, results in the phosphorylation of retinoblastoma protein. This event results in cycle progression from G1 to S phase. Recently, high expression levels of cyclin D1 mRNA and protein have been detected in two-thirds of neuroblastoma cell lines as well as primary human tumours (Molenaar et al, 2003). Other studies have also shown that regulation of cyclin D1 in neuroblastoma cells is critical for the proliferation of these cells (Munoz et al, 2003; Wainwright et al, 2001).

Cyclin D1 knock-out mice have a striking similarity to Brn-3b homozygous null mutants, such that both develop to full term but exhibit a marked reduction in the retinal ganglial cell numbers due to inability of retinal cell precursors to develop during embryogenesis (Sicinski et al, 1995; Gan et al, 1996). Additionally, independent studies have demonstrated that cyclin D1, like Brn-3b, is also over-expressed in human breast cancers (Buckley et al, 1993; Wang et al, 1994; Budhram-Mahadeo et al, 1998). Recent studies have shown Brn-3b also positively affects the CDK 4 promoter (Samady et al, 2004), which acts as a partner for cyclin D1, thereby facilitating G1-S transition.

Our studies (chapter 5) confirm high levels of cyclin D1 protein in IMR-32 cells over-expressing Brn-3b protein whereas concomitant decrease is observed in cells with reduced levels of Brn-3b (data shown in chapter 5 of this thesis). In order to determine whether these similarities result from a direct effect of Brn-3b on cyclin

D1 gene expression, we tested the effect of Brn-3b on the cyclin D1 gene promoter. Indeed, transactivation studies on cyclin D1 promoter show that both isoforms of Brn-3b are able to significantly activate this promoter. In addition, we have also shown that Brn-3b protein is able to bind directly to an octamer sequence in the cyclin D1 promoter between positions -248 and -228 from the transcriptional start site *in vitro*. Moreover, this binding is specific as base mutations between -239 and -237 region in the promoter abolishes the transactivation potential of Brn-3b on this gene. The full physiological relevance of this binding will require additional assays such as site directed mutagenesis and chromatin immunoprecipitation (ChIP), which is a powerful tool for examining the spatial and temporal mapping of chromatin-bound factors *in vivo*. Unfortunately, these could not be carried out due to limitation of time.

Hence, from the studies described in this thesis, we have shown that increasing Brn-3b levels in neuroblastoma cells has a profound effect on the growth and proliferation of these cells both *in vitro* and *in vivo*. This might partly be due to the ability of this transcription factor in transactivating the cyclin D1 gene promoter. The subsequent increase in the levels of cyclin D1, a well-known cell cycle regulator, would allow transition of cells from G1 to S phase and hence promote continued cell division. This is an interesting finding in neuroblastoma cell lines, providing a potentially important mechanism for understanding the aberrant proliferation observed in the neural crest derived tumours in neuroblastoma patients.

Overexpression of Brn-3b causes anchorage independence and enhanced invasion of neuroblastoma cells

Metastasis of cancer cells to distant sites is a severe and generally fatal consequence of tumour progression. In neuroblastomas metastatic disease occurs in approximately 70% of patients. This is indicative of advanced stage of the disease and poor prognosis. The metastatic process is a dynamic process that begins with tumour cell migration by which tumour cells leave the primary site of growth, often penetrating the basement membrane and moving towards the local vasculature. This is followed by intravasation, which describes the process of tumour cell entry into the vasculature, and distribution to distant sites. Subsequently, tumour cells need to extravasate into the surrounding tissue,

survive in the foreign microenvironment, proliferate, and induce angiogenesis, all the while evading apoptotic death or immunological response (for review see Liotta and Stetler-Stevenson, 1993).

In vitro assays for cellular transformation and invasiveness have used the ability of transformed cells to grow on 'soft-agarose' and migration through a reconstituted basement membrane as an indication that cells with these abilities have metastatic potential. Hence, we tested the ability of IMR-32 cells with altered levels of Brn-3b to grow anchorage independently by growing them on soft agar. Wild-type IMR-32 cells require a solid surface on which to flatten out and divide, and therefore, exhibit modest growth on soft-agar, which affords only moderate opportunity to adhere and spread. However, IMR-32 cells with elevated levels of Brn-3b continue to proliferate and form colonies when grown in 0.3% soft agar. Conversely, IMR-32 cells with reduced levels of Brn-3b protein resulted in smaller colonies with markedly reduced proliferative potential. Furthermore, analysis of migration of Brn-3b modified IMR-32 cells through an artificial Matrigel Basement Membrane (BD Biocoat Matrigel™) showed that over-expressing Brn-3b in these cells increased their invasive properties while cells with reduced levels of this factor exhibited reduced migration through a matrix compared to control cells.

These observations are important given the highly metastatic nature of neuroblastoma tumours, suggesting that if Brn-3b levels are aberrantly expressed in primary neuroblastoma tumours, it might affect the metastatic potential of the cancerous cells. Direct mechanisms that contribute to the various stages in metastasis in neuroblastoma are poorly understood at present. However, contributions of other studies aimed at understanding the molecular and cellular processes of cell matrix organization, cell-cell interactions, cell matrix adhesion, migration, cell proliferation, and angiogenesis have highlighted potential mechanisms that may contribute to the metastasis process in cancers. It will be interesting to determine the specific Brn-3b targets in any of these processes such as cell adhesion molecules in neuroblastoma cells, and given the recent finding of Brn-3b expression in trunck neural crest cells (personal communication, Hudson C, ICH, UCL) it is possible that Brn-3b might be involved in the epithelial-mesenchymal cell transition, a well-established event known to occur during

developmentally regulated migration of neural crest cells, (for review see Tucker, 2004), which might contribute to the enhanced migratory properties of neuroblastoma cells. Furthermore, some light is beginning to be shed on the signaling pathways involved in anchorage dependence and it seems that PKA, PAK and the MAP kinases play important roles (Howe and Juliano, 2000), and therefore, it will be interesting to determine the involvement of Brn-3b in these pathways in neuroblastoma cells.

Brn-3b overexpression overcomes differentiation signals induced by all-trans-retinoic acid

Furthermore, data presented in chapter 3 confirms a significant difference between cells with different levels of Brn-3b in their ability to respond to RA compared to controls. All-trans-retinoic acid (RA) is generally growth inhibitory and causes neuroblastoma cell lines to differentiate or undergo apoptosis (Chu et al, 2003; Nagai et al, 2004; Bernardini et al, 1999). While Brn-3b anti-sense cells ceased to proliferate after exposure to this differentiating agent, in a manner comparable to the control cells, Brn-3b over-expressing cells fail to respond to this stimulus and continue to proliferate even when grown in the presence of RA. These results suggest that high levels of Brn-3b can overcome differentiation signals induced by RA in human neuroblastoma cells.

The role of Brn-3a in proliferation, differentiation and apoptosis of IMR-32 cells

Experiments described in chapter 4 of this thesis focus on the effects of Brn-3a in IMR-32 cells. After generating constitutively over-expressing Brn-3a clonal cells, we tested for any change in their growth properties, as well as response to differentiation and apoptotic stimuli such as RA and cisplatin, respectively. Introduction of a plasmid containing Brn-3a cDNA in an anti-sense orientation into IMR-32 cells failed to generate clones that survived in culture. These could be explained by the role of Brn-3a in protecting neurons in culture ascertained in other studies. For instance, increasing levels of this factor in ND7 cells and primary cultures of trigeminal and dorsal root ganglia can protect these neuronal cells against apoptotic stimuli such as withdrawal of nerve growth factor from the medium and sciatic nerve lesions (Smith et al, 1998a; 2001; Ensor et al, 2001).

Conversely, decreasing the levels of Brn-3a causes increased death against the same apoptotic stimuli. In fact, other studies have shown Brn-3a to directly transactivate the anti-apoptotic Bcl-2 promoter in neuronal cells (Smith et al, 1998a; 1998b; Ensor et al, 2001) while it represses the p53-mediated activation of pro-apoptotic factors Bax and Noxa (Budhram-Mahadeo et al, 2003; Hudson et al, 2004). Hence, the requirement of Brn-3a for survival of neuronal cells could help to understand the death of all IMR-32 cells transfected with Brn-3a anti-sense plasmid.

Whilst we managed to obtain and propagate four clonal cells over-expressing Brn-3a, over 75% of transfected cells failed to grow and therefore could not be adequately propagated. It is possible that in our studies neuroblast cells over-expressing Brn-3a failed to proliferate because of the known effect this transcription factor has in inducing differentiation. Hence, cells over-expressing this factor and hence undergoing differentiation must have ceased to proliferate and subsequently, died in culture (Lakin et al, 1995; Smith et al, 1997c; Smith and Latchman, 1996). Furthermore, we utilised a non-homologous recombination technique and, therefore, it is also possible that the clones over-expressing Brn-3a that were propagated, had a growth advantage due to the random site of integration of the plasmid. Random integration of Brn-3a plasmid into the cellular genome may have disrupted a critical gene(s) involved in differentiation and/or survival of IMR-32 cells. A general consensus to overcome this problem is to carry out analyses with more than two clonal cell lines that are selected randomly from a larger pool of positive clones. However, this choice was strictly limited with Brn-3a manipulated clonal cells as only a total of four Brn-3a over-expressing clones survived. Therefore, this reduced our chance of selecting cells that had no disruptions in any of the genes.

Based on the effects observed in other neuronal cells in previous studies, we expected to find that over-expressing Brn-3a in IMR-32 cells would enhance differentiation of these cells whilst increasing survival to apoptotic stimuli. Analysis of rate of growth and proliferation shows that Brn-3a does not alter the rate of growth in these cells. Similarly, we did not observe any significant morphological differences between Brn-3a over-expressing and control cells. Brn-3a over-

expressing cells do not demonstrate altered response upon treatment with differentiation inducing agent, RA, or with apoptosis inducing cisplatin as no significant difference in survival is observed between Brn-3a over-expressing and control cells. In light of the role of Brn-3a in differentiation and survival described in other studies, it is important to repeat these analyses in an inducible stable system, such that Brn-3a expression can be switched on and off upon administration of specific substrate such as doxycyclin. This would overcome the problem of propagation due to the failure of transfected cells to proliferate when Brn-3a levels are either constitutively elevated or decreased.

Summary

In conclusion, the results in this thesis have highlighted the potent proliferative role of Brn-3b as well as enhancing invasive properties of human neuroblastoma cells. In addition, we have also shown that Brn-3b mediates this effect by activating the cyclin D1 gene promoter, causing increased cell cycle progression.

Given the strong proliferative effect of Brn-3b in IMR-32 neuroblastoma cell lines, it is reasonable to speculate that this transcription factor may play a critical role in determining the growth characteristics of neuroblastoma tumours when it is elevated. In addition, Brn-3b expression may serve as a marker to identify a poor prognostic factor for a sub-group of neuroblastomas. Its correlation with the induction of cyclin D1 expression in neuroblastoma cells suggests its possible role as:

a) a prognosticator of neuroblastomas, and

b) a direct or indirect target to develop therapeutic strategies against Brn-3b. A direct strategy would be to ablate its expression while specific targeting of the octamer sequences in the cyclin D1 promoter (required for the positive regulation of cyclinD1 by Brn-3b) could also, indirectly, affect the function of Brn-3b in neuroblastoma cells.

Future Work

It will be interesting to establish if Brn-3b protein levels are altered in a cell cycle dependent manner that might precede the onset of cyclin D1 expression. To this

end, current studies are ongoing in our laboratory aiming at arresting human neuroblastoma cell lines (IMR-32 and SHSY5Y) at specific phases of the cell cycle by using strategies such as treating the cells with inhibitors (e.g. hydroxyurea, fluorouracil and colchicine), or re-initiating the cell cycle after serum withdrawal. Harvesting RNA and proteins from these cells will allow determination of the levels of the Brn-3b at different phases of the cell cycle in relation to known cell cycle markers such as PCNA and cyclin E, and facilitate correlation with the expression of cyclin D1. Alternatively, it is possible that Brn-3b levels remain steady but this protein is subject to post-translational modifications, such as phosphorylation, that might be needed to transactivate targets such as cyclin D1 gene promoter. Results from such studies should help to identify the mechanism of regulation of cyclin D1 by Brn-3b in a physiological context.

In order to determine the importance of this transcription factor in proliferation of neuroblastoma tumours, we need to analyse its expression and function in primary human samples in order to make our data clinically relevant. Preliminary studies carried out by Dr. Budhram-Mahadeo in our laboratory (MMBU, ICH, UCL) have shown a significant increase in the levels of Brn-3b mRNA compared to Brn-3a in 18 primary neurobastoma tumour biopsies. These findings are encouraging but larger cohort studies are required to fully determine the role of these transcription factors in the neuroblastoma tumours.

Special Footnote

Subsequent to the submission and acceptance of this thesis some conflicting evidence has emerged to suggest that the IMR-32 cells used in our research may not be of human origin. IMR-32 cells were obtained from American Type Culture Collection, who characterised these as human neuroblastoma cell lines derived from an abdominal mass removed from a 13-month-old-Caucasian male. Recent preliminary karyotyping carried out by Dr. Tracy Warr's group (Department of Molecular Neuroscience, Institute of Neurology, London) has shown these IMR-32 cells to be tetraploid and led the group to suggest that they may, in fact, be of rat origin. However, in-depth analysis of these cells needs to be carried out using species-specific probes to unequivocally determine the true origin of these cells. At present, it is therefore premature to amend any of the conclusions made in this thesis.

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