CRE Mediated Regulation of the CGRP and Bcl-2 Genes in PC12 Cells

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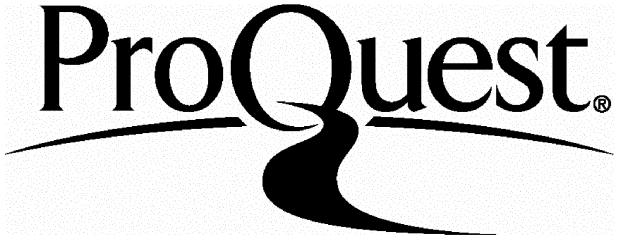

I dedicate this work to my parents and beloved husband in thanks for their unfailing encouragement and support.

ABSTRACT

The calcitonin/calcitonin gene-related peptide (CT/CGRP) gene is expressed in sympathetic and sensory neurons in response to the neurotrophin nerve growth factor (NGF) and the second messenger cAMP. It is believed that the effects of these stimuli are mediated by the transcription factor CREB, which is activated by phosphorylation at serine 133, an event that can be induced by many different intracellular signalling mechanisms. CREB binds constitutively to a cAMP response element (CRE) within the CT/CGRP gene promoter. This CRE site is necessary and sufficient for promoter activation by cAMP and is necessary but not sufficient for activation by NGF. This study shows that this difference is paralleled by a difference in the signalling pathways required for each stimulus to activate the CT/CGRP promoter. Whilst the effects of cAMP are mediated by protein kinase A signalling, NGF-mediated promoter stimulation requires the activation of Ras/Raf/mitogen-activated protein kinase kinases 1 and 2 (MEK-1/MEK-2) and the p42/p44 mitogen-activated protein (MAP) kinases. Other signalling pathways activated by NGF, such as protein kinase C and p38 MAP kinase, are not involved in the NGF response.

CREB has also been implicated in mediating hypoxia-induced tyrosine hydroxylase (TH) promoter activation. The Bcl-2 promoter, which contains a CRE site, is responsive to NGF and hypoxia in certain neuronal cells. This study has shown that the Bcl-2 CRE is responsive to hypoxia in PC12 cells, although the Bcl-2 promoter is not. Indeed, TH, Bcl-2 and CT/CGRP CRE sites respond differentially to NGF, cAMP and hypoxia, and the responses exhibited by the three CRE sites when linked to a heterologous promoter can differ from those they exhibit in the context of their respective promoters. Some of these differences can be attributed to the sequences of the individual CRE sites, whilst others can be attributed to the proteins which bind to them.

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Abbreviations

A	Alanine
Amp	Ampicillin
APS	Ammonium Persulphate
CAMK	Calcium/Calmodulin-dependent Protein Kinase
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB-binding protein
CGRP	Calcitonin Gene-related Peptide
CNS	Central Nervous System
CRE	Cyclic AMP Responsive Element
CREB	CRE-binding Protein
CT	Calcitonin
DAG	Diacylglycerol
db cAMP	Dibutyryl Cyclic AMP
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DRG	Dorsal Root Ganglion
DTT	Dithiothreitol
E	Glutamic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular Signal-regulated Kinase
F	Phenylalanine
FGF	Fibroblast Growth Factor
GEF	Guanine Nucleotide Exchange Factor
HBS	HEPES-buffered Saline
HEPES	*N* – 2 – Hydroxyethylpiperazine – *N'* – 2 – Ethane Sulphonic Acid
IP_3	Inositol Triphosphate
JNK	c-Jun N-terminal Protein Kinase
K	Lysine
LiCl	Lithium Chloride
MAPK	Mitogen-activated Protein Kinase

MAPKK	MAPK Kinase
MAPKKK	MAPKK Kinase
MEK	Mitogen-activated Protein Kinase Kinase
MeOH	Methanol
mRNA	Messenger RNA
MSK	Mitogen- and Stress-activated Kinase
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
Neo	Neomycin
NGF	Nerve Growth Factor
PBS	Phosphate-buffered Saline
PBS-T	PBS-Tween 20
PDGF	Platelet-derived Growth Factor
PIP_2	Phosphatidyl Inositol 4, 5 - Biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl Fluoride
PNS	Peripheral Nervous System
R	Arginine
RNA	Ribonucleic Acid
RSK	p90 kDa Ribosomal S6 Kinases/MAPK-activated Protein Kinase
SAPK	Stress-activated Protein Kinase
SDS	Sodium Dodecyl Sulphate
T	Threonine
TAE	Tris-acetate/EDTA Buffer
TBE	Tris-/EDTA Buffer
TBS	Tris-buffered Saline
TE	Tris/EDTA
TEMED	N, N, N', N' - tetramethylethylenediamine
TH	Tyrosine Hydroxylase
TLC	Thin Layer Chromatography
UV	Ultraviolet
Y	Tyrosine

List of Publications

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1 Introduction

1.1 Regulation of Gene Expression

1.1.1 Eukaryotic Transcription - A General Overview

A multicellular organism is made up of many different cell types, most of which contain the whole complement of DNA that makes up the genome of that organism. Despite this, each cell will have its own specific and variable pattern of gene transcription, mRNA processing and protein synthesis, with strict control mechanisms regulating these processes throughout development and adult life. This control over gene expression allows the formation of the diverse cell and tissue types that make up the whole organism and it is crucial for both individual cell function and for the well being of the organism as a whole.

In eukaryotic cells, regulation of gene expression can occur at a number of different levels. For instance, gene activity can be influenced by chromosome structure as can be seen in mammalian female development, where either one of the X-chromosomes found in somatic cells is inactivated by heterochromatisation (Lyon, 1992; Lee and Jaenisch, 1997). Gene regulation can also occur at a post-transcriptional level during RNA processing. In the case of the calcitonin/CGRP gene, a single transcribed messenger RNA can give rise to two different proteins in different tissue types. Tissue-specific alternative splicing of the message causes synthesis of calcitonin in thyroidal cells and CGRP (calcitonin gene-related peptide) in certain neuronal cells (Rosenfeld *et al*, 1983). However, the primary control mechanism for gene regulation is at the level of transcription. This regulation of gene expression occurs in a cell-specific manner and is mediated by specific proteins that respond to environmental signals by modifying the rate of transcription of their target genes. These protein factors, known as transcription factors, bind to specific control elements found in promoter sequences located in the DNA directly adjacent to a gene and, depending on their function, interact with other transcription factors and/or the basic transcription machinery to either elevate or reduce the level of gene transcription (Latchman, 1998a).

1.1.2 Gene Promoter Sequences

Promoters are those DNA sequences generally found upstream of a gene that contain the regulatory elements required for the transcription of that gene. With respect to transcription, there are three different classes of eukaryotic genes and these gene classes have distinct promoters, each recognised by a specific RNA polymerase. RNA polymerases are those enzymes able to produce complementary RNA sequences from a DNA template by the polymerisation of ribonucleotides. All eukaryotic RNA polymerases are large proteins made up of many subunits, several of which are conserved between enzymes (Woychik *et al*, 1990). RNA polymerase I is responsible for the transcription of genes encoding 28S, 18S and 5.8S ribosomal RNA (rRNA) (Jacob, 1995). RNA polymerase II synthesises the messenger RNA (mRNA), which is subsequently translated into protein (Young, 1991). Finally, RNA polymerase III synthesises transfer RNA (tRNA), as well as small nuclear RNA molecules and 5S rRNA (Gabrielson and Sentenac, 1991). Certain promoter elements are crucial for gene transcription and interact with the basic transcription machinery. These are often found near the transcription initiation site. RNA polymerases I and II and their accessory factors have recognition sites upstream of the transcription initiation site, whilst RNA polymerase III and its accessory proteins may have recognition sites either upstream or downstream of the start point (Latchman, 1998b). However, as well as constitutive promoter elements, promoters recognised by RNA polymerase II also contain other *cis*-acting regulatory elements found only in specific sets of genes. These elements tend to be located further upstream from the transcription initiation site and bind to specific transcription factors. Transcription by RNA polymerase II will be further discussed below, since protein coding is the most subject to regulation.

1.1.3 RNA Polymerase II and Basal Gene Transcription

RNA polymerase cannot initiate transcription alone and requires certain accessory proteins in order to function. The enzyme and accessory factors together make up the basic transcription machinery that is required for the initiation of transcription from a generic promoter, i.e., a promoter containing the minimum sequence from which RNA polymerase II is able to initiate transcription. Sequences that are able to bind RNA polymerase II and the basal transcription machinery are highly conserved throughout many promoters. Most eukaryotic promoters have a TATA box, so-called because its six base pair consensus sequence consists entirely of the

bases adenine and thymidine (5'-TATAAA-3'). This is generally located ~25 base pairs upstream of the initiation site and tends to be surrounded by GC-rich sequences. Single base pair substitutions in the TATA box act as strong down mutations indicating that it is necessary for promoter function (Breathnach and Chambon, 1981). Experiments have shown that deletion of the TATA box causes the positioning of the transcription initiation site to become erratic and often those promoters lacking TATA boxes have multiple initiation sites. TATA-less promoters tend to be those promoters directing the transcription of general housekeeping genes that are expressed in all tissues (Weis and Reinberg, 1992). In place of the TATA box these genes have a sequence known as the initiator element located over the transcription start site. This element appears to aid in determining the transcription initiation point and acts as a minimal promoter to produce basal levels of transcription.

As stated previously, RNA polymerase II requires certain accessory factors in order to function. These factors must assemble into a pre-initiation complex in a defined order at the promoter in order to recruit RNA polymerase II to the initiation site and activate transcription (Nikolov and Burley, 1997). Two alternative pathways for complex assembly have been described and are illustrated in figure 1.1. The first and most rate-limiting step of transcription complex assembly *in vivo*, is the binding of the transcription factor TFIID complex to the TATA sequence of a promoter (Klages and Strubin, 1995). TFIID consists of TBP, the TATA-binding protein (Hernandez, 1993), and TBP-associated factors (TAFs). Whilst TBP binding of DNA is sufficient for basal level transcription by RNA polymerase II, TAF co-activators are important in transcription factor activated transcription (Verrijzer *et al*, 1995). Those gene promoters lacking a TATA box are still able to recruit TBP to the DNA, through interactions with another protein factor that binds to the initiator element at the start site (Weis and Reinberg, 1992).

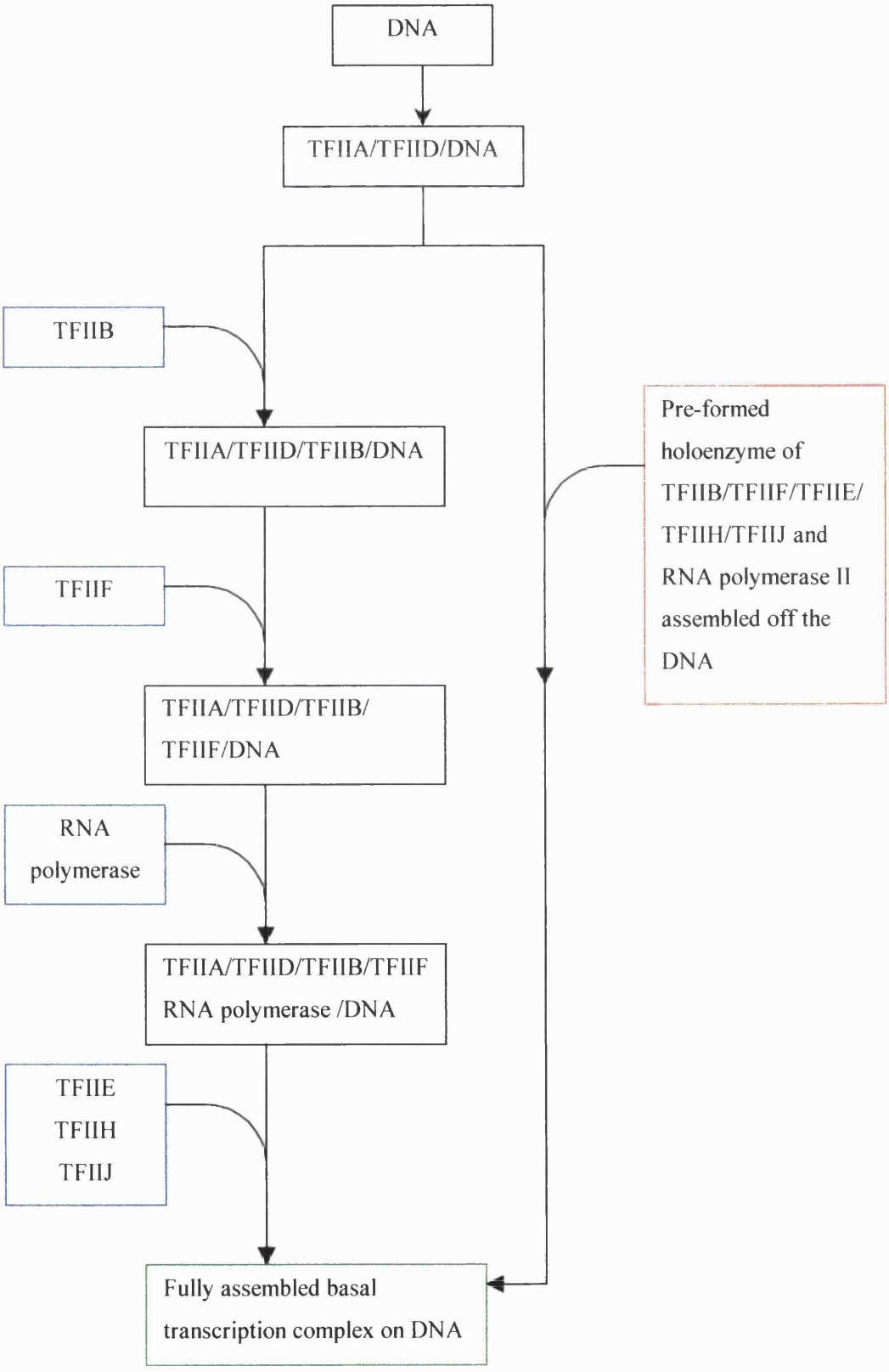
Once TFIID is bound to the TATA element, TFIIA is brought into the complex and enhances the DNA binding affinity of TFIID (Jacobson and Tijan, 1996). It is thought that TFIIA dissociates a repressor protein from TBP (Cortes *et al*, 1992) and, in the step-wise pathway for complex assembly, TFIIB is then able to bind TFIID to form the TATA/TBP/TFIIB ternary complex. RNA polymerase II is then able to recognise the complex and is recruited together with TFIIF thus bringing the polymerase enzyme to the DNA. The pre-initiation complex is then able to bind three further factors known as TFIIE, TFIIH and TFIIJ. TFIIH appears to be critical for transcription initiation (Hoeijmakers *et al*, 1996). TFIIH has a kinase activity that is able to phosphorylate the C-terminus of the largest RNA polymerase II subunit, and it is thought that this phosphorylation allows RNA polymerase II to dissociate from the rest of the transcription machinery and begin the elongation phase of transcription

Fig. 1.1 – Alternative Pathways for the Assembly of the Basal Transcription Complex for RNA Polymerase II

Initially TFIID binds to the DNA promoter region and forms a complex with TFIIA. In the stepwise pathway for complex assembly TFIIB, TFIIF and RNA polymerase II are then sequentially recruited to the DNA-bound complex. Finally they are joined by TFIIE, TFIIH and TFIIJ to complete the formation of the basal transcription complex.

In the alternative pathway for complex assembly, rather than a step-wise recruitment of proteins, it is proposed that a pre-existing complex of RNA polymerase II and its associated factors is recruited to the DNA-bound complex of TFIID and TFIIA.

Adapted from Latchman, 1998a.


DNA
TFIIA/TFIID/DNA
TFIIB
TFIIA/TFIID/TFIIB/DNA
TFIIF
TFIIA/TFIID/TFIIB/
TFIIF/DNA
RNA
polymerase
TFIIA/TFIID/TFIIB/TFIIF
RNA polymerase /DNA
TFIIE
TFIIH
TFIIJ
Fully assembled basal
transcription complex on DNA
Pre-formed
holoenzyme of
TFIIB/TFIIF/TFIIE/
TFIIH/TFIIJ and
RNA polymerase II
assembled off the
DNA

(Orphaneides *et al*, 1996). Recent experiments have also suggested that a pre-assembly complex of the RNA polymerase II and its associated proteins exists within cells prior to DNA binding (Pugh, 1996). Rather than the stepwise assembly of the basal transcription complex as described above, an alternative pathway for the formation of the complex has been proposed. In a wide variety of organisms, RNA polymerase II has now been found to be associated with TFIIB, TFIIF, TFIIH and other factors in the absence of DNA, as a complex known as the RNA polymerase holoenzyme. It is thought that, in some cases, the holoenzyme binds to the TFIIA/TFIID/DNA complex, so reducing the number of steps required for pre-initiation complex assembly (Pugh, 1996).

In order for transcription to proceed the RNA polymerase II must dissociate from the pre-initiation complex so that it can move along the gene sequence transcribing the DNA into RNA. The subsequent basal level of gene transcription occurs at a low level in all cell types, however transcription levels of specific genes within specific cell types can be elevated or depressed by regulating the speed and efficiency of the pre-initiation complex formation. This level of regulation is achieved by the binding of various *trans*-acting transcription factors to *cis*-acting gene promoter elements, often located far upstream of the start site. Once bound to the DNA these factors are then able to interact, either directly or indirectly through other proteins, with the forming pre-initiation complex or RNA polymerase itself to direct gene expression.

1.1.4 Transcriptional Regulation by Transcription Factors

Whilst TATA sequences are responsible for regulation of transcription with respect to the initiation site, other sequences have been shown to be responsible for the efficiency and frequency with which transcription is initiated. These elements tend to be located in enhancers far upstream of the initiation site, although the position of these elements relative to the start site can vary considerably between different genes. Some regulatory elements can even be found in the introns of certain genes (Latchman, 1998b). Each gene promoter has its own unique combination of regulatory elements and its activation will be determined by the expression pattern of the transcription factors that bind to them. For instance, immunoglobulin genes carry enhancers located downstream of a promoter, which are only active in the B-lymphocytes where immunoglobulins are expressed (Garcia *et al*, 1986).

Most of the known activating transcription factors have a modular structure and often function as homo- or heterodimers. They generally consist of a DNA-binding domain, which binds to specific DNA regulatory sequences, and an activation domain, which interacts with other proteins to modulate components of the basic transcription machinery. Common DNA-binding motifs include helix-turn-helix, zinc finger and basic regions (Latchman, 1998b). Dimers often interact through leucine zippers and helix-loop-helix motifs. The regulatory elements are often palindromic in their sequences and can usually function in either orientation. Mutations introduced into these sequences can greatly affect their function and thus reduce the rate of transcription from a promoter. Also gene manipulation experiments have shown that these sequences can stimulate expression from any basic promoter in their vicinity when inserted near to the start site, a property exploited in many promoter studies, including those for CGRP described later (Watson *et al*, 1995; Watson and Latchman, 1995). However, transcription factors are only able to bind to DNA that is transcriptionally active. In its inactive state DNA is packaged around histones to form nucleosome structures (Morse and Simpson, 1988), which are condensed into tightly packed chromatin (Felsenfeld and McGhee, 1988), making the DNA inaccessible to transcription factors. De-acetylation of histones appears to play an important role in the activation of DNA (Turner, 1993) and transcriptionally active regions of DNA have been shown to highly depleted in histone H1 (Nacheva *et al*, 1989). Indeed, part of the TFIID transcription complex crucial for the basal transcription of many genes has been shown to exhibit histone acetyltransferase (HAT) activity (Pennisi, 1997). The cAMP-sensitive transcription factor co-activator CBP also has HAT activity that has been implicated in its ability to activate transcription (Ogryzkio *et al*, 1996).

Once bound to the DNA transcription factors can function in a variety of different ways. They may induce conformational changes in protein structures, or exert kinetic effects to influence the stability and assembly of the complex. Motifs commonly found in activation domains include acidic, proline-rich, glutamine-rich and isoleucine-rich regions. The transcriptional activator SP1 appears to function by indirectly interacting with the factor TBP within the pre-initiation complex. The activation domain of SP1 is rich in glutamine and binds to the glutamine-rich domain of TAFII 110 (Gill *et al*, 1994), which in turn interacts with the TAFII 250 TBP-associated factor (Weinzerl *et al*, 1993). *In vitro* studies have shown that SP1 can increase basal level transcription by at least ten-fold. The CNS transcription factor NTF-1 has an isoleucine-rich motif within its activation domain and also targets TAFII 110 as well as TAFII 150 (Chen *et al*, 1994). The strong activator VP16 from the herpes simplex virus targets TAFII 40 through an acidic region in its activation domain (Goodrich *et al*, 1993).

The switching on of a specific transcription factor can have a dramatic effect on a cell. The MyoD1 transcription factor is normally only expressed in muscle cells. However, when MyoD1 is activated in fibroblasts, the cells undergo a dramatic change and differentiate into muscle cells (Buckingham, 1994). This is due to a cascade effect, as transcription factors often regulate the expression of a whole range of other regulatory proteins and, in some cases, even regulate their own expression.

The rate of gene transcription can be reduced as well as elevated, although such regulation is less defined in eukaryotes. Inhibitor proteins, or repressors, can mediate their effects either directly by de-stabilising the pre-initiation complex, or indirectly by compromising the DNA-binding/activation domains required for transcription or competing with activators for their binding sites (Hanna-Rose and Hansen, 1996).

In summary transcription factors are those proteins required for the initiation of transcription that are not part of the enzyme RNA polymerase. These include both factors associated with RNA polymerase in the basal transcription apparatus, as well as those proteins that interact either directly or indirectly with the basal apparatus to increase the rate of transcription. They recognise *cis*-acting sites in promoters although their binding capability is not limited to DNA. They may interact with other transcription factors, co-activator proteins, RNA polymerase, or be recruited into the initiation complex when linked to another protein. The most common form of regulation by transcription factors seems to be positive, although specific repression of a target promoter also occurs. The combination of active transcription factors present within a nucleus at any time is regulated in a tissue-specific manner, and results in the activation of gene expression from a specific promoter or range of promoters containing a specific target sequence e.g., the TATA box. In other words a transcription factor is a protein required for transcription to occur at a specific promoter or range of promoters. However, rather than having a single control element, most gene promoters have a number of different regulatory elements within their sequences. The transcription factors to which they bind may exert their effects additively, synergistically or antagonistically, and it is the combinatorial effect of those factors present within a cell that allows the fine-tuning and highly specific control of gene expression in different cell types and during development.

1.2 The Nervous System

1.2.1 The Central and Peripheral Nervous Systems

The nervous system is a complex network of cells that extends throughout the body providing a rapid channel for communication. It consists of a variety different cell types, many of which have not yet been characterised and their functions not yet understood. The nervous system functions to accumulate and process information that has been received from all over the body, and converts it into a complex pattern of signals which govern not only the behaviour of a single cell, but also that of the organism as a whole.

The nervous system of vertebrates is composed of two different networks. The cells of the brain and spinal cord are known as the central nervous system. The peripheral nervous system consists of nerves, sensory organs and ganglia, which are able to act as a communications network between the body's many different organs and tissues and the CNS.

1.2.2 Early Development

In early development, after gastrulation, the embryo is made up of three distinguishable layers of tissue. The endodermal layer develops into the digestive tract and all the glands associated with it. The mesoderm divides into three parts. Those cells defining the central axis of the embryo give rise to the notochord, which develops into the spinal column of vertebrates. Those cells to the left and right of the notochord go on to form connective tissues, muscle, bone, cartilage, the dermis and many of the structures forming the urogenital and vascular systems, including cardiac muscle and blood cells. The third layer formed after gastrulation, known as the ectoderm, covers the surface of the embryo and gives rise to both the epidermis and entire nervous system (Alberts *et al*, 1989; Larsen, 1998; Wolpert, 1998).

Neurulation of the embryo is initiated when a large group of cells from the central region of the ectoderm interact with the notocord and mesoderm to thicken, roll up into a tubular formation and break away from the remaining cells of the ectoderm (Alberts *et al*, 1989; Brown *et al* 1991; Wolpert, 1998). The cells of the newly formed neural tube will go on to differentiate into the cells of the brain and spinal cord. Cells from the region of ectoderm that

became separated from the neural tube make up the neural crest (Bronner-Fraser, 1994). These cells migrate away from the ectoderm and through the mesoderm as individuals, where they will give rise to the peripheral nervous system, including the sensory and sympathetic ganglia, glia, certain endocrine cells and skin pigment cells (Brown *et al*, 1991; Anderson, 1994; Anderson, 1997).

The next stage of nervous system development involves the establishment a rudimentary network by the directed extension of axons and dendrites. These connections allow cells to communicate and interact with, and influence, each other as well as their local environment. By receiving and transmitting electrical information the cells can then refine and modify their connections to complete nervous system development, a process that continues into adulthood (Brown *et al*, 1991; Wolpert, 1998).

1.2.3 Cells of the Nervous System

In order to be able to receive information and transmit information over long distances, neurons are highly specialised and elongated cells. They consist of a cell body, which contains the nucleus and is the site of neurotransmitter synthesis, a single long axon for transmitting the signal to its target away from the cell body, and many smaller dendrites extending from the cell body. Dendrites provide a surface for receiving information from neighbouring cells, whilst the synaptic terminal of the axon splits into a number of branches designed to pass information to a target cell by secreting neurotransmitters. All the many different types of neuron that make up the nervous system have their own characteristic distribution of dendrites and terminal branches (Alberts *et al*, 1989; Ycajal, 1995).

Within the nervous system nerve cell bodies cluster together to form ganglia, whilst their axons cluster together to form the nerves. Sensory information from peripheral organs is relayed to the CNS through the ganglia and along the axons of sensory neurons whose synaptic termini are linked to the CNS. Sensory organs include the ears, eyes, taste buds, the nose and the different sensory receptors found in skin, muscle and viscera. Conversely motor signals are relayed from the CNS to the peripheral organs via the ganglia and axons of motor nerves whose synapses terminate in those organs. Although the function of most ganglia is to relay information, certain autonomic ganglia do have control functions and work independently of the CNS, an example being the enteric ganglia that control the peristaltic

contractions of smooth muscle in the gut (Alberts *et al*, 1989; Peters *et al*, 1991; Ycajal, 1995; Wolpert, 1998).

As well as neurons, the nervous system also consists of large numbers of accessory glial cells (Trotter, 1993; Price, 1994; Jessen and Richardson, 1996). These make up the majority of neuronal tissue and vastly outnumber the neurons present. Glial cells fill in the spaces between neurons and surround both axons and cell bodies. Despite the fact that most of these cells share the same developmental origins as neurons, glia are generally not electrically excitable and therefore are able to insulate neurons from each other to prevent undesirable cross-talk between them, and to control their local environment. Oligodendrocytes of the CNS and Schwann cells of the PNS (Mirsky and Jessen, 1999) wrap themselves around axons to provide a protective layer known as the myelin sheath. Also, whilst neuronal cells can no longer divide once differentiated, most glial cells can continue to divide throughout their life span.

Most neuronal cells are derived from the same progenitor cells and yet they are able to develop into many different cell types with highly diversified phenotypes and functions. This is because each neuron has followed a specific differentiation pathway determined by its location and the combination of different extracellular developmental factors directing transcription within the cell. Extracellular signals will stimulate particular intracellular signalling cascades to produce a precisely timed pattern of protein expression. Thus the constantly changing protein expression pattern within the cell will continually alter the behaviour of the cell so that it achieves its own specific pattern of migration and differentiation. For this to be attained the regulatory mechanisms of development must be very strictly controlled.

1.2.4 The Effects of NGF on Developing Sensory and Sympathetic Neurons

The factors responsible for neuronal development and survival are known as neurotrophins (Barbacid, 1995; Lewin and Barde, 1996). They are synthesised and secreted from a number of different cell types and include molecules such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophic factor-3 (NT-3). Each neurotrophin will only promote the survival of certain neuronal populations. They bind to specific receptors located on the cell surface of those neurons, and trigger a specific network of intracellular signal transduction pathways (Mattson, 1998). Through the presence of neurotrophic

factors each neuron is able to successfully migrate towards and form a synapse with its target cell. Once this has been achieved then any redundant nerve cells within a population undergo apoptosis (Deshmukh and Johnson, 1997). NGF is considered the prototypic neurotrophic factor and was first discovered in 1953 (Levi-Montalcini, 1987; Hamburger, 1993). The protein consists of five non-covalently-linked subunits and has a molecular weight of 130kD. The β-subunit consists of two identical 118-residue amino acid chains and is the only subunit displaying neurotrophic activity (Server and Shooter, 1977). NGF has been shown to be very important in promoting the growth and survival of certain neurons in the central nervous system as well as for sensory and sympathetic neurons (Thoenen and Barde, 1980; Mattson, 1998).

NGF is expressed in the skin and any tissue innervated by sympathetic and neural crest-derived sensory neurons to guide axons into making correct connections (Levi-Montalcini, 1987). The salivary (submaxillary) gland of the male mouse also produces large quantities of NGF, making it relatively easy to obtain and study (Cohen, 1960). Studies in knockout mice have shown that approximately 70-80% of sensory and sympathetic neurons are totally dependent upon NGF for their survival during development. Without NGF the mice lose these cells whilst other neuronal cells remain unaffected (Crowley *et al*, 1994). Sensory neurons are those cells responsible for relaying sensory information from the periphery to the CNS whilst sympathetic neurons are a subclass of the peripheral autonomic neurons responsible for smooth muscle control and exocrine gland secretion. When anti-NGF antibodies are injected into mouse neonates whilst the nervous system is still developing, most sympathetic and many sensory neurons will die (Johnson *et al*, 1980). If grown in culture these cells would also normally die, but when NGF is added to the cell culture medium, the cells retain the ability to survive (Barde, 1989; Hamburger 1993). As stated above, during normal development many nerve cells within a population will apoptose. In the case of sensory neurons, this is more than half of the original population (Barde, 1989; Hamburger 1993). If, however, NGF is administered to the developing nervous system of an embryo, then certain sensory and sympathetic cells that would normally die are able to survive (Deshmukh and Johnson, 1997). A second effect of NGF is to promote neurite outgrowth, as the presence of NGF in tissues will promote axon innervation. When neurons are cultured in the presence of NGF they will extend long processes over the course of a few hours. Adult cells, although no longer dependent upon NGF for survival, will also extend more and longer processes in response to NGF (Anderson, 1993).

1.2.5 Neuronal Differentiation

Before a neuronal cell can function properly it must first differentiate into an electrically excitable cell capable of releasing neurotransmitters to relay electrical impulses. Differentiating neurons must extend processes, sometimes at great distances from the cell body, into target tissues. The extension of neurites is strictly controlled by environmental influences and intracellular transcription factors. The neurotrophin NGF is an important regulatory factor in neurite outgrowth, and acts as a stimulator for growth cone activity. NGF switches on a number of intracellular signalling pathways, which in turn activate the transcription factors and transcription of genes required for neurite outgrowth. These and other signalling pathways involved in differentiation have been extensively studied and are further discussed in section 1.4.

Growth of axons and dendrites occurs from the tips of these processes, a region known as the growth cone. Locomotion of the growth cone is achieved using actin and myosin filaments, whilst microtubules and their associated proteins aid in the translocation of proteins synthesised in the cell body to the axon for elongation (Mandelkow and Mandelkow, 1995). The elongated axon is then densely packed with cytoskeletal filaments (Cleveland, 1996) and the cell synthesises many structural proteins. Cell adhesion is also very important to the developing neurite as axons often travel long distances and come into contact with a variety of different surfaces. Neuronal-CAM, or N-CAM, and other cell adhesion molecules are expressed at the growth cone and used to interact with the adhesion molecules of other cells that the innervating neuron encounters. The CAMs have also been implicated in the induction of neurite outgrowth (Williams *et al*, 1994a; Williams *et al*, 1994b). Neurites can also be guided by other factors in the extracellular matrix. For instance netrins are able to attract the growth cones of certain neurites and repel others (Hong *et al*, 1999; Hopker *et al*, 1999). Collapsin prevents neurite elongation by inducing growth cone collapse (Luo *et al*, 1993; Jin and Strittmatter, 1997).

Thus the axons of neuronal cells are guided through the tissues they innervate. Their environment is constantly sending signals to the cell body to strictly control the pattern of proteins they express and to successfully complete their development. Neurons are no longer able to divide once they have differentiated, and no stem cells remain to generate more, although some re-modelling of damaged axons may occur.

1.3 Cells Suitable for Studying the Development and Function of Neuronal Cells

1.3.1 Primary Cultures and Cell Lines

Many cell lines have been established from murine and human neuroblastomas, as well as the rat CNS, in order to provide a model system for the study of the nervous system at the cellular level. This is because cell lines are generally easier to culture and manipulate than primary cultures extracted from animal tissue. Although sensory neurons can be extracted from rat dorsal root ganglia with relative ease, it is difficult to obtain a homogenous culture. Cultures are easily contaminated with non-neuronal cells that continue to divide and increase in number, possibly affecting the conditions of the experiment. Cell lines can usually be cultured for many passages and stored as frozen stocks, so rapidly providing the large number of cells required for gene manipulation experiments.

1.3.2 The Pheochromocytoma Cell Line PC12

PC12 cells are a cell line derived from adrenal chromaffin cells used as a model system for the study of sympathetic neurons. PC12 cells have been used frequently and with success for many promoter studies of NGF-regulated genes including those involving CRE-mediated CGRP promoter activation (Watson *et al*, 1995).

The PC12 cell line was cloned from a transplantable rat pheochromocytoma by Lloyd Greene and Arthur Tischler in 1976 (Greene and Tischler, 1976). PC12 cells display many of the characteristics of their parent adrenal chromaffin cells as well as of the neoplastic pheochromocytoma. They have a near diploid chromosome number of 40 and synthesize, store and are capable of releasing a number of neurotransmitters, largely dopamine and norepinephrine, but not epinephrine. However, although neither normal nor neoplastic chromaffin cells respond to nerve growth factor or extend processes *in vivo*, after exposure to NGF, PC12 cells undergo a dramatic phenotypic change and acquire a number of the characteristics of sympathetic neurons. Chromaffin cells and sympathetic ganglia neurons share a common progenitor cell. Thus it seems that the PC12 cell has the pluripotency of this progenitor stem cell from the embryonic neuronal crest, and that NGF will stimulate its differentiation into a neuronal phenotype. NGF treated PC12 cells no longer proliferate, they

extend neurites, become electrically excitable and show a change in composition similar to that seen in neuronal differentiation (Greene and Tischler, 1976). Hence these cells have proven to be useful in the study of neuronal function and differentiation. PC12 cells also respond to a number of other neurotrophic factors and differentiate in the presence of the signalling compound cyclic adenosine monophosphate (cAMP) (Garrels and Schubert, 1979). The effects of cAMP and NGF stimulation on PC12 cells have been extensively studied, together with the intracellular signalling pathways that they activate. This is discussed in greater detail in section 1.4.

Their responsiveness to cAMP and NGF, and their sympathetic neuron-like phenotype, makes PC12 cells an ideal model for NGF- and cAMP-sensitive promoter studies. However there are limitations to the cell system, which must be considered. Like all proliferating cells, PC12 cells can undergo spontaneous mutations in culture, so providing the potential for creating a heterogeneous culture. To minimise the risk of this the cells are grown for only a limited number of passages. It is also possible that a sub-population may be selected that has lost a property of particular interest. Thus there may be a wide variation of cells used in different laboratories.

1.4 Cellular Signalling

1.4.1 A General Overview

The development of a multicellular organism is a highly complex process and requires strict regulation of cell growth and division, differentiation, cell function and tissue formation. As stated previously, regulation of gene expression is mediated by specific proteins that respond to environmental signals by modifying the rate of transcription of their target genes. In order for all these processes to be successfully co-ordinated the cells making up that organism must communicate with each other throughout development. Cells may have direct contact with each other, either through the expression of cell-surface signalling molecules (Snyder, 1985) or by gap junctions that allow exchange of small molecules (Caveney, 1985; Pitts and Finbow, 1986). Alternatively, cells can secrete various signalling molecules (Snyder, 1985). These messenger molecules can travel longer distances between cells, and bind to target cell surface receptors in order to relay their message. This last method of communication falls into three different categories, which vary in the distances that the signalling molecules are able to travel. In the endocrine system specialised cells are able to secrete hormones, which are widely distributed throughout the body in the bloodstream. Paracrine signalling involves the secretion of local chemical mediators, which exert an effect only on their immediate environment as they are rapidly taken up or destroyed (Heldin and Purton, 1996). Finally, in the nervous system a cell can secrete neurotransmitters across the synaptic cleft to its direct neighbour in order to transmit an electrical impulse (Smith and Augustine, 1988). Although many of the same signalling molecules are used as messengers in all three forms of signalling, the difference between these systems lies largely in the speed of the response and the types of signalling molecules present within the target cells.

In order to exert effects such as secretion, cell division or differentiation the signalling molecules must transmit their message to the nucleus of a cell and alter the DNA expression pattern of that cell. This can be achieved in many ways. For instance, hydrophobic signalling molecules, such as steroid hormones, are lipid soluble and able to traverse the plasma and, in some cases, nuclear membranes to exert their effects directly. Steroid hormones bind to the C-terminal domain of their steroid hormone receptors and the receptors undergo a transformation into an active state. The induced conformational change reveals a central DNA-binding domain and an N-terminal activation domain. Gene promoter sequences containing DNA-binding sites (hormone-response elements) for these receptors will bind the

active receptor and the receptor can modulate the rate of transcription from that gene (Yamamoto, 1985). In the case of thyroid hormone-regulated genes, the thyroid hormone receptor can bind to its DNA binding sites even in the absence of the hormone. A specific inhibitory domain of the thyroid receptor inhibits gene expression in the absence of ligand, but when bound to thyroid hormone the receptor undergoes a conformational change to expose an activation domain. The activation domain is then able to bind certain co-activator proteins and activate transcription (Latchman, 1998a). This primary response to the signal will often result in the transcription of other regulatory proteins such as transcription factors or co-activator proteins, leading to a cascade of activation and repression of other genes. Thus the extracellular signalling molecule can modify the expression pattern of a cell.

Unlike the lipid-soluble messengers, secreted water-soluble signalling molecules (such as neurotransmitters, certain hormones and growth factors), cannot traverse the plasma membrane. They must exert their influence on the expression pattern of a cell more indirectly by binding to a specific receptor on the target cell surface. Certain macromolecules achieve entry into a cell by receptor-mediated endocytosis. Growth factors such as EGF bind to their cell-surface receptors and the receptor-ligand complex is then carried into the cell via endocytosis of clathrin-coated pits (Smythe and Warren, 1991). Other macromolecules bind to target receptors that are linked to the intracellular signalling machinery (Heldin and Purton, 1996); ligand binding activates specific receptors and triggers the activation of signalling cascades that transmit the message to the transcriptional machinery. This use of a cascade mechanism allows the signal to be amplified many times before the signal even reaches the nucleus. One steroid hormone molecule can activate one receptor, which can bind to one DNA-binding site to activate a single gene. The cascade system however allows a single molecule of e.g., growth factor to activate a single receptor, which is able to activate a large number of second messenger molecules in the cell through enzyme activity. In fact, each time an enzyme in the cascade is activated, usually by phosphorylation with a kinase, it is able to activate many molecules of substrate, so amplifying the signal at every stage in the cascade.

This system of signalling requires strict regulation at every stage in the cascade. Most cells are able to regulate levels of second messengers by reversibly 'adapting' to high concentrations of signalling ligand. Rather than responding to an absolute concentration of a signalling molecule, cells can adjust their sensitivity to that molecule and respond rather to changes in its concentration. For instance, the binding of a ligand may induce internalisation of receptors, so reducing the number of receptors at the cell surface (Beguinot *et al*, 1984). Secondly, the receptors and enzymes involved are rapidly inactivatable, often by dephosphorylation with a phosphatase (Sun and Tonks, 1994).

In this way, cells are constantly receiving a whole variety of different signals from their environment, each exerting their own effects on the cell, and it is this combination of signalling events that will determine the pattern of gene expression.

1.4.2 Cell-Surface Receptor Proteins

Cell-surface receptors are proteins found on the target cell plasma membrane that can bind a signalling molecule, or ligand, with high affinity and convey this event to the intracellular signalling machinery. A single signalling molecule may exert very different effects on different target cell types depending on the type of cell-surface receptor to which it is binding and/or the intracellular signalling machinery to which the receptor is linked. The number and distribution of different receptors on a cell's surface varies greatly from cell to cell and will partly determine the form of the signal transmitted, the effects being either transient or sustained for longer periods, depending on the intracellular signalling cascades involved.

There are many different classes of cell-surface receptors. For example, channel-linked receptors are transmitter-gated ion channels that are mainly involved in rapid synaptic signalling between electrically excitable cells (Alberts *et al*, 1989). The channels of these receptors can be transiently opened or closed by their specific neurotransmitter ligands to modulate the ion permeability of the plasma membrane and thus the excitability of the post-synaptic cell.

Protein serine/threonine kinase receptors have serine/threonine kinase intracellular domains. The first to be identified was a transmembrane protein in *C. elegans* in 1990 (Georgi *et al*, 1990) and since then over a dozen others have been cloned. The serine/threonine kinase receptors bind members of the transforming growth factor (TGF)-β superfamily of proteins and appear to be involved in cell-cycle control (Sherr, 1995).

The receptors most relevant to this study are the receptor tyrosine kinases and G-protein-coupled receptors. As their name suggests, receptor tyrosine kinases are transmembrane proteins with tyrosine kinase activity in their cytoplasmic domains (Kavanaugh and Williams, 1996). The extracellular domain of these receptors binds to ligands such as the growth factors NGF, FGF and PDGF. The transmembrane domain acts as a lipid anchor and may also have a role in receptor function. For instance, mutations in the transmembrane domain of the FGF

receptor have been associated with achondroplasia (Rousseau *et al*, 1994). The catalytic domain is responsible for the activation of certain intracellular signalling compounds by phosphorylation. Receptor tyrosine kinases are involved in the activation of a number of signalling pathways, including the Ras (Pronk and Bos, 1994) and PI3 kinase (Coughlin *et al*, 1989) signalling cascades.

G-protein-linked receptors were first identified in 1977 (Lin *et al*, 1977). Ligands binding to their extracellular domains include acetylcholine, epinephrine and substance P (Cascieri *et al*, 1996). The transmembrane domain is made up of seven transmembrane helices, and the cytoplasmic activation domain is linked to a GTP-binding regulatory protein. The G-protein linked receptors often act by causing a change in the concentration of certain intracellular messengers e.g., cyclic adenosine monophosphate (Northup *et al*, 1980) and the calcium divalent cation Ca^{2+} (Berridge, 1993). Consequent phosphorylation events caused by the activation of specific protein kinases or phosphatases will then transmit the signal to the nucleus. The second messengers cAMP and Ca^{2+} will now be discussed.

1.4.3 Cyclic Adenosine Monophosphate and Protein Kinase A Signalling

The secondary messenger molecule cyclic adenosine monophosphate, or cAMP, was first identified in 1959 as the intracellular mediator that could activate glycogen breakdown in skeletal muscle and liver in response to the hormone epinephrine (Alberts *et al*, 1989). Since then it has been shown to be essential for the regulation of many intracellular reactions in all animal cells. Cellular responses mediated by cAMP include thyroid hormone synthesis and secretion in response to thyroid-stimulating hormone, increase in heart rate in response to epinephrine, and progesterone secretion in the ovaries in response to luteinising hormone (Alberts *et al*, 1989). Cyclic AMP signalling is also important in proliferation of the anterior pituitary somatotroph cells (Struthers *et al*, 1991), follicle stimulating hormone regulation of spermatogenesis (Foulkes *et al*, 1993), circadian rhythms (Ginty *et al*, 1993) and memory (Huang *et al*, 1994).

In order to function successfully as a signalling compound, cellular levels of cAMP must be readily alterable for it to cause rapid responses to extracellular stimuli. The primary regulator of cellular cAMP concentration is the plasma-membrane-bound enzyme adenyl cyclase. In animal cells adenyl cyclase is activated by stimulatory G-proteins (G_s) and inhibited by inhibitory G-proteins (G_I). These G-proteins are linked to specific cell-surface receptors that

recognise specific extracellular ligands (McKnight *et al*, 1988). When ligands such as the hormones glucagon, epinephrine and vasopressin and the neurotransmitter acetycholine, bind to their G-protein-linked receptors, the stimulatory G-protein simultaneously exchanges a bound molecule of GDP for GTP (Collins *et al*, 1992). This changes the protein structure and enables it to associate with and activate adenyl cyclase as long as the GTP is bound. However, the G-protein is a GTPase and will eventually hydrolyse the GTP to GDP to terminate the adenyl cyclase activation. The adenyl cyclase enzyme catalyses the conversion of ATP to cAMP, causing levels of cAMP within the cell to rise (Taussig and Gilman, 1995). In order to create a transient signal a rapid increase in cAMP concentration must be followed by a rapid decrease, and so cAMP is continually destroyed by the cyclic AMP phosphodiesterases which hydrolyse it to 5'-AMP (Charbonneau, 1990).

The most important target of cAMP signalling is the serine/threonine kinase known as cAMP-dependent protein kinase (PKA). PKA is a tetrameric enzyme consisting of two regulatory subunits and two catalytic subunits. As the intracellular levels of cAMP rise, cAMP binds to the two regulatory subunits of the enzyme, so activating and releasing the enzyme's two catalytic subunits (Krebs and Beavo, 1979; McKnight *et al*, 1988). Substrates for PKA include many regulatory proteins, such as glycogen synthase, pyruvate kinase, and tyrosine hydroxylase (Heldin and Purton, 1996). Another known target of PKA is the transcription factor CREB, which regulates the transcription of cAMP-sensitive genes (Karin and Smeal, 1992). The active regulatory subunits of PKA are able to migrate to the nucleus of a cell in order to phosphorylate this and other nuclear proteins (Mellon *et al*, 1989). The cAMP/protein kinase A signalling pathway is illustrated in figure 1.2.

Of the CREB/ATF family of transcription factors (see section 1.6), CREB, CREM and ATF-1 can be phosphorylated by PKA directly (Sassone-Corsi, 1995). These proteins bind constitutively to specific sequences within the promoters of cAMP-sensitive genes (CRE sites, see below) and become active when phosphorylated; therefore phosphorylation of these transcription factors by PKA will modify their effects upon gene transcription. Thus cAMP is able to mediate the regulation of cAMP-sensitive genes.

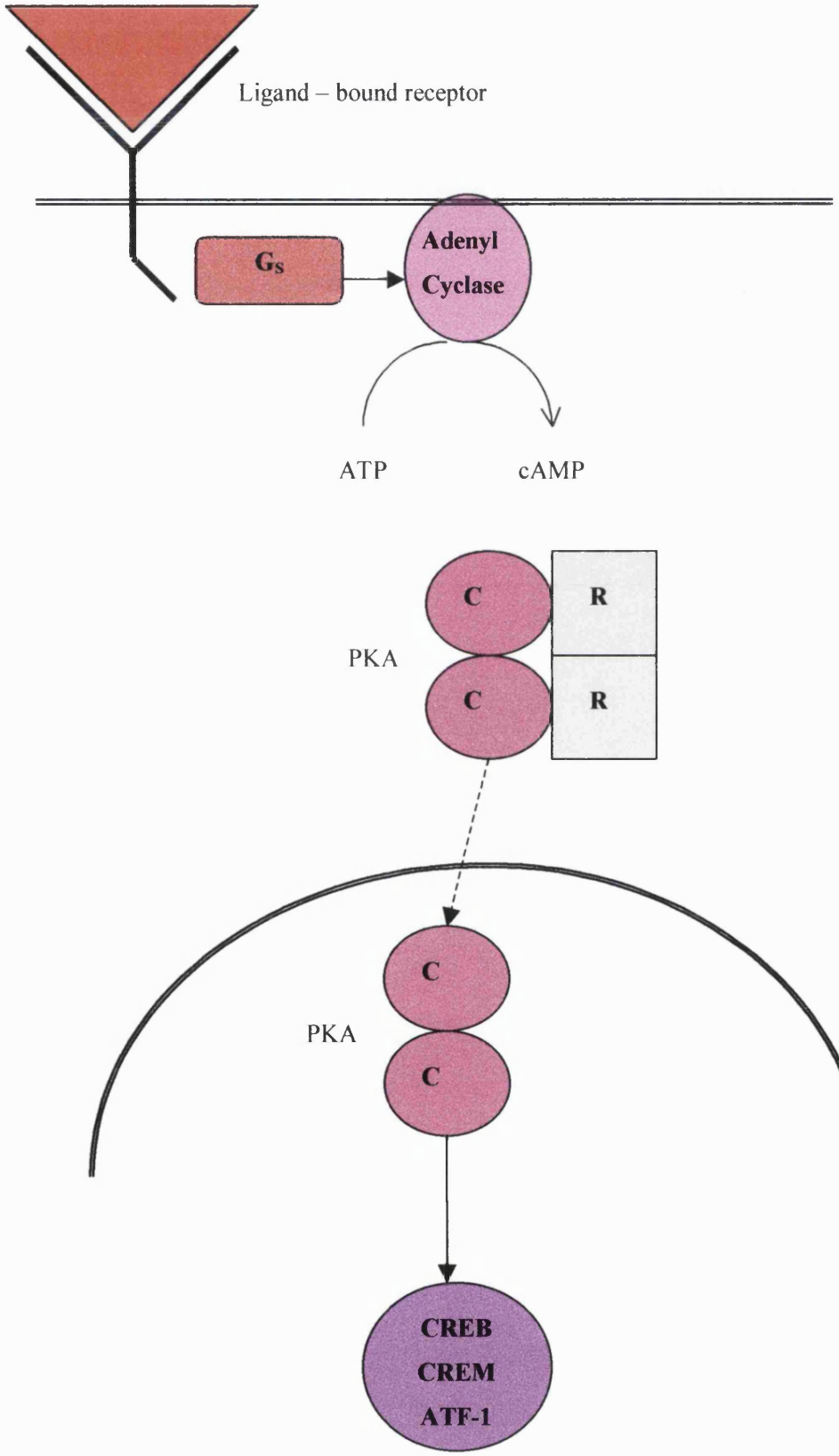
One of the first genes found to be responsive to cAMP was that of somatostatin (Andrisani and Dixon, 1990). Experiments in the pheochromocytoma-derived PC12 cell line showed that expression of a gene fused to the somatostatin promoter was inducible by cAMP (Montminy *et al*, 1986a) and occurred in wild-type cells, but not in protein kinase A-deficient cells (Montminy *et al*, 1986b). Since then many other cAMP-sensitive genes and their protein

products, such as c-fos, tyrosine hydroxylase, parathyroid hormone and calcitonin/calcitonin gene-related peptide, have been identified in many different cell types.

Genes responsive to cAMP have a common element in their promoter sequences. This sequence is known as the cAMP response element (CRE) and consists of the 8bp palindromic consensus sequence 5'-TGACGTCA-3'. Many genes containing CRE sites have been identified, for instance the genes encoding proenkephalin, fibronectin and c-jun (Sassone-Corsi, 1995); however such genes are not necessarily cAMP-inducible since other intracellular signalling cascades also regulate CREB and the ATF transcription factors.

Fig. 1.2 – Cyclic AMP-mediated Transcription Factor Activation

When certain hormones and neurotransmitters bind to their cell-surface receptors, G-proteins linked to those receptors activate the membrane-bound enzyme adenyl cyclase. Adenyl cyclase catalyses the conversion of ATP to cyclic AMP and thereby increases the concentration of cyclic AMP within the cell. Cyclic AMP binds to the tetrameric enzyme PKA and this causes the regulatory (R) subunits of the enzyme to separate from the catalytic (C) subunits. The active catalytic subunits of PKA are then able to migrate to the nucleus and phosphorylate the transcription factors CREB, CREM and ATF-1.


Ligand – bound receptor
Gs
Adenyl
Cyclase
ATP
cAMP
C
R
PKA
C
R
C
PKA
C
CREB
CREM
ATF-1

1.4.4 Calcium Signalling and the Calmodulin-dependent Protein Kinases

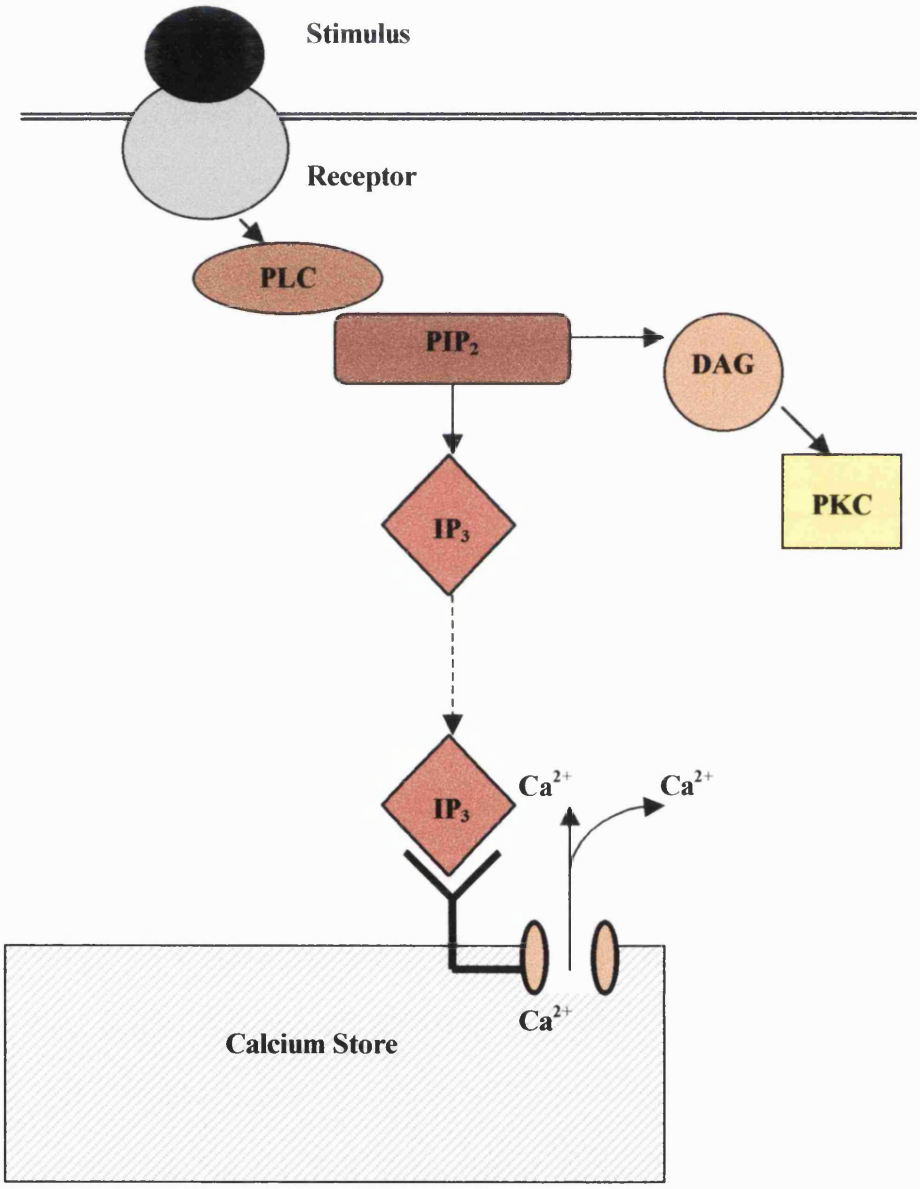
In 1947 it was demonstrated that Ca^{2+} could act as an intracellular mediator of signal transduction when skeletal muscle was induced to contract after injection with a small amount of the cation (Heilbrunn and Wiercenski, 1947). Since then Ca^{2+} has been implicated in a wide variety of cellular responses including neuronal excitability, secretion and proliferation.

In a normal eukaryotic cell the concentration of free Ca^{2+} inside the cytosol is much lower than that in the environment surrounding the cell, creating a steep concentration gradient, which would drive Ca^{2+} into the cell. In many cells a second gradient exists between the cytosol and the endoplasmic reticulum (or sarcoplasmic reticulum in muscle), which is able to sequester cellular calcium (Heldin and Purton, 1996). Calcium signalling is dependent upon the concentration of cytoplasmic calcium. When an extracellular signal causes the cell to open its membrane Ca^{2+} channels, Ca^{2+} is driven into the cytoplasm and the intracellular concentration increases, so activating any Ca^{2+} sensitive mechanisms. To switch the signal off, the cytoplasmic Ca^{2+} must be pumped back out of the cell or into the intracellular storage compartments.

There are many types of plasma membrane calcium channels including those operated by membrane depolarisation (voltage-operated channels) and those operated by specific receptors (McCleskey, 1994; Clapham, 1995). Intracellular calcium channels belong to one of two families: inositol 1,4,5-triphosphate receptors and ryanodine receptors (Berridge, 1993; Meissner, 1994). Activation of these receptors can occur by two different mechanisms. In skeletal muscle a signal is sent by direct protein-protein interactions from the cell surface to the receptor (Meissner, 1994). The alternative pathway involves the activation of second messengers by G-proteins. When extracellular signalling molecules, such as certain hormones, bind to cell surface receptors that are linked to low-molecular-mass G-proteins, the enzyme phospholipase C is activated. Phospholipase C will then cleave the plasma membrane lipid phosphatidyl inositol 4,5-biphosphate (PIP_2) to give diacylglycerol and inositol triphosphate (IP_3). Diacylglycerol goes on to activate protein kinase C (PKC), which is discussed in section 1.4.9. IP_3 diffuses into the cytoplasm and binds to its receptor on the calcium-sequestering compartment to open gated-channels and cause an influx of Ca^{2+} to the cytosol (Berridge, 1993). This is illustrated in figure 1.3. Ryanodine receptors are activated by cellular NAD^+ (Lee, 1994). Elevating levels of calcium itself can also trigger the activation of

Fig. 1.3 – Release of Calcium from Intracellular Stores

Receptors linked to phospholipase C (PLC) are able to activate the enzyme when bound by their extracellular ligand. PLC cleaves PIP_2 to generate diacylglycerol (DAG) and inositol triphosphate (IP_3). Whilst DAG activates the enzyme protein kinase C (PKC), IP_3 diffuses through the cytoplasm and binds to IP_3 receptors located in the membranes of intracellular calcium storage compartments. The transmembrane domain of the activated receptor forms a channel, thus allowing the release of calcium from the compartment to raise the intracellular calcium concentration.


Stimulus
Receptor
PLC
PIP2
DAG
PKC
IP3
IP3
Ca2+
Ca2+
Ca2+
Calcium Store

intracellular calcium channels.

However, to ensure that the calcium signal is transient, there are also mechanisms for the rapid removal of the cation. The normal intracellular Ca^{2+} concentration is maintained at low levels by the activity of Ca^{2+} channels in the plasma membrane, which are able to pump Ca^{2+} into the extracellular fluid in an ATP-dependent manner (Carafoli, 1994). ATP-dependent channels also exist in the sarco/endoplasmic reticulum membrane (Pozzan *et al*, 1994). As Ca^{2+} signalling is particularly important in nerve and muscle cells, these cells often have an additional set of specialised plasma membrane channels that are able to couple the efflux of Ca^{2+} with the influx of sodium ions, known as Na^{+}/Ca^{2+} exchangers (Philipson and Nicoll, 1992). Intracellular messengers such as IP_3 are down-regulated by the actions of a phosphatase.

When the concentration of Ca^{2+} of a cell is high, the Ca^{2+} is able to form complexes with several Ca^{2+}-binding proteins, the most important of which is calmodulin. Calmodulin is ubiquitously expressed and can account for as much as 1% of a cell's protein composition. It acts as a type of intracellular Ca^{2+} receptor that changes conformation on Ca^{2+} binding and is then able to interact with other proteins such as kinases and transport proteins (Alberts *et al*, 1989). The primary targets of Ca^{2+}-bound calmodulin are the Ca^{2+}/calmodulin-dependent protein kinases (CAMK), which are a group of serine/threonine protein kinases that can have very specific substrates (e.g., myosin light chain kinase) or exert more general effects on the cell (e.g., CAMKII and CAMKIV). The distinct types of CAMKs present in the target cell will determine the type of response the cell gives to the increase in cytosolic Ca^{2+} (Heldin and Purton, 1996).

To add further complexity to the calcium and cAMP signalling pathways, Ca^{2+} and cAMP are able to interact with each other. For instance, in some cells CAMKs are able to alter the activities of adenyl cyclase and cAMP phosphodiesterase to alter the levels of intracellular cAMP. Protein kinase A and cAMP can also regulate certain Ca^{2+} channels found in cellular membranes, as well as being able to phosphorylate certain CAMKs (Heldin and Purton, 1996). Some cellular proteins are substrates for both PKA and CAMKs and so can be regulated by both Ca^{2+} and cAMP. For instance, CREB is activated effectively by both PKA and CAMKIV, for instance (Sun *et al*, 1994). In neuronal cells CAMKIV is activated and translocated to the nucleus and phosphorylates CREB at serine 133 in response to electrical excitation (Ghosh *et al*, 1994; Deisseroth *et al*, 1998).

1.4.5 The Involvement of Growth Factors in Cellular Signalling

The second messenger signalling pathways described above are largely activated by hormones, neurotransmitters and membrane depolarisation. However, growth factors such as fibroblast growth factor (FGF), nerve growth factor (NGF) and epidermal growth factor (EGF) are also able to trigger many other receptor-mediated intracellular signalling cascades.

Growth factors are molecules, which influence cell growth, differentiation and survival. For instance, FGF is involved in directing the division of many cell types including endothelial cells, fibroblasts and myoblasts. EGF promotes proliferation in epidermal cells and other cell types (Alberts *et al*, 1989). NGF is important in promoting the survival and development of certain neurons (Thoenen and Barde, 1980), and is able to stimulate neurite outgrowth (Anderson, 1993). The growth factors are able to direct tissue specific gene expression by binding to receptors expressed on the surface of their target cells and transmitting a signal through the cytoplasm to the nuclear transcription factors (Cadena and Gill, 1992). Different growth factors can activate similar signalling pathways within their target cells and yet exert very different effects upon that cell. Both EGF and NGF, for example, are able to activate the signalling molecules Ras and p42/p44 MAP kinase in PC12 cells. However the effects of EGF are transient whereas NGF can persistently activate the signalling cascade over a much longer period (Marshall, 1995).

The many different growth factors stimulate very similar signalling cascades within a variety of cell and tissue types. Signalling molecules have also proved to have homologues in many species from yeast to man. However, for the purposes of this study, only the effects of NGF on the intracellular signalling cascades of mammalian neuronal cells will be discussed.

1.4.6 Nerve Growth Factor and Intracellular Signalling

As described in section 1.2.4, NGF is a neurotrophin important for the growth and survival of certain sensory and sympathetic neurons. It was the first neurotrophin to be identified and the mechanisms by which it exerts its effects have been extensively studied in the pheochromocytoma cell line PC12. NGF stimulation of PC12 cells induces a dramatic change in phenotype; the cells extend neurites, become electrically excitable, cease to proliferate and differentiate into a sympathetic neuron-like phenotype over several days (Greene and

Tischler, 1976). The extended processes are very similar to those of sympathetic neurons, and NGF withdrawal causes rapid neurite degeneration.

In order to promote complex cellular processes such as differentiation and neurite outgrowth NGF interacts with specific receptors in the plasma membrane that can relay signals to the nucleus. There are two known NGF receptors, the low affinity neurotrophin receptor $p75^{NTR}$ and the tyrosine kinase receptor Trk-A. Trk-A is specific for NGF and can mediate most of its biological effects (Denchant *et al*, 1994; Ip and Yancopoulos, 1994); $p75^{NTR}$ is a common receptor for all neurotrophins and is involved in general neurotrophin signalling (Barbacid, 1995). There is evidence that some co-operation between the two receptors does exist in NGF signalling (Maliartchouk and Sargovi, 1997; Powers *et al*, 1998); however the growth and survival-promoting effects of NGF on neurons and PC12 cells are generally mediated by the Trk-A receptor (Denchant *et al*, 1994; Ip and Yancopoulos, 1994). Little is understood about $p75^{NTR}$–mediated signalling, although it may play a role in apoptosis (Rabizadeh *et al*, 1993; Taglialatela *et al*, 1997). Activation of Trk-A leads to the phosphorylation, and therefore activation, of transcription factors such as CREB, and the expression of many different genes sensitive to those transcription factors (Riccio *et al*, 1997).

When NGF binds to Trk-A, the receptor dimerises and is autophosphorylated by its own tyrosine kinase activity (Jing *et al*, 1992). The activated receptor is then able to trigger a number of different intracellular signalling events, some of which have been well characterised (Kaplan and Stephens, 1994). In brief, the tyrosine kinase activity of Trk-A activates guanine nucleotide exchange factors (GEF) through an adaptor protein (Schlessinger, 1994). These then activate GTPases (Chardin *et al*, 1993; Gale *et al*, 1993), which activate downstream effectors known as MAP kinase kinase kinases (MAPKKKs). These are serine/threonine kinases that, as their name suggests, phosphorylate their downstream targets at serine and threonine residues. The target proteins are known as MAP kinase kinases (MAPKKs). These dual-specificity kinases phosphorylate MAP kinases (MAPKs) at specific threonine and tyrosine residues within a conserved TEY motif in the activation domain. MAPKs are serine threonine kinases capable of activating transcription factors by phosphorylating them directly or indirectly through other proteins (Marshall, 1994; Heldin and Purton, 1996; Minden and Karin, 1997). Thus the NGF signal is transmitted to the nucleus by a series of phosphorylation events. Naturally the down-regulation of these signals is mediated by the action of specific phosphatases that can dephosphorylate the individual signalling molecules (Heldin and Purton, 1996).

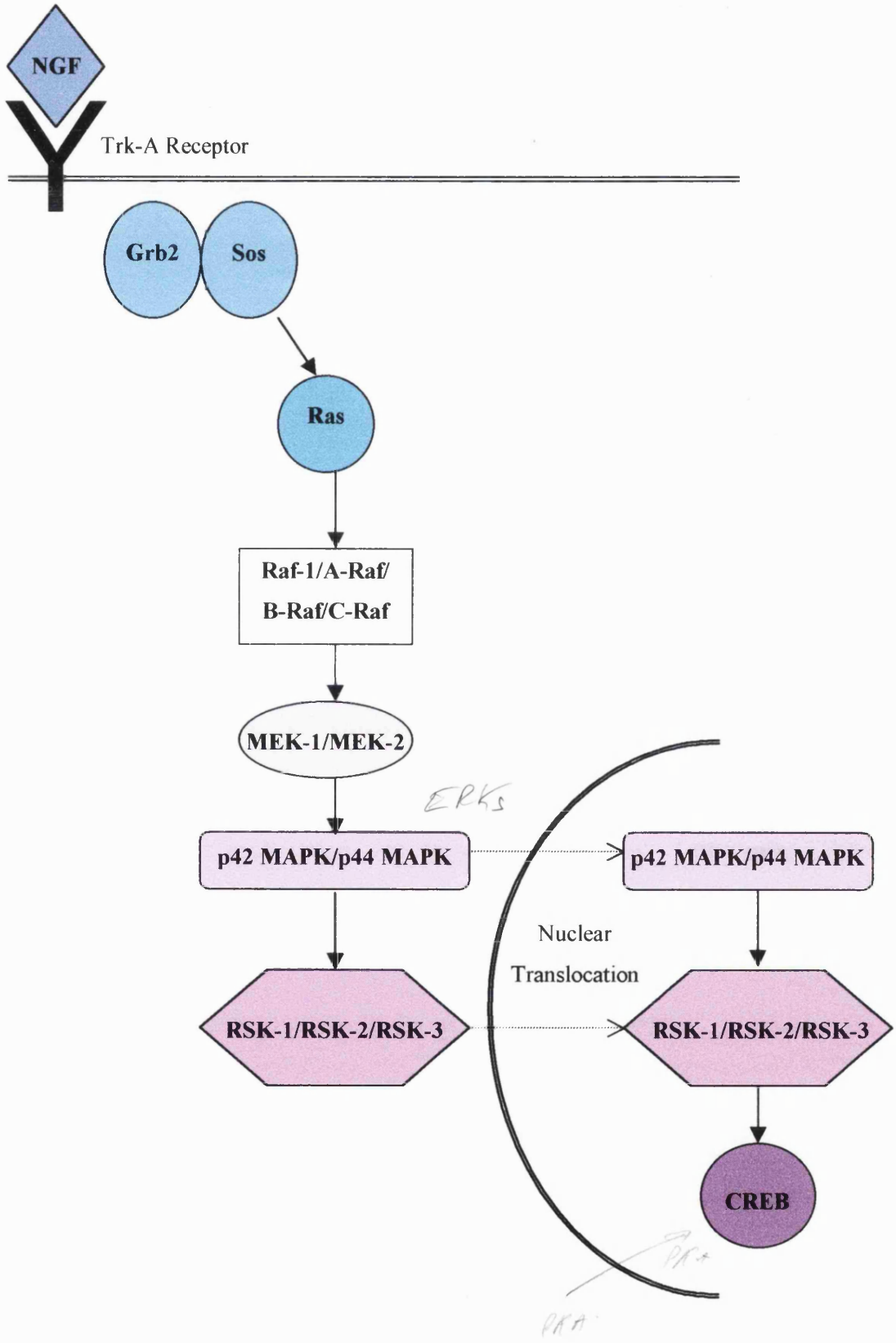
NGF can activate three major MAPK signalling cascades, including the stress-activated MAP kinases (SAPK) p38 and JNK and the extracellular signal-regulated kinase (ERK) p42/p44. The individual signalling pathways are described in more detail below. NGF-mediated PKC signalling is also discussed since PKC is a regulator of p42/p44 MAP kinase.

1.4.7 The Ras/p42 and p44 MAP Kinase Signalling Pathway

The first MAP kinase signalling pathway to be identified in mammals was that of the p42 and p44 MAP kinases (see figure 1.4). It is generally activated in response to mitogenic stimuli, such as growth factors, by receptor tyrosine kinases. When NGF binds to the Trk-A receptor the subsequent cytosolic autophosphorylation recruits the adaptor protein Grb2 to the receptor (Chardin *et al*, 1993). This protein, like all adaptors, has no enzyme activity but serves to bring a second protein, Sos, to the receptor through its SH3 and SH2 domains. Sos is a GEF and therefore catalyses the exchange of GDP for GTP on guanine nucleotide-binding proteins. The target of Sos is the GTPase Ras, which is activated by binding to GTP (Chardin *et al*, 1993; Gale *et al*, 1993; Bos, 1997). Activated Ras binds to members of the Raf family of serine/threonine kinases (Vojtek *et al*, 1993; Marshall, 1996; Bos, 1997). Thus Raf is brought to the plasma membrane and is subsequently modified by phosphorylation. The exact mechanisms behind Raf activation remain unknown, although other protein factors are believed to be involved. Raf is effectively the MAPKKK of this signalling cascade, although its sequence is not homologous to the family of MAPKKKs required for other MAPK signalling pathways (Kyriakis *et al*, 1992). Activated Raf phosphorylates the cytosolic MAPKKs MEK-1 and MEK-2 (also known as MKK1 and MKK2) at specific serine and threonine residues within their activation domains (Huang *et al*, 1993; Zheng and Guan, 1994). This activates the MAPKKs and causes them to phosphorylate their downstream MAPK targets. MEK-1 activates the MAPK p42 (also known as ERK2), whilst MEK-2 activates the MAPK p44 (ERK1). Both p42 and p44 are phosphorylated at specific tyrosine and threonine residues within a Thr-X-Tyr motif (Haystead *et al*, 1992; Mordret, 1993). This motif is conserved throughout all the known MAP kinases, and phosphorylation of both residues is required for activation. The activated MAP kinases are serine threonine/kinases and have specific target proteins known as MAPK-activated kinases (RSKs, also called MAPKAP1) as their substrates (Froedin and Gammeltoft, 1999). Both MAPK and RSKs can be translocated to the nucleus, where RSKs can activate certain transcription factors (Chen *et al*, 1992). The nuclear translocation of MAPK has recently been reported to depend upon

Fig. 1.4 – NGF-mediated Activation of CREB by p42 and p44 MAP Kinases

When NGF binds to its receptor Trk-A, the receptor is able to interact with the guanine nucleotide exchange factor Sos via the adaptor protein Grb2. Sos then activates its downstream effector, the Ras GTPase. Activated Ras binds to the serine/threonine kinase Raf, which is then phosphorylated. Activated Raf phosphorylates the MAPK kinases MEK-1 and MEK-2, which subsequently phosphorylate the MAP kinases p42 and p44. The activated MAP kinases are translocated to the nucleus, together with their downstream effectors the RSKs. The MAPKs phosphorylate the RSKs, which in turn phosphorylate CREB. Thus NGF is able to regulate the transcription of CREB-sensitive genes.


NGF
Trk-A Receptor
Grb2
Sos
Ras
Raf-1/A-Raf/
B-Raf/C-Raf
MEK-1/MEK-2
ERKs
p42 MAPK/p44 MAPK
p42 MAPK/p44 MAPK
Nuclear
Translocation
RSK-1/RSK-2/RSK-3
RSK-1/RSK-2/RSK-3
CREB

PKA (Yao *et al*, 1998). Transcription factors activated by RSKs are able to modify gene expression within the nucleus and thus NGF can exert an effect on the regulation of gene expression through the MAPKs p42 and p44.

Down-regulation of the signalling cascade occurs at a number of different levels. As with all the MAP kinase signalling pathways, each kinase can be de-activated by specific protein phosphatases, particularly protein phosphatase 2A (Heldin and Purton, 1996). The GTPases are down-regulated by the exchange of bound GTP for GDP (Heldin and Purton, 1996). Ras has a very low intrinsic GTPase activity, but this can be enhanced by the action of the GTPase activating proteins (GAPs).

The transcription factor targets of p42/p44 include CREB (Xing, 1996), Elk-1 (York *et al*, 1998), c-Fos (Chen *et al*, 1993) and the co-activator CBP (Liu *et al*, 1998). Consequently this signalling cascade is able to alter gene expression within cells to have a dramatic effect on a number of cellular processes. In PC12 cells, for instance transient activation of the p42/p44 MAPKs by epidermal growth factor (EGF) will cause PC12 cells to proliferate, whilst sustained activation of the ERKs by NGF is partly responsible for neurite outgrowth and the differentiation process (Marshall, 1998). This pathway has also been implicated in the NGF-induced expression of survival factors such as Bcl-2 in neuronal cells (Liu *et al*, 1999).

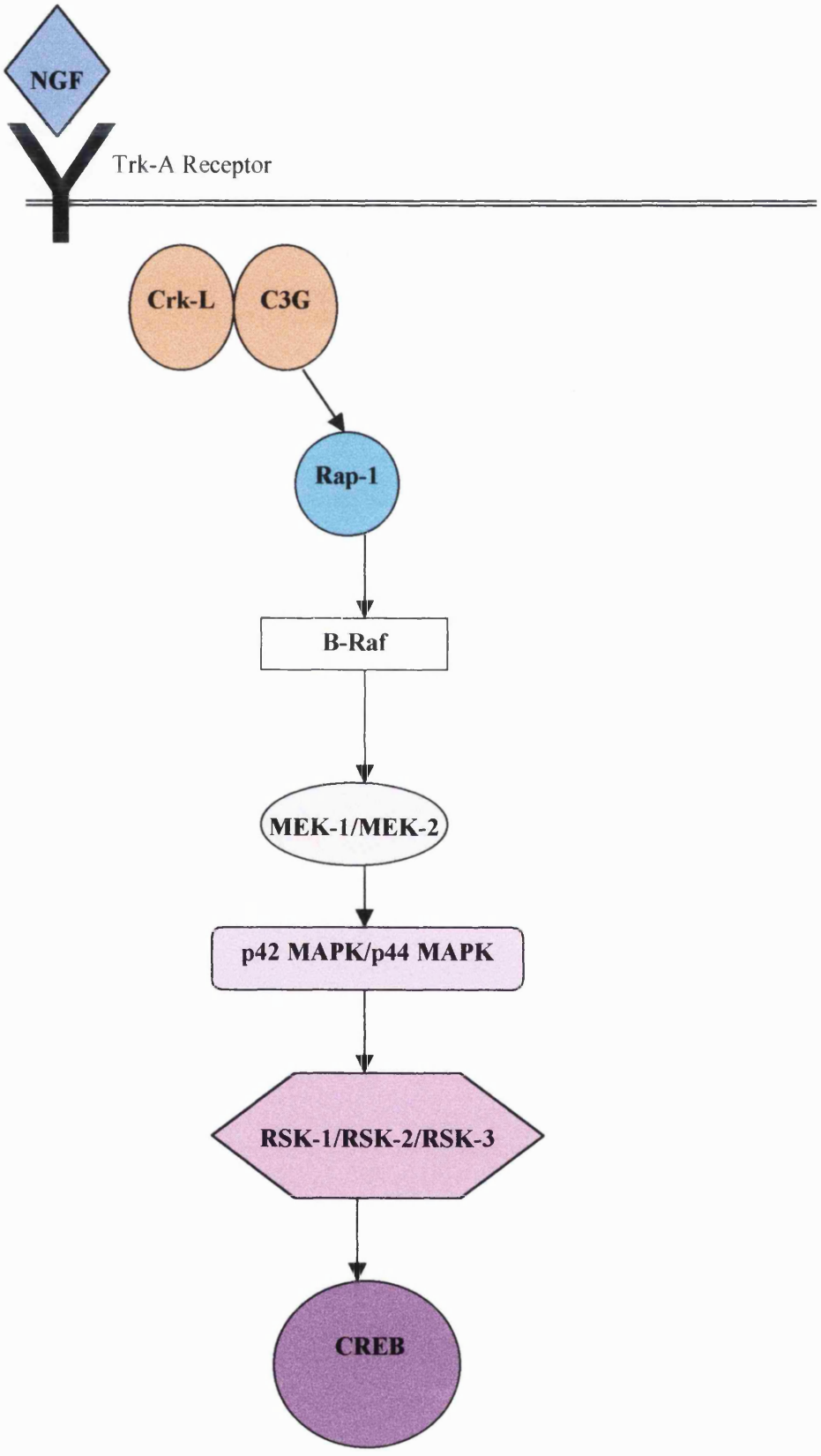
1.4.8 The Rap Pathway

As with the GTPase Ras, the signalling pathway mediated by the GTPase Rap1 also involves the activation of p42 and p44 MAP kinases. When NGF binds to Trk-A, it not only triggers the activation of Ras by Grb2 and Sos, but also the activation of Rap1 by the Trk-A –linked CRK adaptor proteins, predominantly Crk-L, and the guanine nucleotide exchange factor C3G (York *et al*, 1998). C3G will bind to the SH3 domain of CRK proteins but not to any other SH3 containing proteins such as Grb2 (Knudsen *et al*, 1994; Feller *et al*, 1995). Once activated by GTP binding, Rap 1 is able to form a stable complex with B-Raf (York *et al*, 1998), which can subsequently phosphorylate MEK-1 and MEK-2, which in turn activate p42 and p44 MAPK (figure 1.5).

Studies in PC12 cells have indicated that both Ras and Rap could be required for the NGF-induced activation of transcription factors and that Ras and Rap are activated by parallel pathways. It is believed that Ras is responsible for the initial NGF-mediated activation of the

Fig. 1.5 – NGF-mediated Rap Activation of p42/p44 MAP Kinase

When NGF binds to Trk-A a signal is transmitted to the Trk-A –linked adaptor protein Crk-L and the GEF C3G. Activated C3G induces the binding of GTP to Rap1 and this in turn activates B-Raf. Active B-Raf phosphorylates its MAPKK downstream targets MEK-1 and MEK-2. These activate p42 and p44 MAPK respectively. The MAP kinases activate RSK proteins, which in turn activate the phosphorylation of transcription factors such as ATF-1 and CREB.


NGF
Trk-A Receptor
Crk-L
C3G
Rap-1
B-Raf
MEK-1/MEK-2
p42 MAPK/p44 MAPK
RSK-1/RSK-2/RSK-3
CREB

p42/p44 MAP kinases, whilst Rap1 maintains sustained activation (York *et al*, 1998). Interestingly, Crk-L and C3G are also able to activate the MAP kinase JNK; however this interaction is blocked in the presence of NGF (Tanaka *et al*, 1996).

In other cell types, such as fibroblasts, the relationship between Rap and Ras is antagonistic rather than synergistic (Cook *et al*, 1993). In these cells Rap is unable to induce MAP kinase activation and is believed to sequester Raf by trapping it in an inactive complex. Rap activation requires protein kinase A by an unknown mechanism, and therefore mediates cAMP-elicited responses (Burgering *et al*, 1993).

The function of Rap in PC12 cells is likely to be very different to that in other cells and appears to be closely linked with Ras. For instance, like Ras, Rap is able to mediate neurite outgrowth (Vossler *et al*, 1997). Unlike Ras though, Rap is not necessary for this. Rap is also able to mediate NGF induction of the transcription factor Elk-1 and the PC12 differentiation marker stromelysin in co-operation with Ras (York *et al*, 1998). However, experiments have also shown that Rap1, but not Ras, is required for the induction of sodium currents in these cells (York *et al*, 1998).

1.4.9 The Protein Kinase C Signalling Pathway

Protein kinase C (PKC) was so named because of its dependence upon calcium. As previously described (see figure 1.3), PKC is activated by diacylglycerol (DAG). NGF can mediate DAG activation of PKC since phospholipase C, the enzyme responsible for the cleavage of phosphatidyl inositol 4,5-biphosphate (PIP_2) into DAG and IP_3, is activated by Trk-A (Kaplan and Stephens, 1994). When DAG is activated it binds to phosphatidyl serine in the plasma membrane and forms a complex with PKC. It was thought that formation of the complex increased the affinity of the kinase for Ca^{2+} and so activated the enzyme (Alberts *et al*, 1989). However many new isoforms of PKC have since been discovered, many of which do not require calcium for their activation (Heldin and Purton, 1996). At present there are at least eleven known PKC isotypes. Isotypes α, β and γ are calcium-dependent and most isotypes have a dependence upon DAG for kinase activation, although the PKCs ζ, λ and ι are neither calcium- nor DAG-dependent. The mechanisms activating these atypical PKCs are unclear. The activation of PKC by DAG is transient since DAG is rapidly phosphorylated to a phosphatidate or cleaved to form arachidonic acid (Alberts *et al*, 1989).

In addition to activation by DAG, many isoforms of PKC can also be activated by the phosphorylated inositol phospholipids PIP_2 and PIP_3, suggesting a role for the phosphoinositide 3-kinase (PI3K) in PKC signalling (Toker *et al*, 1994; Palmer *et al*, 1995). This enzyme is thought to be a downstream effector of the growth factor-activated Ras G-protein (Rodriguez-Viciana *et al*, 1994; Kodaki *et al*, 1994) and mediates PKC activation by the generation of phosphoinositol 3,4,5-triphosphate (PIP_3) (Carpenter and Cantley, 1990; Parker and Waterfield, 1992). Growth factors, cytokines, G-proteins and cell-cycle control proteins have all been implicated in PI3K activation. Nerve growth factor is able to activate the GTPase Ras, which in turn is able to interact with PI3K and activate PKC (see figure 1.6). Thus PKC can be further linked to NGF-stimulated intracellular signalling.

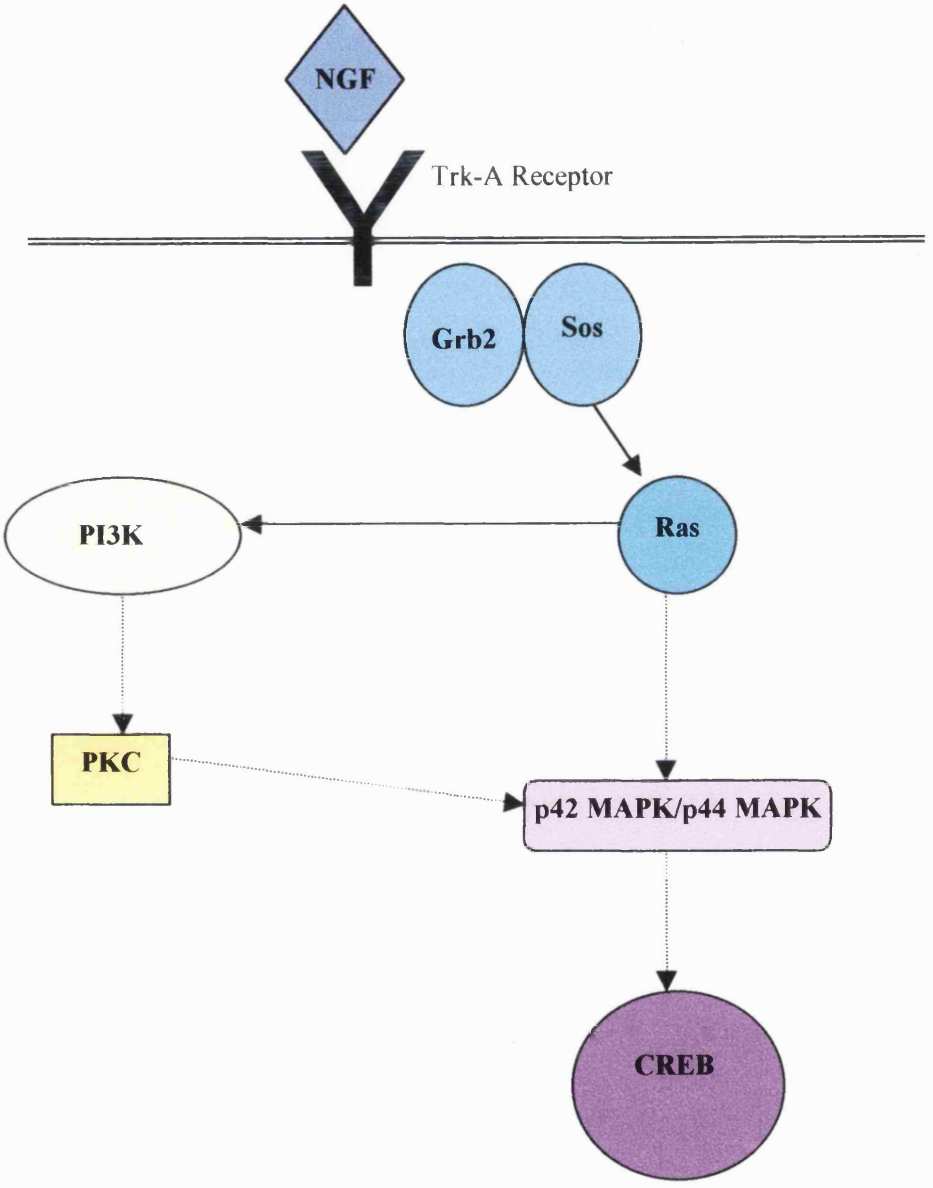
Active PKC is able to phosphorylate the serine/threonine residues of many target proteins to carry out a variety of functions, depending on cell type and PKC isotype. For instance, knock-out mice lacking PKC-γ, an isotype normally found only in the brain, are generally healthy although they have defects in long-term potentiation and learning (Abeliovich *et al*, 1993). In the brain, PKC can alter the excitability of nerve cells by phosphorylating certain ion exchange channels. A study of PKC-α supression in human a549 cells suggested a role for this isotype in cell adhesion (Dean *et al*, 1994). Overexpression of PKC δ in NIH3T3 cells led to growth arrest, whereas overexpression of PKC-ε in the same cells resulted in transformation (Mischak *et al*, 1993). In a gliablastoma cell line PKC-γ accelerated growth, whereas PKC-δ reduced their rate of growth (Mishima *et al*, 1994). Recent studies have also implicated PKC-α, β, δ and ε in the differentiation of non-neuronal cells (Hundle *et al*, 1995). As with other signalling kinases, PKC can affect the pattern of gene expression within a cell as many genes contain regulatory elements that are sensitive to PKC. These elements include the TRE and NFκB sites (Heldin and Purton, 1996).

PKC-ε is expressed largely in the nervous system and is thought to have a role in neuronal development (Hundle *et al*, 1995). In PC12 cells PKC-ε is activated by nerve growth factor and it is the predominant isotype found in differentiating neurons. Although PC12 cells also contain PKC-α, δ and ζ, NGF stimulation of these cells activates the PKC-ε isotype alone, and this activation is mediated by DAG (Ohmichi *et al*, 1993). The mechanism behind this selective activation of PKC-ε is unclear, however it does not coincide with an increase in intracellular calcium levels, therefore is unlikely to involve phosphatidylinositols. Studies in PC12 cells have shown that PKC-ε, but not PKC-δ, can stimulate differentiation and neurite outgrowth by increasing NGF-induced p42/p44 MAP kinase activation (Hundle *et al*, 1995).

Fig. 1.6 – A Potential Role for PKC in the NGF-mediated Activation of p42/p44 MAP Kinase

The enzyme PI3K can be activated by the action of upstream regulators such as NGF and Ras. Activated PI3K is able to induce the activation of its downstream target PKC, which in turn is able to up-regulate the NGF-mediated activation of the p42/p44 MAP kinases. These MAP kinases are able to regulate the CREB transcription factor; therefore PKC may potentially participate in the NGF-mediated activation of CREB.

Solid arrows denote direct protein interactions and dashed lines represent incomplete pathways.


NGF
Trk-A Receptor
Grb2
Sos
PI3K
Ras
PKC
p42 MAPK/p44 MAPK
CREB

In some non-neuronal cell types certain PKC isoforms activate Raf-1 (Kolch *et al*, 1993), an upstream effector of MEK in the p42/p44 signalling pathway. Therefore it is possible that PKC-ε may increase the activity of an upstream effector of p42/p44 in neuronal cells.

In summary, PKC may be responsible for an increase in the activation of p42/p44 in response to NGF, and therefore may be responsible for the up-regulation of MAP kinase-sensitive proteins such as the transcription factor CREB (see figure 1.6).

1.4.10 The Jun Kinase Signalling Pathway

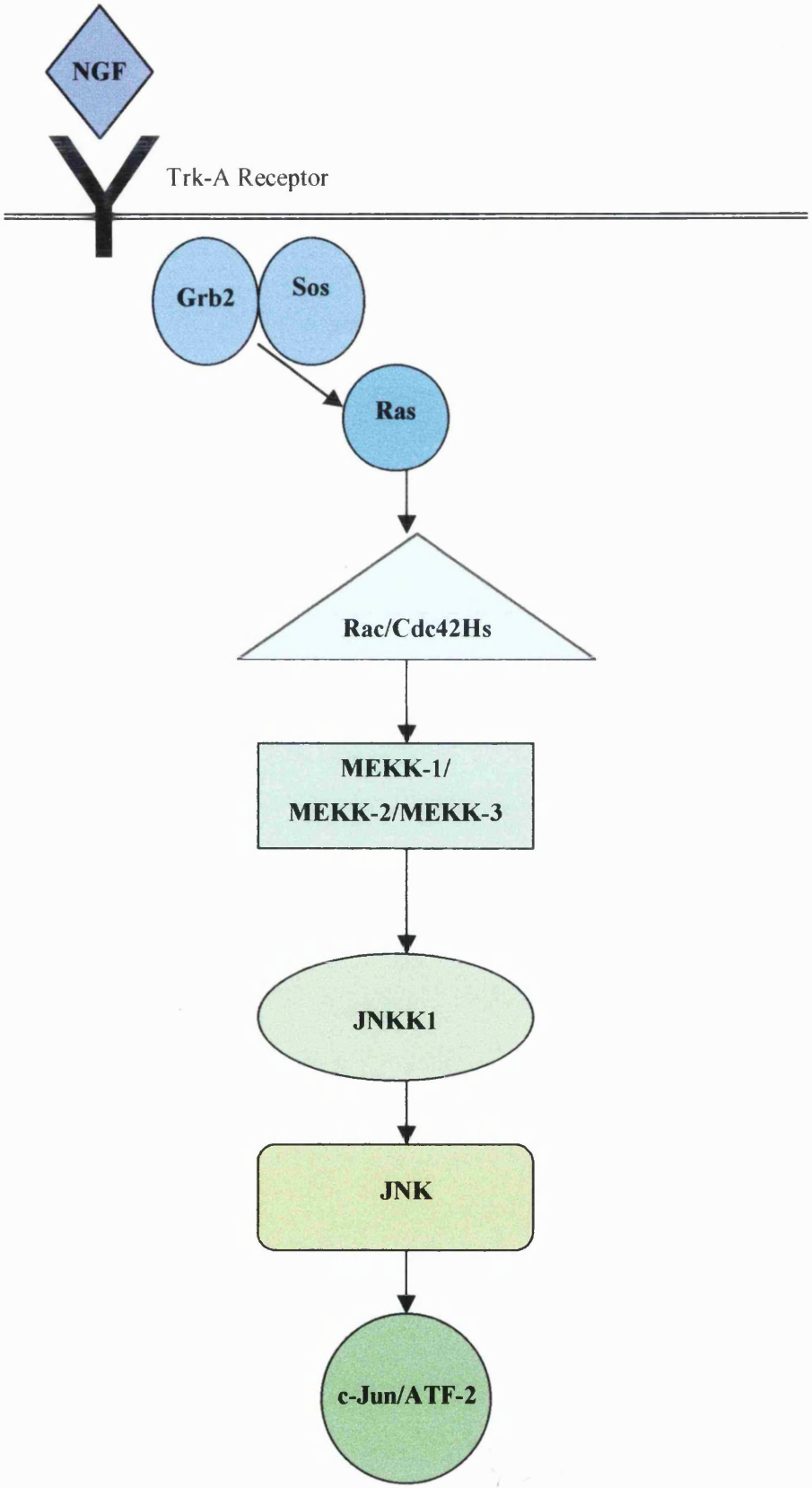
As well as activating p42/p44 MAP kinases and PKC, the activation of Trk-A by NGF can also stimulate the stress-activated MAP kinase (SAPK) signalling cascades. These kinases were so-called because of their response to stress stimuli, such as UV irradiation and growth factor withdrawal, although they are also responsive to extracellular stimuli such as growth factors and cytokines. There are two major SAPK signalling pathways; the first involves the Jun N-terminal kinases (JNK) p46 and p54 and the second involves the p38 MAP kinase.

NGF-mediated activation of the JNK pathway is illustrated in figure 1.7. It requires stimulation of Trk-A and the activation of Ras GTPase. Ras activated Raf activates p42/p44 but cannot activate JNK; however Ras can also activate the small GTPases Rac and Cdc42Hs (Coso *et al*, 1995). By an unknown mechanism these two proteins interact to induce the activation of the MAPKKKs known as MEKK-1, MEKK-2 and MEKK-3 (Minden and Karin, 1997). These kinases then phosphorylate their downstream effector Jun kinase kinase (JNKK1, also known as SEK-1 or MEK-4), at specific serine and threonine residues (Sanchez *et al*, 1994). Active JNKK1 is able to directly phosphorylate the enzyme c-Jun N-terminal protein kinase (JNK) at the conserved MAP kinase activation site on threonine and tyrosine (Lin *et al*, 1995). As its name suggests, JNK is then able to stimulate the activity of the transcription factor c-Jun by phosphorylating specific serines in the amino-terminal activation domain (Hibi *et al*, 1993). JNK can also activate, among others, the transcription factor ATF-2 (Gupta *et al*, 1995), which is able to dimerise with c-Jun to activate expression from the c-jun promoter (Minden and Karin 1997). Thus NGF can induce gene expression of JNK-sensitive genes.

The JNK signalling pathway is closely linked to other NGF-sensitive signalling cascades. For instance, the JNKK1 activators MEKK-1/2/3 also activate MEK components of the p42/p44

Fig. 1.7 – NGF-mediated Activation of the JNK Signalling Pathway

Activation of the Trk-A receptor by NGF leads to the activation of the Ras GTPase. Ras interacts with the small GTPases Rac and Cdc42Hs to activate the MAP kinase kinase kinases MEKK-1, MEKK-2 and MEKK-3. These kinases phosphorylate the MAP kinase kinase JNKK1, which in turn phosphorylates the MAP kinase JNK. Phosphorylated JNK then up-regulates the activity of c-Jun and ATF-2 transcription factors and thus modulates gene transcription.


NGF
Trk-A Receptor
Grb2
Sos
Ras
Rac/Cdc42Hs
MEKK-1/
MEKK-2/MEKK-3
JNKK1
JNK
c-Jun/ATF-2

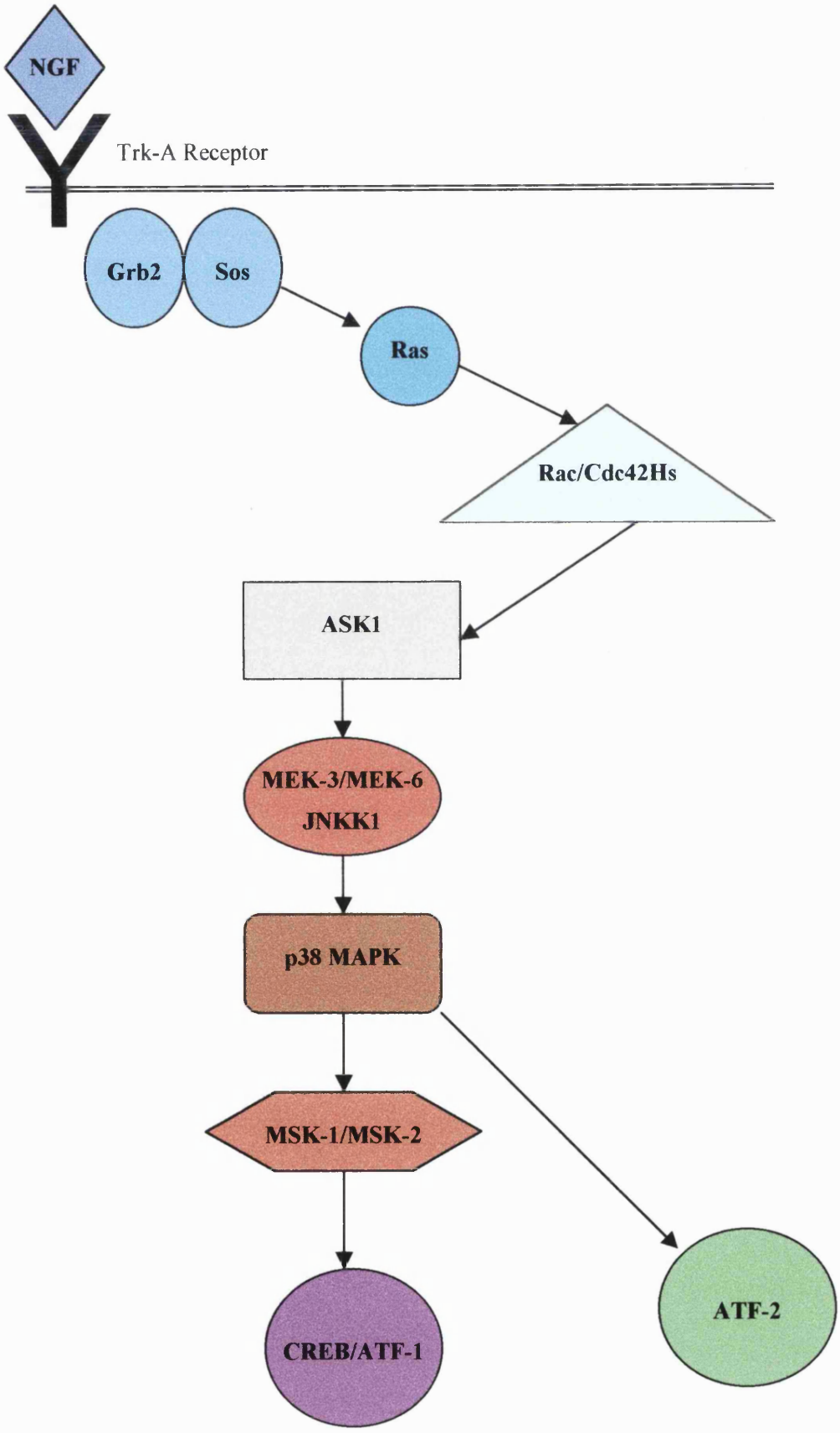
signalling pathway (Minden and Karin, 1997). The JNK activator JNKK1 can also activate p38 MAP kinase (Lin *et al*, 1995). The stimuli activating JNK appear to have a broad range of physiological effects ranging from cell growth and proliferation to cell death; therefore it is probable that the biological functions of JNK involve co-operation with other signalling pathways. For instance, in PC12 cells JNK can trigger apoptosis in response to NGF-withdrawal in co-operation with p38 MAP kinase, whilst p42/p44 MAP kinases inhibit apoptosis (Xia *et al*, 1995). NGF itself does not appear to activate JNK in PC12 cells (Xing *et al*, 1998). Conversely, the activation of JNK in B-cells rescues cells from apoptosis and activation of p42/p44 MAP kinase may cause apoptosis (Minden and Karin, 1997). JNKK1 null chimeric mice thymocytes also have reduced resistance to apoptosis suggesting a protective role for JNK (Minden and Karin, 1997). Experiments in cardiomyocytes showed that JNK is able to suppress hypertrophy, whereas p38 MAP kinase promotes hypertrophy. Again this suggests a protective role for JNK, however the effect of p38 MAP kinase on cell survival is negative (Nemoto *et al*, 1998). In conclusion, the protective effects of SAP kinases are unclear and are likely to be cell specific.

1.4.11 The p38 MAP Kinase Signalling Pathway

The second and least understood SAP kinase signalling pathway involves the MAP kinase p38. The p38 MAP kinase can be activated by many of the same extracellular stimuli as JNK, such as cytokines and growth factors as well as environmental stresses such as UV irradiation. NGF-mediated activation of the p38 MAP kinase signalling pathway is illustrated in figure 1.8. As with JNK activation, NGF stimulated activation of p38 MAP kinase is also mediated by the Trk-A receptor, Ras, Rac and Cdc42Hs (Minden and Karin, 1997). Unlike JNK, however, p38 MAP kinase is not activated by the MEKK proteins (Derijard *et al*, 1995; Xia *et al*, 1995). The serine/threonine kinase known as ASK1 has been implicated as the target of Rac here, since it acts as an effective MAPKKK for this pathway (Ichijo *et al*, 1997). The downstream targets of ASK-1 are the MAPKKs MEK-3, MEK-6 and JNKK1 (Han *et al*, 1996; Raingeaud *et al*, 1996; Lin *et al*, 1995), and these are able to phosphorylate the p38 MAP kinase directly at the MAP kinase activation site on threonine and tyrosine residues. Phosphorylated p38 MAP kinase is able to activate the kinases known as MSKs, or MAPKAP2 (see section 1.5), and these are able to phosphorylate the transcription factors CREB and ATF-1. The transcription factor ATF-2 is also phosphorylated by p38 MAP kinase directly (Raingeaud *et al*, 1995). Thus NGF is able to direct gene expression by a third MAP

Fig. 1.8 – NGF-mediated p38 MAP Kinase Activation

Activation of the Trk-A receptor by NGF leads to the activation of the Ras GTPase. Ras interacts with the small GTP-binding proteins Rac and Cdc42Hs to activate the MAP kinase kinase kinase ASK1. This then phosphorylates the MAP kinase kinases MEK-3/MEK-4 and JNKK1, which in turn phosphorylate p38 MAP kinase. This kinase is able to activate the transcription factor ATF-2 directly and the transcription factors ATF-1 and CREB indirectly via the MSK proteins.


NGF
Trk-A Receptor
Grb2
Sos
Ras
Rac/Cdc42Hs
ASK1
MEK-3/MEK-6
JNKK1
p38 MAPK
MSK-1/MSK-2
CREB/ATF-1
ATF-2

kinase signalling cascade and NGF does activate the p38 MAP kinase signalling cascade in PC12 cells (Xing *et al*, 1998).

As stated previously, p38 appears to be involved in apoptosis although its exact role is likely to vary between cell types. The kinase p38 has also been implicated in the cellular response to inflammation. Both JNK and p38 are strongly responsive to cytokines and inhibitors of p38 block certain inflammatory responses (Minden and Karin, 1997).

1.4.12 A Summary of MAP Kinase Signalling

Growth factors such as NGF are able to transmit signals from the extracellular environment to the nucleus in order to activate target proteins, including certain endogenous transcription factors. This is achieved by the activation of specific cell surface receptors that are linked to intracellular signalling molecules. The MAP kinases are a large family of such signalling molecules. These proteins can be divided into three main groups, each with their own specific signalling cascade. These are the p42/p44 MAP kinases, the p38 MAP kinase and JNK. The cross talk between the various MAP kinase signalling cascades is extensive and is summarised in figure 1.9. For instance, in SAPK signalling JNKK1 is able to phosphorylate both JNK and p38 MAP kinase and ASK1 is able to activate both JNKK1 and MEK-3/6. Further cross talk exists between p42/p44 MAP kinase and JNK pathways since MEKK1, 2 and 3 can activate JNKK1, MEK-1 and MEK-2. It is also possible that PKC may affect the activity of p42/p44 MAP kinases. Finally the GTPases Ras and Rap are both able to activate p42/p44 in response to NGF.

The MAP kinases themselves can also have similar transcription factor targets. For instance, JNK and p38 MAPK both activate ATF-2. The p42/p44 and p38 MAP kinases are able to activate CREB and ATF-1. Despite these relationships, however, each MAP kinase still displays a high degree of substrate specificity.

The MAP kinase proteins themselves are highly conserved and share many similar features in their structures and activation mechanisms. For instance, all MAP kinases are serine/threonine kinases and are themselves activated at highly conserved threonine and tyrosine residues within a TXY motif in their activation domains (Haystead, *et al* 1992). Activtion of MAP kinases occurs by the action of dual-specificity MAP kinase kinases (MAPKKs), and these too are very similar between pathways. MAPKKs are activated at conserved serine and

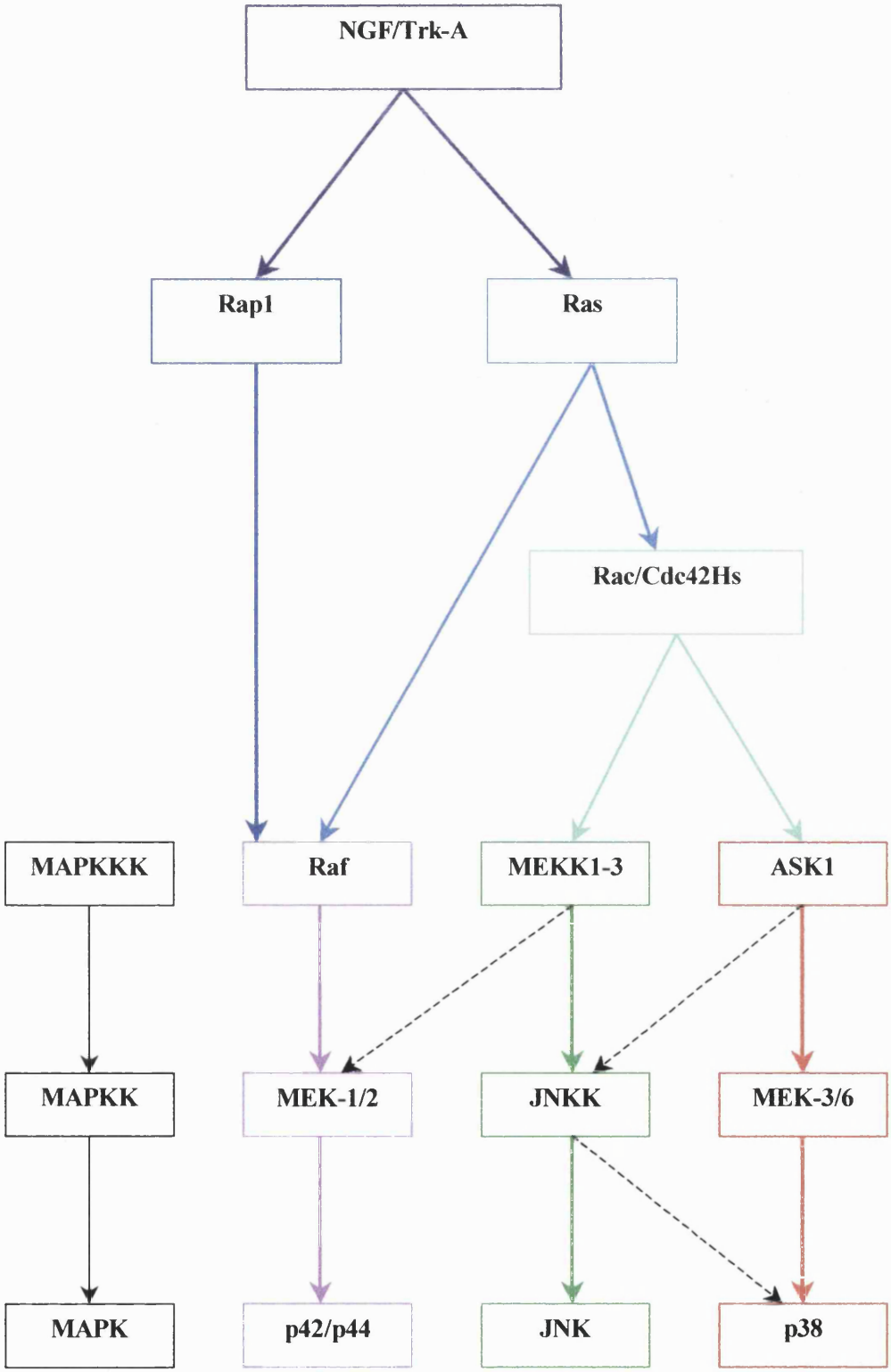
Fig. 1.9 – The MAP Kinase Signalling Pathways

Trk-A –bound NGF is able to activate three different MAP kinase signalling pathways in PC12 cells; p42/p44, p38 and JNK. Trk-A receptor activation leads to the activation of the GTPases Ras and Rap1. Ras is able to trigger the activation of all three types of MAP kinase kinase kinase (MAPKKK), namely Raf, the MEKK proteins and ASK1. Rap is only able to activate Raf. Whilst Raf can be activated directly by both Rap1 and Ras, the MEKK proteins and ASK1 are activated by the interaction of Ras with two other GTP-binding proteins, Rac and Cdc42Hs.

Once activated MAPKKKs are able to phosphorylate their downstream MAP kinase kinase (MAPKK) targets. The MAPKKs MEK-1 and MEK-2 are primarily activated by Raf, but also by MEKK1, 2 and 3. JNKK is primarily activated by MEKK1, 2 and 3, but also by ASK1. MEK-3 and MEK-6 are activated by ASK1.

The activated MAPKKs are able to phosphorylate their MAP kinase (MAPK) targets. MEK-1 and MEK-2 activate the MAPKs p42 and p44 respectively. JNKK primarily activates JNK, but is also able to phosphorylate the p38 MAPK. MEK-3 and MEK-6 are p38 MAPK activators.

Thus, by sending a signal through the cytoplasm in the form of a protein-phosphorylation cascade, NGF is able to activate the MAPK proteins.


NGF/Trk-A
Rap1
Ras
Rac/Cdc42Hs
MAPKKK
Raf
MEKK1-3
ASK1
MAPKK
MEK-1/2
JNKK
MEK-3/6
MAPK
p42/p44
JNK
p38

threonine residues by MAP kinase kinase kinases. These are more diverse and can be activated by other protein kinases or GTP-binding proteins. Thus the kinases tend to be highly conserved in their catalytic domains, but vary in their regulatory domains and in substrate specificity (Minden and Karin, 1997). The effects of these MAP kinases on their downstream effectors will now be discussed.

1.5 RSK and MSK Family Proteins

The family of 90kDa ribosomal S6 kinases, or RSK proteins, was first discovered in *Xenopus laevis* in 1985, upon the identification of a kinase phosphorylating the ribosomal protein S6 (Erikson and Maller, 1985). RSK proteins are now known to be substrates for the p42/p44 MAP kinases, and the family comprises of three known isoforms; RSK-1, RSK-2 and RSK-3 (Moller *et al*, 1994; Zhao *et al*, 1995). These proteins exhibit 75-80% amino acid homology and are expressed in a variable pattern in different tissues (Froedin and Gammeltoft, 1999). They are serine/threonine kinases and have two functional kinase domains within a single polypeptide. The N-terminal kinase domain of RSK is required for the phosphorylation of substrates (Leighton *et al*, 1995), whilst the C-terminal domain is involved in RSK activation by regulating the N-terminal kinase (Froedin and Gammeltoft, 1999). Co-immunoprecipitation experiments have shown that p42/p44 MAP kinases preferentially associate with RSK-3 and less so with RSK-1 and RSK-2 (Zhao *et al*, 1996). The RSK proteins are activated by almost all extracellular stimuli that activate the Ras/Mek/p42/p44 signalling pathway, such as growth factors, cytokines and neurotransmitters; substrates for RSK include the transcription factor CREB, c-Fos and the estrogen receptor-α (Xing *et al*, 1996; Froedin and Gammeltoft, 1999). Both *in vivo* and in PC12 cells, NGF is able to activate all three RSK proteins to induce CREB activation (Xing *et al*, 1998). The RSK family has also been shown to interact with the transcription co-activators CREB-binding protein (CBP) and p300 (Nakayima *et al*, 1996), as well as the Ras GTP/GDP exchange factor, Sos (Douville and Downward, 1997). RSK phosphorylates Sos in response to EGF, creating a negative feedback loop on Ras activation, indicating that RSK proteins may also play a role in the down-regulation of the growth factor response.

RSK is able to phosphorylate many other cellular proteins, including glycogen synthase kinase-3, the cell adhesion molecule L1-CAM, the cell-cycle kinase Myt-1 and certain proteins within the ribosomal complex (Sutherland *et al*, 1993; Wong *et al*, 1996; Palmer *et al*, 1998; Angenstein *et al*, 1998). In addition to their role in intracellular signalling, these substrates suggest that RSK proteins may be involved in regulation of the cell cycle, growth control and even neurite outgrowth. In humans, mutations in *rsk-2* cause the X-linked Coffin-Lowry syndrome, symptoms of which include mental retardation and progressive skeletal malformations. This suggests a role for RSK-2 in brain function and bone formation during development and in adult life. As patients only have defects in the nervous and skeletal systems, this suggests that RSK-2 is important in these tissues but redundant to RSK-1 and RSK-3 in others.

In Hela cells, RSK proteins are present in both the cytoplasm and the nucleus, and growth factor stimulation will activate RSK in both locations (Chen *et al*, 1992). Some cytoplasmic RSK translocates to the nucleus on activation and nuclear translocation is followed by immediate-early gene expression of which CREB is an important mediator. CREB is activated by extracellular signals at serine 133 and recruits the co-activators CBP and p300 to activate the transcription of genes containing a cAMP response element (CRE). As described above, CREB can be activated via a number of different signalling pathways, including cAMP, Ca^{2+} and the Ras signalling cascade. Purification of the factor that activated CREB after NGF stimulation of the Ras signalling pathway (Ginty *et al*, 1994) revealed that it was RSK-2 (Xing *et al*, 1996). In a fibroblast cell line derived from a patient with Coffin-Lowry syndrome, EGF is no longer able to activate CREB, although cAMP activation of CREB is unaffected (De Cesare *et al*, 1998). These cells are deficient in RSK-2 but have normal levels of other RSK proteins.

CBP and p300 are also important targets of RSK. These transcriptional co-activators associate with transcription factors, including the RSK-binding factors CREB and c-Fos, in response to extracellular stimuli. It has been shown that a large proportion of cellular CBP rapidly forms a complex with RSK in response to serum or insulin in a number of different cell types, and that this complex formation is Ras-dependent (Nakajima *et al*, 1996). RSK specifically recognises the E1A-binding domain of CBP/p300, but not the phospho-CREB-binding domain. In PC12 cells, the ectopic expression of the RSK-binding site of CBP/p300 inhibits c-fos transcription and neurite outgrowth in response to nerve growth factor, indicating that the CBP/RSK complex is important for the induction of Ras-responsive genes. Interestingly, the CBP/RSK complex formation can also inhibit the transcription of cAMP-responsive genes. This shows a possible mechanism for negative cross-talk between the Ras and PKA signalling pathways at the transcriptional level (Froedin and Gammeltoft, 1999). CBP itself is discussed in greater detail in section 1.7.

Recently a novel family of RSK-like proteins has been identified that includes the mitogen- and stress-activated kinase, MSK (Deak *et al*, 1998; Pierrat *et al*, 1998). MSK proteins are 40% identical to RSK and also have two kinase domains. The MSK proteins MSK-1 and RSK-B are substrates for p38 MAPK, as well as p42 and p44 MAPK, and can therefore mediate stress responses. It is thought that MSK regulates the activity of the transcription factors CREB, ATF-1 and the c-Jun/c-Fos heterodimer AP-1 and, *in vitro*, CREB is a better substrate for MSK-1 than RSK-1 or RSK-2 (Froedin and Gammeltoft, 1999). In PC12 cells, NGF activates all three RSKs and MSK-1, and MSK-1 is largely activated by the p38

signalling cascade. Both the p42/p44/RSK and p38/MSK signalling pathways contribute to NGF-induced CREB activation (Xing *et al*, 1998).

MSK proteins associate with p42/p44 MAPK and p38 MAPK, but not JNK (Pierrat *et al*, 1998), whereas RSK proteins bind specifically to p42/p44 (Smith *et al*, 1999), and so it is believed that the specificity of action of each RSK/MSK family member may be dependent upon a specific association with individual MAP kinases.

1.6 The CREB/ATF Transcription Factors

1.6.1 A General Overview

As described above, members of the CREB/ATF family of transcription factors can be activated by numerous extracellular stimuli and intracellular signalling cascades, including cAMP. There are at least eight known members of the CREB/ATF family, including CREB, CREM and the ATFs, all of which belong to the superfamily of b-zip proteins. These proteins all have conserved, highly basic DNA binding domains and leucine zipper domains required for protein-protein interactions in dimerisation (Karin and Smeal, 1992; Hurst, 1996). They generally have distinct and separable DNA-binding, dimerisation and transcription activation domains, and dimerization is a pre-requisite for DNA binding (Delmas *et al*, 1994). A proposed model suggests that dimerization brings the two basic regions of the two monomers adjacent to each other so that there is optimal contact with the binding site (Vinson *et al*, 1989). Although the CREB family members have highly conserved dimerization and DNA-binding domains, there is little or no homology between their transactivation domains. Thus the ability to form heterodimers diversifies their effects on gene transcription still further (Lee and Masson, 1993). Different CREB isoforms can be created by various post-translational modifications. Alternative splicing events can produce inhibitor forms of CREB transcription factors, which lack an activation domain but have the same DNA-binding capabilities as the activating CREB transcription factors. These inhibitory factors are able to compete for the same binding sites as active factors, but are unable to stimulate transcription, so exerting indirect repression of transcription (Walker *et al*, 1996). As well as producing repressor forms of CREM, alternative splicing can also produce CREM factors with differing DNA-binding capabilities. This is possible because the CREM gene contains two distinct exons for two different DNA-binding domains (Latchman, 1998).

1.6.2 The Cyclic Adenosine Monophosphate Response Element (CRE)

The DNA-binding domains of CREB family members recognise specific DNA sequences found in the promoter regions of cAMP-responsive genes. This site is known as a cyclic AMP element (CRE), and has an 8bp palindromic consensus sequence of 5'-TGACGTCA-3' (Delmas *et al*, 1994). When transferred to a gene that is not normally cAMP-sensitive, the

CRE is able to confer cAMP-responsiveness to that gene. The basic lysine- and arginine-rich domains of the CREB dimer interact with the two regions of the palindromic sequence. Flanking sequences to a range of 18-20bp have also been shown to be necessary for the transcription of certain genes, either because they facilitate transcription factor binding, or act as control elements (Deutsch *et al*, 1988). For a large number of genes the CRE is located within the first 200bp upstream of the transcription initiation site, and usually there is only one CRE site per gene, although there are exceptions (Delmas *et al*, 1994).

Within the consensus sequence itself, the TGACG motif is highly conserved through known CRE sites, whilst the TCA motif shows greater variation between genes. Two types of CREB binding site have been proposed; symmetric sites show a high affinity binding of CREB, whether phosphorylated or not, and asymmetric sites display low affinity binding of unphosphorylated CREB and high affinity for phosphorylated CREB, so basal transcription is of a higher level in the presence of a symmetric site (Lee and Masson, 1993). Thus DNA binding assays suggest that phosphorylation of CREB by PKA has little effect on binding of CREB to high affinity binding sites, whereas binding to low affinity sites is enhanced by phosphorylation, i.e., DNA binding activity of CREB increases on phosphorylation in a CRE-dependent manner.

Certain CREB family transcription factors contain CRE sequences themselves and thus their own transcription is regulated by changes in cAMP levels. For instance, the CREM gene has a promoter sensitive to cAMP, which produces inhibitor CREM molecules lacking an activation domain (Latchman, 1998a). As with the alternatively spliced inhibitor CREM molecules, these inducible cAMP early repressors, or ICERs, can bind to the CRE and repress activation of transcription from cAMP-responsive genes. This suggests that they may play a role in the down-regulation of cAMP signalling, making the response a transient one.

1.6.3 Activation of Transcription by CREB

The 43kD cAMP response element binding protein, or CREB, was the first transcription factor shown to bind to a CRE site. The CREB gene is expressed ubiquitously and has been cloned from human placenta and rat brain libraries (Hoeffler *et al*, 1988; Gonzales *et al*, 1989). The gene is partly regulated by alternative splicing and the protein exists in numerous isoforms (Lee and Masson, 1993). CREB appears to have functions in a variety of processes, such as nerve cell excitation, setting off circadian rhythms and glucogenesis (Delmas *et al*,

1994). Many genes induced by neuronal activity have CRE-like sequences in their promoters and neuronal activity has been shown to increase the phosphorylation of CREB in the rat hippocampus and cortex (Moore *et al*, 1996). In the cortex CREB activation is preceded by an increase in the activity of auto-phosphorylated calmodulin-dependent kinases, whilst in the hippocampus there is an increase in intracellular cAMP levels. The result is an increase in the expression of c-Fos and AP1 transcription factors, which have a CRE in their promoters. In contrast GABA, an inhibitor of neuronal activity, reduces the activity of CREB. CREB has also proved to be important in a number of fundamental developmental processes including pituitary development (Delmas *et al*, 1994), spermatogenesis (Walker *et al*, 1996) and long-term memory (Stevens, 1994; Frank and Greenberg, 1994). CREB is also involved in opiate tolerance (Nestler, 1993). The known primary targets of CREB include, among others, genes for somatostatin, c-fos, U-type plasminogen activator, DNA polymerase β and several receptors (Lee and Masson, 1993). By regulating a number of transcription factors and other regulatory molecules, CREB is then able to amplify the effects of the signalling cascades to which it responds beyond these primary responses.

Transcriptional activity of CREB has been studied both *in vitro* and *in vivo*. To remove the effects of endogenous CREB in *in vivo* studies, two methods have been described. One employs the yeast Gal 4 system, whereby CREB was fused to the yeast activator Gal 4 DNA binding domain, and a reporter gene was fused to Gal4 binding sites (Lee *et al*, 1990). Others have used exogenous CREB in F9 embryonal carcinoma cells, which lack a functional cAMP signalling pathway (Gonzalez and Montimony, 1989). These experiments led to the identification of several functional elements involved in transcriptional activation.

The CREB gene has 12 exons, which can be spliced into a number of different isoforms (Walker *et al*, 1996). Of these, the 2 activator forms CREBα and CREBΔ appear to be ubiquitously expressed in somatic cells in a ratio of 1:3. The CREB isoforms αγ, γ, Ω and ω lack DNA binding domains and nuclear translocation signals, and can be seen in the cytoplasm of cells in the adult testis (Lee and Masson, 1993). These therefore cannot activate transcription without the assistance of other factors.

In somatic cells CREB-regulated transcription is not regulated at the level of its own expression. It is bound to the CRE prior to cAMP stimulation and may contribute to basal expression. In order to be able to increase the rate of transcription in response to cAMP, CREB has a kinase inducible domain (KID), or P-box, which contains multiple phosphorylation sites. The KID is flanked by two glutamine-rich regions characteristic of

other transcription factor activation domains, e.g., SP1, AP1 (Delmas *et al*, 1994). The serine at position 133 of the P-box can be phosphorylated by PKA, both *in vivo* on stimulation of the adenylate cyclase pathway, and *in vitro* (Gonzalez and Montminy, 1989). The phosphorylation of CREB at serine 133 causes an alteration in the structure of the transactivation domain of CREB, and induces transcriptional activation. Phosphorylation is generally necessary for activation, and it has been shown that replacing serine 133 with other negatively charged amino acids or alanine abolishes the ability of CREB to activate transcription (Yamamoto *et al*, 1988). Interestingly though, it was possible to activate CREB-mediated transcription in Tu6 pancreatic islet cells without phosphorylating CREB (Leonard *et al*, 1992). As well as serine 133, a second serine further downstream within the KID at position 142 has also been implicated in CREB-mediated transcription (Sun and Maurer, 1995). When this serine is mutated to an aspartic acid residue, which mimics phosphorylation, the response to PKA is greatly reduced. This suggests that the phosphorylation of CREB at serine 142 has a negative effect on CREB activity.

However, PKA is not the only regulator of CREB activity. CREB can be phosphorylated by a number of signalling molecules, including RSK, MNK and MSK proteins, in response to the p42/p44 and p38 signalling pathways (see section 1.5). Recent reports have shown that intracellular calcium levels can also have an effect on CREB. Transfection of a number of cell lines with mutant forms of two different calcium/calmodulin-dependent protein kinases (CAM kinases) showed that an influx of calcium resulted in the phosphorylation of CREB at serine 133 (Sun *et al*, 1994). CAMKIV phosphorylates CREB at serine 133 and the expression of CREB-regulated genes is activated. CAMKII phosphorylates not only serine 133, but also serine 142. However, it was shown that the phosphorylation of CREB at serine 142 has the effect of greatly reducing CREB-induced transactivation of gene expression. Thus it was demonstrated that the phosphorylation of serine 133 by CAMKIV has a positive effect on CREB-mediated transcription, whereas phosphorylation of serine 142 by CAMKII has a negative effect. The CREM transcription factor, which is phosphorylated at serine 117 by PKA, is also sensitive to the action of CAM kinases and a kinase known as p70 S6 kinase (de Groot *et al*, 1994). A further demonstration of cross-talk between cAMP and other signalling pathways can be seen with the transcription factor NF-IL6. Co-transfection studies investigating the CRE-containing enhancer of the human prointerleukin 1β gene using NF-IL6 and CREB showed that expression was not stimulated by cAMP alone. Rather there is a co-stimulation between lipopolysaccharides and cAMP. NF-IL6 activates the enhancer in the presence of LPS and forms a heterodimer with phosphorylated CREB (Tsukada *et al*, 1994).

Down-regulation of CREB occurs by a dephosphorylation mechanism. In PC12 cells serine 133 is dephosphorylated by the enzyme protein phosphatase 1 (PP1). For example, following a forskolin stimulated response to cAMP the transcription of somatostatin is reduced by the PP1-mediated inactivation of CREB (Hagiwara *et al*, 1992). In human HepG2 cells protein phosphatase 2A is the major CREB phosphatase (Wadzinski *et al*, 1993).

Although CREB, CREM, ATF-1 and ATF-2 all have a consensus PKA phosphorylation site, ATF-2 was demonstrated to be insensitive, and ATF-1 only weakly responsive, to cAMP *in vitro* (Flint and Jones, 1991). As shown in sections 1.4.10 and 1.4.12, ATF-2 can be activated by JNK and ATF-1 by the p38 signalling pathway.

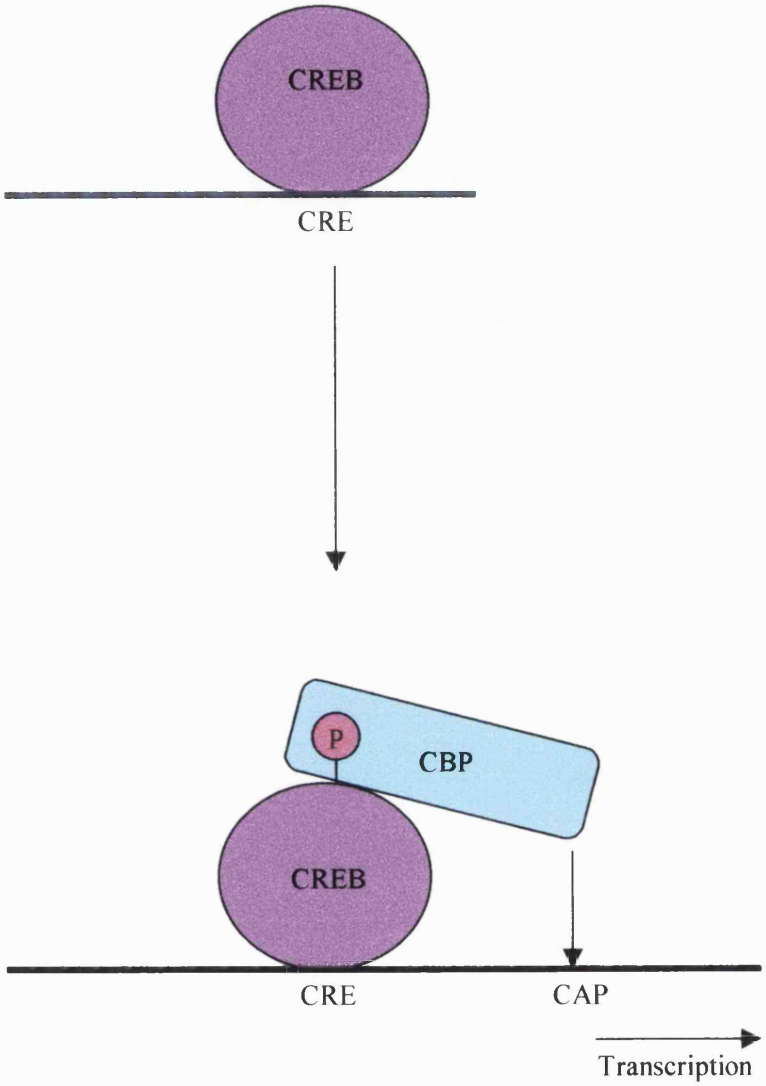
1.7 CREB-binding Protein and CREB

As stated above, phosphorylation of CREB at serine 133 is necessary for CREB-mediated activation of transcription. A recently characterised 265kD protein has been shown to bind to CREB phosphorylated at this position, but not to dephosphorylated CREB (Parker *et al*, 1996; Shikama *et al*, 1997). This protein, named CREB-binding protein or CBP, plays a critical role as a co-activator of CREB in the transcription activation mechanism (see figure 1.10), since microinjection of an anti-CBP antiserum results in the blocking of transcription from a cAMP-responsive promoter (Arias *et al*, 1994), and transfection of cells with a CBP expression vector potentiates CREB activity in a phosphorylation-dependent manner (Kwok *et al*, 1994). The region of the protein involved in CREB binding has been termed the KIX domain (Parker *et al*, 1996). A number of hydrophobic amino acids lying on one face of an α-helix within the KIX domain contribute to the formation of a CBP/CREB complex. CREB also has a series of hydrophobic amino acids flanking serine 133, which strongly suggests that the interactions between the two proteins are hydrophobic. CBP itself can constitutively activate gene expression when brought to a promoter as a Gal4 fusion protein, and there are two proposed mechanisms for CBP-mediated transcription activation. Firstly, CBP is able to interact with several components of the basal transcription machinery, including TFIIB, and has been shown to be a part of the RNA polymerase II holoenzyme complex (Nakajima *et al*, 1997). It is therefore possible that CBP acts as a bridge between CREB and the basal transcription machinery, either by recruiting the holoenzyme to the DNA or by modulating the activity of the individual components within the complex (Latchman, 1998a). Secondly, CBP has been shown to have histone acetyltransferase (HAT) activity, therefore it may affect the rate of transcription by modifying chromatin structure (Ogryzko *et al*, 1996). Acetylated histones are generally found associated with transcriptionally active DNA, rather than the densely packaged inactive chromatin structures. This suggests that CBP may be recruited to DNA by CREB in order to modify the chromatin structure and make it accessible to the basal transcription machinery (Wade *et al*, 1997).

Whilst the phosphorylation of CREB serine 133 is important for CREB/CBP complex formation and transcriptional activation by whichever mechanism, serine 142 appears to affect transcription without disrupting the interaction with CBP (Sun and Maurer, 1995). In fact, mutation of serine 142 reduces the ability of PKA to activate CREB. This suggests the possibility of an additional co-activator being involved in the induction of gene expression from a cAMP-regulated promoter.

Fig. 1.10 – Initiation of Transcription by CREB and CBP

CREB is bound to DNA even when inactive. When activated by phosphorylation at serine 133, CREB is able to bind to the co-activator CBP. CBP is then able to initiate transcription from the start site (CAP), either by interacting with the pre-initiation complex or by altering chromatin structure.


CREB
CRE
P
CBP
CREB
CRE
CAP
Transcription

As well as being a co-activator in the expression of cAMP-responsive genes, CBP and its close relative p300 are involved in gene activation via a whole host of different signalling pathways and they are able to interact with many different protein factors, including AP1, nuclear receptors, STAT factors, MyoD, p53 and RSK (Shikama *et al*, 1997). For instance, as stated previously, CBP/RSK complex formation is important for the induction of Ras-responsive genes. In microinjection experiments anti-CBP antiserum blocked the expression of serum-responsive reporter genes (Nakajima *et al*, 1996). The cellular pool of CBP and p300 is relatively low therefore the different signalling cascades must compete for available co-activators, often antagonising each other as a result. It is proposed that the cAMP and Ras signalling pathways antagonize each other due to their requirement of the common co-activator CBP (Nakajima *et al*, 1996).

1.8 The Calcitonin/Calcitonin Gene-related Peptide Gene

1.8.1 Calcitonin and Calcitonin Gene-related Peptide

The calcitonin (CT)/ calcitonin gene-related peptide (CGRP) gene is one of many genes known to be regulated by cAMP and NGF, in all probability via the CREB transcription factor. Calcitonin was first discovered in 1961 as a peptide expressed in the endocrine system, in the thyroid C cells derived from the neural crest (Copp, 1994). It consists of a single peptide chain of 32 amino acid residues and is considered to be a neuropeptide hormone involved in calcium regulation. Calcitonin has many clinical applications in the treatment of bone disorders such as osteoperosis, as it is able to prevent bone resorption by inhibiting osteoclasts and conserves skeletal mass in times of calcium deficiency (Zaida *et al*, 1990). Calcitonin also acts as a powerful analgesic with a potency far greater than morphine. It is therefore used for the relief of bone pain (Copp, 1994).

However, in 1983 it was discovered that the gene encoding calcitonin in thyroid cells, also encodes a second and entirely different peptide in neuronal cells (Rosenfeld *et al*, 1983). This 37 amino acid neuropeptide was given the name calcitonin gene-related peptide.

The discovery of CGRP showed that a single gene was able to generate different protein products in different tissue types by the generation of different mRNAs. This is achieved by the mechanism of alternative splicing whereby, as well as being regulated at the transcriptional level in both thyroid and neuronal cells, the CT/CGRP gene primary transcript also undergoes post-transcriptional modification in a tissue-specific manner, probably due to the presence of tissue-specific splicing factors (Latchman, 1998b). The primary transcript of the CT/CGRP gene consists of six exons; in thyroid cells alternative splicing generates calcitonin mRNA, which consists of exons 1-4, and in neuronal cells alternative splicing generates CGRP mRNA, which consists of exons 1-3, 5 and 6 (Rosenfeld *et al*, 1983; Latchman, 1998b).

CGRP is only expressed in certain neuronal cells of the CNS and PNS (Rosenfeld *et al*, 1983). It is synthesised in a subset of dorsal root and trigeminal ganglia neurons and in their termini in the brain stem and spinal cord. CGRP immunostaining also shows expression in the tongue and in motor neurons of the facial nucleus, those involved in tongue movement and those innervating the heart and muscles of the larynx and pharynx. Immunostaining further

reveals the presence of CGRP in thin fibres that are associated with the smooth muscle of blood vessels in tissues such as the heart, lung and gastrointestinal tract. Interestingly, although the thyroid gland is innervated by sympathetic and parasympathetic neurons no CGRP expression is detectable there. Likewise calcitonin expression is not activated in the brain or sensory ganglia (Rosenfeld *et al*, 1983). The immunostaining pattern of CGRP is very similar to that of the neuropeptide substance P, as the two are often expressed in the same cells (Carlton *et al*, 1987).

CGRP is often expressed in cells that are involved in pain transmission and the peptide is believed to be involved in mechano- and thermo-nociception and neurogenic inflammation (Taylor and Pierau, 1991). For example, CGRP has been shown to promote nociceptive transmission in the spinal dorsal horn of the rat during conditions of inflammation (Kawamura *et al*, 1989), and in the adjuvant arthritic rat, changes to CGRP levels are largely restricted to areas of neurogenic inflammation and can be reduced by treatment with the anti-inflammatory diclofenac sodium (Kuraishi *et al*, 1989). In addition, whilst substance P is involved in increasing vasopermeability, CGRP is a highly potent vasodilator (Holzer, 1998). The primary afferent neurons originating in the dorsal root ganglia form a perivascular network around the arterial system and, when stimulated, are able to release CGRP to induce vasodilatation, often in co-operation with nitric oxide (Holzer *et al*, 1995). In man, a slight increase in the physiological levels of CGRP will cause flushing, hypotension, secondary catecholamine release and subsequent tachycardia. Intravenous injections of CGRP cause systemic vasodilatation and a re-distribution of blood to the brain and skin. It is also believed that CGRP is involved in blood pressure modulation as CGRP levels decrease in hypertension (Raynaud *et al*, 1994). Other roles of CGRP include glucose metabolism and the regulation of electrolyte and water flow in the colon.

1.8.2 CGRP and NGF

In vivo experiments on neonates indicated that NGF regulates the expression of substance P in sensory neurons (Kessler and Black, 1980; Otten *et al*, 1980). It has since been shown that NGF also regulates the neuronal expression of CGRP; NGF treatment of cultured adult DRG neurons increases both CGRP mRNA levels and the amount of CGRP secreted (Lindsay, 1988; Lindsay *et al*, 1989; Lindsay and Harmar, 1989). NGF treated DRG neurons in culture produce increased levels of CGRP within three days and continue to do so for up to 18 days. Indeed, NGF can increase CGRP mRNA levels in cultured cells as much as fifteen fold

(Lindsay *et al*, 1989). Thus, although adult neurons are no longer dependent upon NGF for survival, NGF is still able to modulate neuronal CGRP expression, suggesting that CGRP activation is not merely a survival effect of the NGF, but continues into adulthood. As a regulator of Substance P and CGRP, NGF has been implicated in the maintenance of chronic pain states. Heterozygotes for NGF exhibit defects in nociception whilst the overexpression of NGF in a transgenic mouse model leads to the ectopic expression of the neuropeptides CGRP and Substance P (Ma *et al*, 1995). Systemically introduced antibodies against NGF reduce neuropeptide up-regulation (Otten, 1984) and the behavioural hypersensitivity that would normally occur during induced inflammation states. NGF antibodies applied to nociceptive afferent neurons reduce neurogenic inflammation (Tonra and Mendell, 1998).

1.8.3 The CT/CGRP Promoter

The CT/CGRP gene has been sequenced in both rat and human and the promoter region analysed for key regulatory elements. Many key sequences involved in tissue-specific transcription have already been identified and these are discussed below.

The CT/CGRP promoter is sensitive to glucocorticoid, retinoic acid, cAMP and NGF, and the specific CT/CGRP promoter elements responsible for this sensitivity have been identified and extensively studied. CT/CGRP transcription in thyroid C cells is regulated by an element in the gene promoter located between –1127 and –957 in rat and –941 and –898 in human (Stolarsky-Fredman *et al*, 1990; Ball *et al*, 1992). In normal conditions this element strongly induces calcitonin expression; experiments in thyroid C cell lines showed that an 18bp element at –1025 to –1043 in the rat gene was able to confer a 50 fold induction of a luciferase reporter when linked to a heterologous promoter. This enhancer element contains binding sites for helix-loop-helix (HLH) and octamer transcription factors. When a point mutation was introduced into either binding motif, induction of gene expression was reduced ten fold. Separation of the motifs also caused down-regulation of the promoter. Thus it was identified that in thyroidal cells the HLH and octamer transcription factors form a cell-specific complex and act synergistically to activate the CT/CGRP promoter (Tverberg and Russo, 1993).

Further experiments with retinoic acid showed that nuclear retinoic acid receptors down-regulate activity of the CT/CGRP promoter and that this down-regulation could be attributed to the 18bp enhancer containing the HLH and octamer binding sites. Retinoic acid repression

is dependent upon both octamer and HLH binding motifs, and disrupts binding of the HLH/octamer enhancer complex (Lanigan *et al*, 1993). The 18bp element was also shown to have a glucocorticoid receptor half-site which is responsible for down-regulation of promoter activity in thyroid C cell lines in response to glucocorticoids. Again glucocorticoids appear to repress gene expression by the inhibition of cell-specific transcription factors (Tverberg and Russo, 1992).

The CT/CGRP promoter can also be regulated by cAMP at CRE sites located within the region –132 to –252 (de Bustros *et al*, 1990). This enhancer contains two CRE sites, two octamer binding sites and two C-rich AP2-like elements. In a thyroid carcinoma cell line the downstream CRE combined with CT/CGRP promoter sequences was accountable for 70% of the cAMP-mediated activation of the CT/CGRP promoter, whilst the upstream CRE conferred 10% (de Bustros *et al*, 1992). Therefore, in thyroid C cell lines at least, the cAMP activation of the CT promoter is the additive effect of a number of different enhancer elements within the promoter sequence. This, however, does not appear to be the case for the cAMP-induced expression of CGRP in neuronal cells and cell lines, as described below.

In order to be able to carry out promoter studies with respect to CGRP expression in neuronal cells, several new plasmids were created (Symes *et al*, 1992; Watson *et al*, 1995; Watson and Latchman, 1995). The Calgcat plasmids contain various regions of the CT/CGRP promoter linked to the bacterial chloramphenicol acetyl transferase gene in the Gcat vector (Calgcat 1-6 and 10) or pBLCAT2 (Calgcat 9). Deletion mapping experiments using these plasmids identified that CT/CGRP regulation by cAMP in neuronal cells involves an element located downstream of the thyroidal cAMP-sensitive sites, at –103 to –109 (Watson *et al*, 1995; Watson and Latchman, 1995). This element has the sequence 5'-TGACGCA-3' and bears a strong resemblance to the consensus CRE. When the CRE site in Calgcat 4 (-140 to +8 of the CT/CGRP gene) was mutated, cAMP-responsiveness of the promoter in DRG neurons was totally abolished (Watson and Latchman, 1995). This suggests that the CRE site is necessary for the response to cAMP in neuronal cells. When the CRE alone was linked to a heterologous promoter, the CRE site was able to confer cAMP-responsiveness to the test promoter. This indicates that the CRE is sufficient as well as necessary for the cAMP-induced activation of the promoter. It is probable that cAMP induces the phosphorylation of CRE-bound CREB at serine 133, presumably via the activation of PKA, to stimulate transcription in co-operation with CBP, although the exact mechanism has not yet been investigated.

As well as being responsive to cAMP, the CT/CGRP promoter is also sensitive to NGF in neuronal cells. In PC12 cells, Calgcat 1 (-1670 to +8 of the CT/CGRP gene) expression can

be induced up to six fold by NGF. As with cAMP, promoter studies have shown that NGF-mediated CT/CGRP promoter activation involves the CRE site located at –103 to –109. As with cAMP a mutation at this site abolishes the NGF response, indicating that the CRE is essential for NGF-mediated transcription. However, when linked to a heterologous promoter, the isolated CRE is no longer NGF-sensitive in cultured DRG neurons (Watson *et al*, 1995; Watson and Latchman, 1995). This suggests that the CRE alone is not sufficient for NGF-mediated promoter activation, but rather some other regulatory factor is required for this. Indeed, the region –140 to –72 (Calgcat 9), which contains the CRE site, is the minimum sequence necessary for a heterologous promoter to be activated by NGF in DRG neurons. In PC12 cells Calgcat 1, but not Calgcat 9 or Calgcat 4, will respond to NGF. Again as with cAMP, it is likely that NGF-mediated expression of the CT/CGRP promoter involves the transcription factor CREB, and possibly CBP. However, NGF can initiate a number of different signalling cascades in neurons and PC12 cells, many of which result in the phosphorylation of CREB. The exact mechanism for CRE-mediated activation of CGRP is as yet unknown.

In conclusion, the CRE is necessary and sufficient for the cAMP-mediated CT/CGRP promoter activation, and necessary but not sufficient for activation by NGF. The signalling mechanisms controlling these processes have not yet been identified.

1.9 The Tyrosine Hydroxylase and Bcl-2 Genes

1.9.1 The Tyrosine Hydroxylase Gene

Tyrosine hydroxylase (TH) is an enzyme expressed in neuronal tissues that is involved in the synthesis of catecholamines. It catalyses the oxidative hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine, the first and most rate-limiting step in the biosynthesis of neurotransmitters such as dopamine, norepinephrine and epinephrine in neuronal cells and epinephrine in adrenal chromaffin cells (Yoon and Chikaraishi, 1992). TH is expressed in the PNS in sympathetic ganglia and in adrenal medulla chromaffin cells, which are derived from the neural crest. Both NGF-stimulated and non-stimulated PC12 cells also synthesise TH (Greene and Tischler, 1976). In the CNS, TH is expressed in certain neurons of the midbrain, brainstem, diencephalon, retina and olfactory bulb (Hoekfelt *et al*, 1984). TH enzyme activity is regulated both at the protein level and transcriptional level. TH protein modulation is achieved by the phosphorylation of existing TH molecules, so increasing their affinity for the co-factor tetrahydropterin (Lloyd and Kaufman, 1974). Phosphorylation of TH is mediated by the cAMP-sensitive enzyme PKA (Zigmond *et al*, 1989).

TH transcription is sensitive to many stimuli, including cAMP, NGF, EGF, and glucocorticoids (Lewis and Chikaraishi, 1987; Lewis *et al*, 1987; Gizang-Ginsberg and Ziff, 1990). Analysis of the TH gene regulatory sequences revealed the presence of many potential transcription factor binding sites, including AP2, AP1, E2A/MyoD, POU and SP1 (Fung *et al*, 1992; Yang *et al*, 1998). A consensus CRE site, located at –38 to –45 in the rat gene, is essential for basal expression from the TH gene as well as cAMP-activated expression (Fung *et al*, 1992; Kim *et al*, 1993; Lazaroff *et al*, 1995). Interestingly, gel-shift assays using PC12 cell nuclear extracts revealed that both the transcriptional activator CREB and the repressor CREM were bound to the consensus CRE site. Also transfection experiments showed that the CREM family member ICER can repress the transcriptional activity of the TH promoter. This suggests that cAMP regulation of the TH promoter can be both positive and negative, with ICER rapidly reversing the increase in TH transcription in response to increases in cAMP levels (Tinti *et al*, 1996). The activation of TH transcription by cAMP is mediated by the PKA signalling pathway and activation of CREB transcription factor, rather than any other CREB family member (Piech-Dumas and Tank, 1999).

So, as with the CT/CGRP gene, transcription of the TH gene is inducible by cAMP, a response mediated by the CRE sequence. Unlike the CT/CGRP gene, however, NGF-induced expression of the TH gene does not require the CRE element. NGF-activated TH expression is mediated by an AP1 site (Gizang-Ginsberg and Ziff, 1990), and studies in PC12 cells revealed that this TH 'fat specific element' (TH-FSE) binds to the Fos/Jun transcription factor complex (Ghee *et al*, 1998). Like the CRE, the FSE is also important in basal expression of the gene. In addition, immunohistochemical staining of the rat brain revealed that Fos and CREB contribute to TH activation differentially in different tissues. TH regulation in the dopaminergic cells of the midbrain is likely to be regulated by the CRE-associated factors CREB and CBP, whereas TH regulation in the olfactory bulb appears to be mediated by Fos (Ghee *et al*, 1998).

1.9.2 Tyrosine Hydroxylase and Hypoxia

A reduction in arterial oxygen tension (hypoxia) is a powerful physiological stimulus and can induce the synthesis and release of dopamine from certain O_2-sensitive cells. As well as being sensitive to cAMP and NGF, the TH promoter is also sensitive to a reduction in oxygen levels, as hypoxia can both increase the rate of transcription, and increase the stability of TH mRNA in PC12 cells (Czyzyk-Krzeska *et al*, 1994). Several promoter sequences involved in the TH response to hypoxia have been identified, including the AP1 site (Norris and Millhorn, 1995) and the CRE (Beitner-Johnson and Millhorn, 1998). Promoter studies of the TH gene in PC12 cells have shown that the TH promoter can be activated approximately three fold in conditions of hypoxia. However, if the CRE is mutated, most of this response is lost. Furthermore, hypoxia induces the rapid and persistent phosphorylation of CREB at serine 133 in PC12 cells (Beitner-Johnson and Millhorn, 1998). This CREB activation is mediated by an as yet unidentified signalling mechanism, since the inhibition of numerous signalling molecules known to mediate CREB activation, e.g., MAPK, PKA, PKC, RSK-2, has no effect on hypoxia-induced CREB activation. This suggests that a novel signalling mechanism triggered in response to hypoxia may be responsible for the phosphorylation of CREB and the transcription of TH and, potentially, other CRE-regulated genes.

1.9.3 The Bcl-2 Gene

Bcl-2 is a protein involved in the regulation of programmed cell death. Expression of Bcl-2 has a protective effect preventing apoptosis in many cell types, including B cells and neurons (Reed, 1994; White, 1996). During normal neuronal development many cells die by apoptosis and Bcl-2 plays an important role in the survival of the remaining cells. Knock-out mice carrying an inactive Bcl-2 gene lose motor, sensory and sympathetic neurons (Michaelidis *et al*, 1996), and culture of neurons derived from these mice is difficult as their ability to survive is compromised (Greenlund *et al*, 1995). Conversely the overexpression of Bcl-2 in animal models leads to enhanced neuronal survival and an increase in neuronal cell numbers during development (Dubois-Dauphin *et al*, 1994; Farlie *et al*, 1995). Overexpression of Bcl-2 is also able to improve the survival of neurons in culture, as well as protect them from apoptotic stimuli such as neurotrophic factor withdrawal (Allsopp *et al*, 1993; Middleton *et al*, 1996). Over-expression of Bcl-2 in PC12 cells enhances neuronal survival after NGF withdrawal (Batistatou *et al*, 1993). When antisense constructs are used to reduce the Bcl-2 levels in cultured neurons, then the survival effects of neurotrophic factors are inhibited (Allsopp *et al*, 1995). A reduction in the Bcl-2 levels of PC12 cells inhibits the survival effects of NGF treatment (Katoh *et al*, 1996). PC12 cells grown in serum-free medium in the absence of neurotrophin also undergo apoptosis unless protected by Bcl-2 (Sato *et al*, 1994). Thus Bcl-2 mediates the anti-apoptotic effects of neurotrophic factors such as NGF in neuronal cells.

It has also been reported that certain neuronal cells alter the rate of Bcl-2 synthesis in response to hypoxia. During hypoxia treatment, the Bcl-2 of rat cortical neurons is actually down-regulated, and this is accompanied by apoptosis (Tamatani *et al*, 1998). Cultured neurons from the foetal rat brain are able to up-regulate the survival factor Bcl-2 during a 24-hour re-oxygenation period following 6 hours of hypoxic injury. This up-regulation can be further increased if the cells are pre-conditioned with a one-hour exposure to hypoxia 24 hours prior to the 6-hour treatment. This reduces the level of hypoxia-induced cell death (Bossenmeyer-Pourie and Daval, 1998).

Bcl-2 gene transcription is regulated by two promoters; an upstream promoter known as P1 and a downstream promoter located entirely downstream of –1280 known as P2. The predominant promoter in non-neuronal cells, such as B cells, is P1 (Chen and Boxer, 1995; Wilson *et al*, 1996), whereas P2 is the major transcriptional promoter in neuronal cells (Smith *et al*, 1998a; Smith *et al*, 1998b). Several regulatory elements involved in Bcl-2 promoter activation in neuronal cells have been identified. For instance, the neuronally expressed

transcription factor Brn-3a mediates Bcl-2 P2 promoter activation via a sequence located between –584 and –594 base pairs relative to the start site (Smith *et al*, 1998a; Smith *et al*, 1998b). The Bcl-2 gene also has a CRE site located at –1546 to –1537, which was first identified as a regulatory element for Bcl-2 expression in B cells (Wilson *et al*, 1996). The CRE site lies in a region of P1 known to be important for the NGF-responsiveness of the isolated P1 promoter in neuronal cells (Liu *et al*, 1999). It was thought that, as with the CT/CGRP promoter, the CRE may mediate this NGF-sensitivity. In fact, mutational analysis showed that the CRE was not required for the NGF response, and deletion-mapping experiments in PC12 cells located the NGF-sensitivity to a site downstream of the CRE at –1472 to –1414 (Liu *et al*, 1999). However, more recent conflicting evidence suggests that NGF-mediated regulation of Bcl-2 in PC12 cells may actually require the CRE (Riccio *et al*, 1999); therefore the involvement of the CRE-binding transcription factor CREB in Bcl-2 promoter activation in response to NGF is still under debate. Interestingly, promoter studies have also suggested that the Bcl-2 promoter is not sensitive to cAMP, despite the presence of the CRE site (Liu *et al*, 1999). The role of the CRE in Bcl-2 transcription regulation in neuronal cells has not been investigated further; therefore it is not yet known whether the CRE is involved in the up-regulation of Bcl-2 in response to hypoxia, as with the tyrosine hydroxylase gene. It would be interesting to see if the up-regulation of Bcl-2 in response to hypoxia could be due to CREB phosphorylation and CRE-mediated transcription from the Bcl-2 promoter.

1.10 Aims of the Project

The Bcl-2, CT/CGRP and tyrosine hydroxylase genes all have CRE sites in their promoter sequences. In the case of the CT/CGRP promoter, a CRE approximately 100bp from the start site is necessary for the activation of transcription in response to both NGF and cAMP, but not sufficient for the response to NGF in neuronal cells. The signalling pathways mediating these responses are as yet unknown, however it is believed that the cAMP response involves the enzyme PKA and that both responses involve the activation of CREB transcription factor by phosphorylation of serine 133. The tyrosine hydroxylase gene has a CRE site that is sensitive to cAMP and hypoxia. The cAMP response is mediated by PKA and CREB and the response to hypoxia involves the activation of CREB by an uncharacterised novel signalling mechanism. Unlike the CT/CGRP CRE site, the TH CRE is not sensitive to NGF. Similarly, the Bcl-2 gene CRE site is probably not involved in the activation of transcription in response to NGF.

It is proposed that the NGF- and cAMP-mediated transcription of the CT/CGRP gene involves different and distinct signalling pathways that lead to phosphorylation of the transcription factor CREB. It is also proposed that the Bcl-2 and CT/CGRP genes may be sensitive to hypoxia at their CRE sites. The first aim of this project was to identify the signalling mechanisms involved in the NGF- and cAMP-mediated induction of CT/CGRP transcription in PC12 cells. The second aim of this project was to investigate the differential regulation of the CT/CGRP, Bcl-2 and TH CRE sites in PC12 cells by:

a) Investigating the sensitivity of the Bcl-2 promoter to NGF, cAMP and hypoxia.

b) Comparing the effects of NGF, cAMP and hypoxia on an isolated CT/CGRP CRE linked to a heterologous promoter with the effects of these stimuli on isolated Bcl-2 and TH CRE sites

c) Comparing the protein-binding capabilities of the CT/CGRP, Bcl-2 and TH CRE sites using electrophoretic mobility shift assays.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

All centrifuges used were from Beckman Instruments Inc., Palo Alto, CA 94304, USA. These include Micro Centaur bench-top micro-centrifuges, Beckman model J-6B for pelleting bacterial/cell cultures and Beckman model J2-21 used in the large-scale preparation of DNA.

The concentrator used was a Labconco Centrivap Concentrator, Labconco Corporation, Kansas City, MO 64132-2696, USA.

The bacterial incubator shakers were from New Brunswick Scientific Co. Inc., Edison, N. J. USA.
The Gallenkamp Plus II bacterial incubator oven was supplied by Sanyo Gallenkamp plc., Loughborough, Leicestershire LE11 5XG, UK.

The class III microbiological safety cabinet was a Microflow HLFB4 from Labcaire Systems, Clevedon, Avon BS21 6XU, UK.
The cell culture incubator was a model GA2SM incubator from LEEC Ltd., Colwick, Notingham, UK.
The tissue culture incubator medical CO_2 and the hypoxia chamber 5% CO_2 /95% argon gas mix were supplied by BOC Gases (Medical), Priestly Road, Worsley, Manchester M28 2UT.
The microscope used during cell culture work was a Nikon TMS from Microinstruments Ltd., Long Hanborough, Oxford OX7 2LH, UK.
The hypoxia chamber was custom-made at the Department of Physiology, University College London, UK.

The luminometer used was a Turner TD-20e, Turner Designs, Sunnyvale, CA 94086, USA.
The densitometer used was a Bio-Rad model GS-670 Imaging Densitometer, and the phosphoimager used was a Bio-Rad model GS-250 Molecular Imager from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. HP2 7TD, UK.

The plate reader was a Labsystems multiskan RC microtitre plate reader from Labsystems, Helsinki 00811, Finland, using Genesis software v1.87 from Life Sciences International (UK) Ltd., Basingstoke, Hampshire RG21 6YH, UK.

The Vertical Gel Electrophoresis System, Model V16, used for polyacrylamide gel electrophoresis was manufactured by BRL Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD 20877, USA.
Western blotting was carried out using the Bio-Rad Trans-blot ™ Cell, Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., HP2 7TD, UK.
The agitator used for western blotting was an R100 Rotatest Shaker, Luckham Ltd., Burgess Hill, Sussex, England.
The Model 583 Gel Dryer was from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. HP2 7TD, UK.
The agarose gel electrophoresis tanks were from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts. HP2 7TD, UK.
High-voltage power packs (P30) were from Biometra GmbH, 37079 Goettingen, Germany.
The transilluminator and 6405 UV/Vis. Spectrophotometer were produced by Jenway Ltd., Dunmow, Essex CM6 3LB.
The polaroid camera used was a Polaroid MP.4 land camera from Polaroid, Cambridge, MA, USA.

2.1.2 Consumables

Microfuge tubes (0.5 ml and 1.5 ml) were supplied by Anachem Ltd., Luton, Beds., UK.
Gilson pipette tips were supplied by Elkay products Ltd., Boston, MA, USA.
Falcon tubes (15 ml an 50 ml) were purchased from Greiner Labortechnik Ltd., Stonehouse, Glos., UK.
0.2 μm acrodisc filters were from Gelman Sciences, Ann Arbor, MI, USA.
Silica gel coated TLC plates were supplied by Whatman Ltd., Maidstone, Kent, UK.
Luminometer tubes were purchased from the Promega Corporation, Madison, WI, USA.
Hybond C nitrocellulose membrane was from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.
Polaroid black and white film (type 667) was from Polaroid, Cambridge, MA, USA
Kodak Xomat R autoradiographic film was from Eastman Kodak Co., Rochester, NY, USA.

2.1.3 Chemical Reagents

With the exception of those chemicals listed below, general wet and dry laboratory chemicals were supplied by Merck Ltd., Poole, Dorset, UK, or Sigma Chemical Company, St. Louis, MO, USA.
Electrophoresis grade agarose was purchased from Gibco BRL, Life Technologies Ltd., Paisley, Scotland.
Phenol was supplied by Fisher Scientific UK Ltd., Loughborough, Leics., UK.
5x reporter lysis buffer and luciferase assay reagent were supplied by the Promega Corporation, Madison, WI, USA.
Non-denaturing 30% Acryl/Bis solution (37.5:1) was from Amresco, Solon, OH, USA.
Marvel dried skimmed milk was from Premier Beverages, Adbaston, Stafford, UK.

All solutions were made up in double-distilled, de-ionised water (Milli-Q Plus 185, Millipore UK Ltd, Watford, Herts. UK) and autoclaved for at least 10 minutes at 120°C, or filtered through a 0.2 μm filter, except for those solutions containing sodium dodecyl sulphate (SDS).

2.1.4 Biological Reagents

1 kb ladder DNA molecular weight markers were purchased from both MBI Fermentas, Sunderland, Tyne and Wear, UK and Gibco BRL, Life Technologies Ltd., Paisley, Scotland.

The Rainbow™ coloured protein molecular weight markers, high molecular weight range (14.3 kD - 220 kD), were from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.

The radioactive isotopes [γ-^{32}P] ATP (10 mCi/ml, 3000 Ci/mmol) and [^{14}C] chloramphenicol (25 μCi/ml, 50-62 mCi/mmol) were supplied by Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.

ATP was from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.
Poly(di-dC)·poly(dI-dC) double stranded sodium salt was supplied by Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.

RNase A, proteinase K and acetyl co-enzyme A were supplied by Roche Diagnostics Ltd., Lewes, East Sussex BN7 1LG, UK.

All restriction endonucleases, T4 polynucleotide kinase, alkaline phosphatase and T4 DNA ligase, together with their respective 10x buffers, were supplied by the Promega Corporation, Madison, WI, USA.

2.1.5 Kits

Galacto-Light Plus™ reporter gene assay for β-Galactosidase was from Tropix, 47 Wiggins Avenue, Bedford, MA 01730 USA.
Enhanced Chemiluminescence system (ECL™) was from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, UK.
BCA Protein Assay Reagent was from Pierce and Warriner (UK) Ltd., Chester, UK.

2.1.6 Bacterial Culture

All plasmids were grown up in *Escherichia coli* strains DH5α and XL-1 Blue purchased from Gibco BRL, Life Technologies Ltd., Paisley, Scotland.
Antibiotics were obtained from Sigma Chemical Company, St. Louis, MO 63178, USA.
Yeast extract, tryptone and bacto-agar were obtained from Difco Laboratories, Detroit, MI, USA.

2.1.7 Tissue Culture

PC12 cells came from cell banks at the European Collection of Cell Cultures (ECACC), CAMR, Porton Down, Salisbury, SP4 0JG, UK.
Foetal bovine serum, horse serum, Leibowitz L-15 medium, Dulbecco's Modified Eagle Medium (DMEM), Hanks' Bal. Salt (HBSS), 7.5% sodium bicarbonate, penicillin/streptomycin (10000 IU/ml, 10000 μg/ml) and 200 mM L-glutamine were supplied by Gibco BRL, Life Technologies Ltd., Paisley, Scotland. Sera were heat-inactivated at 56°C for 30 minutes before use.
Tissue culture plastics (flasks and multi-well plates) were supplied by Nalgene Nunc International, Poole, Dorset, UK

2.1.8 Plasmid DNA

All plasmids shown below are ampicillin resistant unless otherwise stated.

Name of Plasmid	Description	Source
pRc/CMV	Empty expression vector containing CMV promoter. Neo and amp resistant.	Invitrogen, 9351 NV Leek, The Netherlands
pRc/RSV	Empty expression vector containing RSV promoter. Neo and amp resistant.	Invitrogen, 9351 NV Leek, The Netherlands
RSV β-gal	β-galactosidase expression vector containing an RSV promoter.	Laboratory stocks
pBLCAT2	CAT expression vector with tk promoter derived from pUC18.	Dr G. Schuetz, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Germany
Calgcat 1	CAT expression vector containing –1670 to –1 of the CT/CGRP promoter	Dr. Paul Brickell, Windeyer Institute, London W1P 6DB UK
TH272CAT	CAT expression vector containing –272 to +27 of the TH promoter	Dr. D. M. Chikaraishi, Tufts University, Boston, MA 02111, USA
pRcRSV Gal4(1-147) [RpxG4]	Gal4 DNA binding domain (DBD) expression vector.	Dr. J. C. Chrivia, Saint Louis University School of Medicine, St. Louis, MO 63104, USA
Gal4CBP(1678-2441)	Expression vector of Gal4 DBD linked to the CBP C-terminal domain.	
5xGal4-E1B-Luciferase	Luciferase expression vector containing 5x Gal4 binding sites and E1B element.	Dr. R. A. Maurer, Oregon Health Sciences University, Portland, OR 97201-3098, USA
LB322	Promega pGL2 luciferase expression vector containing bases –3934 to –8 of the Bcl-2 promoter	All LB constructs were from Prof. Linda Boxer, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5112, USA
LB329	Luciferase expression vector containing bases –1642 to –8 of the Bcl-2 promoter	
LB335	Luciferase expression vector containing bases –1281 to –8 of the Bcl-2 promoter	
LB330	Luciferase expression vector containing bases –751 to –8 of the Bcl-2 promoter	
LB124	Luciferase expression vector containing bases –3934 to –1280 of the Bcl-2 promoter	

Name of Plasmid	Description	Source
LB170	Luciferase expression vector containing bases –2857 to –1280 of the Bcl-2 promoter	All LB constructs were from Prof. Linda Boxer, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5112, USA
LB347	Luciferase expression vector containing bases –2337 to –1280 of the Bcl-2 promoter	
LB135	Luciferase expression vector containing bases –1795 to –1280 of the Bcl-2 promoter	
LB351	Luciferase expression vector containing bases –1746 to –1280 of the Bcl-2 promoter	
LB334	Luciferase expression vector containing bases –1644 to –1280 of the Bcl-2 promoter	
LB595	As LB334 but with mutated CRE at –1546 to –1537	
LB375	Luciferase expression vector containing bases –1526 to –1280 of the Bcl-2 promoter	
LB360	Luciferase expression vector containing bases –1337 to –1280 of the Bcl-2 promoter	
LB374	Luciferase expression vector containing bases –1644 to –1521 of the Bcl-2 promoter	
LB367	Luciferase expression vector containing bases –1526 to –1332 of the Bcl-2 promoter	
ACREB	Expression vector for the dominant negative form of CREB, ACREB, in RcCMV	Dr Charles Vinson, National Institutes of Health, Bethesda, MD 20892, USA
KCREB	Expression vector for the dominant negative form of CREB, killer CREB, on an RSV promoter	Dr Richard Goodman, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201-3098, USA
RSVPKI	Expression vector for the PKA inhibitor PKI on an RSV promoter	Dr. R. Maurer, Oregon Health Sciences University, Portland, OR 97201-3098, USA.
Active PKA	Expression vector for constitutively active PKA	
Active PKCδ	Expression vector for constitutively active PKCδ	Dr. M. Marber and Dr. P. Parker, Department of Cardiology, United Medical and Dental Schools of Guy's and St. Thomas' Hospitals, London SE1 7EH, UK
pCAGGS	Empty expression vector with CMV-IE enhancer and chicken β-actin promoter	
Active PKCε	Expression vector for constitutively active PKCε in pCAGGS	
DN p42Y185F	Expression vector for a dominant negative mutant of p42 map kinase (ERK-2) in pRcCMV. Amp and neo resistant.	Dr. M. H. Cobb, University of Texas, Dallas 75235-9041, USA

Name of Plasmid	Description	Source
DN MEK-6	Expression vector for a dominant negative mutant of the p38 activator MEK-6	Dr Roger Davis, Howard Hughes Medical Institute, University of Massachusetts Medical School, MA, USA
DN p54b	Expression vector for the dominant negative JNK p54	
DN MEK-1	Expression vector for a dominant negative mutant of the p42 activator MEK-1	Dr. J. Pouyssegur, Universite de Nice, 06108 Nice, France
MEK-1 SS/DD	Expression vector for a constitutively active mutant of p42 activator MEK-1	
DN p44Y194F	Expression vector for a dominant negative mutant of p44 MAP kinase (ERK-1) where Y is mutated to F. Kanamycin resistant.	
DN p44T192A	Expression vector for a dominant negative mutant of p44 MAP kinase, where T192 is mutated to A. Kanamycin resistant.	
DN RasN17	Expression vector for a dominant negative mutant of the Raf activator Ras	Dr. P. J. Stork, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201-3098, USA
DN RapN17	A dominant negative mutant of Rap-1	
PMT-2	Empty expression vector	Prof. David Ginty, Harvard Medical School, Boston, MA 02115, USA
PMT-2- HA- RSK-2-KR100	A dominant negative mutant of CREB activator RSK-2 in PMT-2	

2.1.9 Bioactive Chemicals

Chemical	Carrier	Concentration Used	Supplier
Anisomycin	MeOH	3 μg/ml	Sigma Chemical Company, St. Louis, MO, 63178 USA.
H-7 dihydrochloride, PKC inhibitor	H_2O	20 μM	Tocris Cookson Ltd., Bristol, BS18 7DY, UK
H89 dihydrochloride, PKA inhibitor	H_2O	20 μM	Calbiochem-Novabiochem Corp., La Jolla, CA 92039-2087 USA
PD98059, MEK1 inhibitor	DMSO	50 μM	New England Biolabs, Beverly, MA 01915, USA
Ro318220 RSK-2 inhibitor	DMSO	0.3 μM	Calbiochem-Novabiochem Corp., La Jolla, CA 92039-2087 USA
SB203580, p38 inhibitor	DMSO	20 μM	Calbiochem-Novabiochem Corp., La Jolla, CA 92039-2087 USA
NGF-2.5S from mouse submaxillary glands	L-15	25 ng/ml	Sigma Chemical Company, St. Louis, MO, 63178 USA.
Di-butyryl cAMP	L-15	1 mM	Sigma Chemical Company, St. Louis, MO, 63178 USA.

2.1.10 Oligonucleotides

TH and Bcl-2 CRE oligonucleotides were obtained from Genosys Biotechnologies (Europe) Ltd., Pampisford, Cambs., CB2 4EF, UK, and were adapted from TH and Bcl-2 CRE oligonucleotides described previously (Tinti *et al*, 1996; Wilson *et al*, 1996).

CGRP CRE oligonucleotides were obtained from ammonia stocks of oligonucleotides synthesised in our own laboratory.

Oligonucleotide	Sequence
Bcl-2 CRE	5'-GATCAACCGTGTGACGTTACGCACAG-3' 3'- TTGGCACACTGCAATGCGTGTCCTAG-5'
CGRP CRE	5'-GATCCAGGGTGACGCAACCCA-3' 3'-GTCCCACTGCGTTGGGTCTA G-5'
TH CRE	5'-GATCCAGGGGCTTTGACGTCAGCCTGA-3' 3'-GTCCCCGAAACTGCAGTCGGACTCTAG-5'

2.1.11 Antibodies

Antibody	Type	Use	Concentration/ Amount Used	Supplier
Anti-ATF-1 (200 μg/0.1 ml)	Mouse monoclonal IgA	EMSA	1.4 μg	Santa Cruz Biotechnology inc., Santa Cruz, CA, USA
Anti-CREB-1 (200 μg/ml)	Mouse monoclonal IgG_{2b}	EMSA	1.4 μg	Santa Cruz Biotechnology inc., Santa Cruz, CA, USA
Anti-Gal4 (DBD) (200 μg/ml)	Rabbit polyclonal IgG	EMSA	1.4 μg	Santa Cruz Biotechnology inc., Santa Cruz, CA, USA
Anti-Phosphorylated CREB (25 μg/ml)	Rabbit polyclonal Igs	Western blotting	1:1000	New England Biolabs Beverly, MA 01915, USA
Anti-actin (200 μg/ml)	Goat polyclonal IgG	Western blotting	1:2000	Santa Cruz Biotechnology inc., Santa Cruz, CA, USA
HRP-conjugated anti-goat Igs	Polyclonal rabbit Igs	Western blotting	1:2000	Dako A/S DK-2600 Glostrup, Denmark
HRP-conjugated anti-rabbit Igs	Polyclonal goat Igs	Western blotting	1:2000	Dako A/S DK-2600 Glostrup, Denmark

2.2 Microbiology

2.2.1 Culturing Bacteria

Solid phase plate cultures of *Escherichia coli* from glycerol stocks or transformations were grown up overnight at 37°C on LB-agar in 10 cm petri dishes. Antibiotic resistant strains were grown on LB-agar containing the appropriate antibiotic. Ampicillin, kanamycin and tetracycline were used at final concentrations of 100 μg/ml, 10 μg/ml and 12 μg/ml respectively.

Liquid cultures of ampicillin or kanamycin resistant strains were grown up overnight at 37°C in LB broth, containing either 100 μg/ml ampicillin or 10 μg/ml kanamycin, as appropriate, in an orbital shaker at 200rpm.

Mini-cultures of up to 10 ml were grown by inoculating LB with a single bacterial colony from an LB agar plate. Maxi-cultures of 500 ml were grown by inoculating 500 ml of LB with 10 ml of mini-culture.

1L LB:	10 g bacto-tryptone
	5 g yeast extract
	10 g NaCl
1L LB-agar:	As LB with additional 15 g bacto-agar

2.2.2 Preparation of Competent Cells

Host bacteria were streaked out on an LB-agar plate (DH5α with no supplements, XL-1 blue with 12 μg/ml tetracycline) and grown up overnight at 37°C. 50ml of LB broth was then inoculated with one colony from the plate and grown to a density of 0.4 - 0.55 OD_{580}. Cells were cooled on ice for 5 minutes and pelleted at 4°C for 5 minutes at 2500g. The pellet was resuspended in 15 ml of freezing-buffer RFBI and left on ice for 15 minutes before a second

centrifugation. RFBI was removed and the pellet resuspended in 4 ml of ice-cold freezing-buffer RFBII. Cells were snap frozen and stored in 100-500 µl aliquots at -70°C until needed.

RFBI:
0.3 g Potassium acetate
0.99 g Manganese chloride
1 ml 1M $CaCl_2$
2.4 ml RbCl (0.5 g/ml)
15 ml Glycerol
1 ml 0.2M Acetic acid
Filter sterilised in 100 ml sterile water - final pH 5.8, stored at 4°C

RFBII:
5 ml 0.1 M Mops pH 6.8
3.75 ml 1M $CaCl_2$
120 µl RbCl (0.5 g/ml)
7.5 ml Glycerol
Filter sterilised in 50 ml sterile water - final pH 6.8, stored at 4°C

2.2.3 Transforming Competent Cells

Competent cells were thawed at room temperature and immediately divided into 50 µl aliquots. These were left on ice for 3 minutes before the addition of 50 ng of plasmid DNA, in no more than a 5 µl volume. Transformations were incubated on ice for a further 40 minutes. Samples were then heat-shocked for 3 minutes at 42°C, left to cool on ice, and then incubated with 250 µl LB for 30 minutes at 37°C. The cells were then plated onto LB agar containing the appropriate antibiotic.

For each transformation a negative control (no DNA) and positive control (control plasmid) were carried out.

2.2.4 Long Term Storage of Bacterial Cultures

Samples of overnight bacterial cultures were stored as glycerol stocks in 40% glycerol (v/v), at -70°C.

2.3 Preparation and Analysis of Plasmid DNA

2.3.1 Small-scale DNA Preparation – Miniprep

Plasmid DNA was extracted from 3 ml of liquid bacterial culture using the alkaline lysis method (Sambrook *et al*, 1989). Bacterial cells were pelleted in a micro-centrifuge at 12000g for 5 minutes and resuspended in 100 µl lysis buffer (solution 1). Cells were lysed by vortexing, and gently mixed with 200 µl of NaOH/SDS mix (solution 2). Cellular debris and chromosomal DNA were precipitated with 150 µl of 5 M/3 M potassium acetate (solution 3) on ice for 5 minutes, and pelleted in a micro-centrifuge for 5 minutes at 12000g. The supernatant containing plasmid DNA was purified further by phenol/chloroform extraction. An equal volume of phenol was added to precipitate any protein, and the aqueous (top) layer recovered by centrifugation in a microfuge for 2 minutes at 12000g. The aqueous layer was then mixed with an equal volume of chloroform to remove traces of phenol, and again recovered by centrifugation for 2 minutes at 12000g. Plasmid DNA was recovered by precipitation from the aqueous layer with 2 volumes of 100% ethanol, on ice for 15 minutes, and by centrifugation for 5 minutes at 12000g. The DNA was then washed with 70% ethanol and resuspended in 50 µl sterile H_2O containing 10 µg RNase A. DNA was stored at -20°C until required.

Solution 1: 50 mM Glucose
25 mM Tris pH8
10 mM EDTA in sterile H_2O
This solution was not autoclaved.

Solution 2: 10 ml 10% SDS solution
(100 ml) 4 ml 5 M NaOH
86 ml H_2O
This solution was not autoclaved and was stored for no longer than 24 hours.

Solution 3: 60 ml 5 M potassium acetate
(500 ml) 11.5 ml glacial acetic acid
28.5 ml H_2O

2.3.2 Large-scale DNA Preparation – Maxiprep

Plasmid DNA was obtained from 500 ml bacterial cultures using the alkaline lysis method (Sambrook *et al,* 1989), with the omission of the lysozyme step. Bacterial cultures were pelleted by centrifugation for 15 minutes at 2500g and 4°C. The cells were resuspended in 10 ml of ice cold solution 1 and gently mixed with 20 ml of solution 2. The lysed cells were then treated with 15 ml of solution 3 and incubated on ice for a further 15 minutes, in order to precipitate out cellular debris and chromosomal DNA. The precipitate formed was pelleted for 15 minutes at 2500g and 4°C with no brake, and the supernatant filtered through a fine mesh to be mixed with an equal volume of ice-cold iso-propanol. Centrifugation for 15 minutes at 5000g and 4°C formed a DNA-containing pellet that was subsequently washed with 70% ethanol and dissolved in 3 ml of TE pH 8.0 buffer. This solution was treated with 3 ml of ice-cold 5 M LiCl to remove high molecular weight RNA, and the precipitate pelleted for 15 minutes at 12000g and 4°C. The supernatant was re-precipitated with an equal volume of ice-cold iso-propanol and again centrifuged for 15 minutes at 12000g and 4°C. The impure plasmid DNA pellet was washed with 70% ethanol, dissolved in 500 µl of 0.1 M Tris containing 0.1 mg of RNaseA and incubated at 37°C for 30 minutes to degrade any residual RNA. This was followed by a 1 hour incubation with an equal volume of PEG solution on ice. The PEG precipitate formed was pelleted for 15 minutes in a microfuge at 12000g and 4°C, and dissolved in 400 µl 10 mM tris/0.5 M NaCl. This solution was purified by repeated phenol/chloroform extractions until no more white precipitate was formed. Finally the aqueous phase was incubated with 2 volumes of ethanol for 15 minutes at -20°C, and the DNA precipitate pelleted for 10 minutes at 12000g. The purified plasmid DNA was washed with 70% ethanol, dissolved in 100 µl of sterile H_2O and spectrophotometrically quantified. DNA was generally diluted with sterile H_2O to a working concentration of 1 µg/µl.

Solutions 1, 2 & 3: As for mini-prep method

PEG solution: 10 mM Tris pH 8.0
1 mM EDTA
20% PEG 6000 in sterile H_2O
This solution was not autoclaved.

2.3.3 Spectrophotometric Quantification of DNA

DNA concentration and purity was analysed in a spectrophotometer at 260 nm and 280 nm using a quartz cuvette. Concentration and purity were calculated using the following equations:

1 OD = 50 μg/ml double-stranded DNA

$OD_{260}/OD_{280} \approx 1.7$ in a pure sample

2.3.4 Restriction Digests

To analyse the prepared plasmid, 1 μg of maxiprep DNA was digested with 10 units each of appropriate enzyme in a 20 μl volume, for 1hr - overnight as necessary, in conditions as recommended by the enzyme supplier. The digest was then separated into fragments electrophoretically on an agarose gel.

2.3.5 Agarose Gel Electrophoresis

Plasmid DNA was resolved according to size and charge on a 0.8 - 1% (w/v) agarose gel whilst smaller DNA fragments were separated on 2% (w/v) agarose gels. A molten gel was formed by heating the agarose in a solution of 1x TAE. This was then cooled to 60°C and the DNA-intercalating fluorescent dye ethidium bromide was added to a final concentration of 5 μg/ml. The gel was then cast in a gel former with a comb to form wells, and placed in an electrophoresis tank submerged in 1x TAE running buffer. DNA samples were loaded into the wells in 1x loading buffer together with an appropriate DNA size-marker. Gels were run at 80V, towards the anode, until the loading dye in the loading buffer had migrated no further than to within 2 cm of the end of the gel. The DNA was visualised on a transilluminator under ultraviolet light using appropriate eye protection, and photographed on polaroid film.

1x TAE:	0.04 M Tris-acetate
	0.001M EDTA

1L 50x TAE:	242 g Tris base
	57.1 ml Glacial acetic acid
	100 ml 0.5 M EDTA pH 8.0
DNA loading buffer:	0.25% Bromophenol blue
	0.25% Xylene cyanol FF
	30% Glycerol in H_2O

2.4 Cloning of CRE Oligonucleotides into pBLCAT2

2.4.1 Annealing Oligonucleotides

Each strand of an oligonucleotide pair was dissolved in sterile water to give a concentration of 10 μg/μl. 10 μg (1 μl) of each strand was then added to 18 μl 1x Maniartis medium salt buffer (Sambrook *et al,* 1989), and heated to 95^0C for 5 seconds. The strands were allowed to anneal by cooling slowly to room temperature.

10x Maniatis medium salt buffer:
- 0.1 M Tris pH 7.9
- 20 μM $MgCl_2$
- 0.5 M NaCl
- 10 μM EDTA

2.4.2 Kinase Treatment of Oligonucleotides

Double-stranded oligonucleotides (DS oligos) were activated by the addition of 5'-terminal phosphate groups. 200 ng of DS oligo were incubated with of 1 mM ATP in 1x T4 polynucleotide kinase buffer and 10 units of T4 polynucleotide kinase in a 20 μl volume for 30 minutes at 37°C. The oligonucleotides were then purified by a 2 minute centrifugation at 400g through a G50 Sephadex column. The phosphorylated oligos were stored at -20°C until required.

2.4.3 BamHI-digestion of pBLCAT2

The plasmid pBLCAT2 was digested by the enzyme BamHI at a restriction site within the multiple cloning region. 1 μg of plasmid was digested in a 30 μl volume of 1x BamHI restriction buffer and 20 units of BamHI, for 1 hour at 37°C.

2.4.4 Phosphatase Treatment of pBLCAT2

The BamHI-digested vector 5'-terminal phosphate groups were removed by the action of calf intestinal alkaline phosphatase in order to minimise self-religation in the later ligation reaction. 1μg of the digested vector was incubated for 1 hour at 55°C in 1x alkaline phosphatase reaction buffer and 2 units of alkaline phosphatase in a 50 μl volume. The enzyme was subsequently inactivated by a 2 minute incubation at 95°C and removed with a phenol/chloroform purification step. The vector was precipitated from solution with 2 volumes of 100% ethanol, pelleted at 12000g, washed with 70% ethanol and dissolved in sterile water. Phosphatased plasmid was stored at –20°C until required.

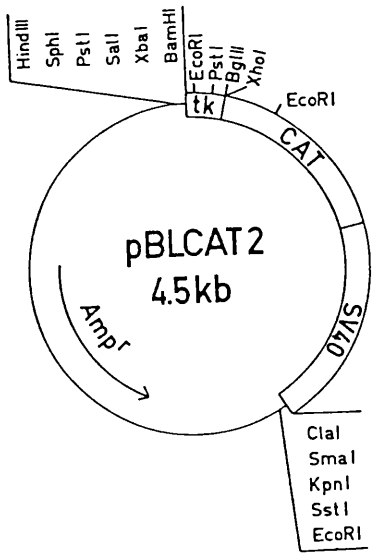
2.4.5 Ligation

The phosphorylated oligonucleotides containing the CRE sequences of different genes were inserted into the BamHI site of the BamHI digested and phosphatased plasmid pBLCAT2. In order to improve the chances of creating a clone with a single insert, three different ligation reactions were carried out for each oligonucleotide with various vector:oligo ratios. 100 ng plasmid was incubated overnight at 16°C with either 10 ng, 50 ng or 100 ng of phosphorylated oligo, together with 3 units of T4 DNA ligase and 10 μl of 1x DNA ligase buffer. A control ligation with the vector alone was also carried out to determine the level of self-religation.

Once the ligation reaction was complete 50 ng of the ligated vector was transformed into 50 μl of competent XL-1 blue bacteria and grown up on ampicillin-containing LB-agar plates overnight.

Once grown, 10 colonies derived from each ligation reaction were selected and grown up in 5 ml liquid culture overnight for mini-prepping. DNA obtained from each mini-prep (3 ml of bacterial culture) was then restriction digested and separated electrophoretically on a 2% agarose gel in order to determine a) the number of inserts (oligos) that had been incorporated into pBLCAT2 and b) the orientation of those inserts.

a)


HindIII
SphI
PstI
SalI
XbaI
BamHI
EcoRI
PstI
BglII
XhoI
tk
EcoRI
CAT
pBLCAT2
4.5kb
Ampr
SV40
ClaI
SmaI
KpnI
SstI
EcoRI

a) To determine the number of inserts incorporated

Oligonucleotide	Digest Carried Out	Fragment Size (bp)	
		No Insert	1 Insert
Bcl-2 CRE	XbaI/XhoI	174	196
CGRP CRE	XbaI/XhoI	174	191
TH CRE	XbaI/XhoI	174	197

b) Insert orientation

Oligonucleotide	Digest Carried Out	Fragment Size (bp)	
		Sense Oligo 5'-3'	Antisense Oligo 5'-3'
Bcl-2 CRE	BamHI/XhoI	168	190
CGRP CRE	BamHI/XhoI	185	168
TH CRE	BamHI/XhoI	191	168

Once those bacterial cultures containing plasmid with a single insert in the correct orientation were identified, 200 μl of these cultures was used to inoculate 10 ml of fresh LB and grown up overnight. These mini-cultures were then used to make glycerol stocks and to inoculate maxi-cultures for the large-scale preparation of DNA.

2.5 Tissue Culture

2.5.1 Culture of the PC12 Cell Line

The pheochromocytoma cell line PC12 cells were maintained in 80 cm^2 tissue culture flasks in PC12 cell culture medium, consisting of Dulbecco's modified Eagle Medium (DMEM) supplemented with 5% (v/v) foetal bovine serum, 10% (v/v) horse serum, 2 mM L-glutamine and penicillin and streptomycin at 100 units/ml and 100 μg/ml respectively. Cells were passaged at 70% confluence by incubating the cells in Hanks' HBSS at room temperature for 5 minutes to detach them from the flask surface. This was followed by centrifugation at 145g for 5 minutes. The cell pellet was resuspended in growth medium and divided as required. Cells were stored in the above growth medium containing 10% (v/v) DMSO at -70°C for up to six months, and in liquid nitrogen for longer periods.

2.5.2 Calcium Phosphate Transfection of PC12 Cells

Transfections of PC12 cells were performed with 4x 10^5 cells per well on six-well tissue culture plates, at least 16 hours after plating, according to the calcium phosphate precipitation method (Gorman, 1985). The transfection mix for each well was prepared by mixing plasmid DNA with 248 mM $CaCl_2$ in a 50 μl volume, and adding this drop-wise to 50 μl of 2x HBS (HEPES-buffered saline). The precipitate was left to form at room temperature for 30 minutes. Cells were incubated for 5 hours with 100 μl of precipitate in 1ml of Dulbecco's modified Eagle Medium (DMEM) containing 10% (v/v) foetal bovine serum and penicillin and streptomycin at 100 units/ml and 100 μg/ml respectively and 2 mM L-glutamine. Experiments were generally carried out using 4 μg of reporter plasmid. Where appropriate, 2 μg of inhibitor expression vector was added per well, with 2 μg of equivalent empty vector being added to control samples. For each transfection 1 μg of β-galactosidase expression vector was used as an internal control. Each experiment was carried out using triplicate samples.

Cells were subsequently subjected to glycerol shock for 45 seconds in 15% glycerol/2x HBS (v/v) and washed twice with DMEM supplemented with 10% (v/v) foetal bovine serum. NGF/cAMP/hypoxia treated cells were deprived of serum overnight, for 16 hours prior to treatment, in L-15 culture medium supplemented with 1% horse serum, 0.37% sodium bicarbonate, 2 mM L-glutamine and penicillin and streptomycin at 100 units/ml and

100 μg/ml respectively. Biochemicals were added to cells in 1 ml of the supplemented 1% horse serum medium. 2.5S NGF was added to a final concentration of 25 ng/ml. Di-butyryl cAMP was added to a final concentration of 1 mM. Chemical inhibitors were added to cells 1 hour prior to NGF/cAMP treatment. PD98059 was added to a final concentration of 50 μM and SB203580, H89 and H7 were each added to a final concentration of 20 μM. Anisomycin was added at 3 μg/ml and Ro318220 was added to a final concentration of 0.3 μM. In those experiments using PD98059, SB203580, Ro318220 and anisomycin, an equivalent volume of the DMSO/MeOH carrier was added to control samples. SB203580 is light sensitive, therefore culture plates containing cells treated with the compound were wrapped in foil to minimise their exposure to light.

Cells were harvested 48 hours after transfection by washing twice in PBS and lysing cells in Promega 1x Reporter Lysis Buffer. The cell lysate was stored at -20°C for up to 1 month until assayed for chloramphenicol acetyl transferase (CAT) or luciferase activity. Results were normalised against β-galactosidase activity.

10x HBS:	8.18% (w/v) NaCl
	5.94% (w/v) HEPES
	0.2% (w/v) Na_2PO_4
	Filter-sterilised in a tissue-culture hood and stored at 4°C.
2x HBS:	1:5 dilution of 10x HBS in H_2O, adjusted to pH 7.12 with 1 M NaOH.
1L 1x PBS:	8 g NaCl
	0.2 g KCl
	1.44 g Na_2HPO_4
	0.24 g KH_2PO_4
	Adjusted to pH 7.4 and stored at room temperature.

2.5.3 Hypoxia Treatment of PC12 Cells

Cells were incubated for 4 hours in 1 ml of supplemented 1% horse serum medium in a hypoxia chamber fed with a constant flow of gas consisting of 5% CO_2 and 95% argon. After treatment cells were fed with 1 ml fresh media and harvested 24 hours later.

2.6 Reporter Gene Assays

2.6.1 Chloramphenicol Acetyl Transferase (CAT) Assays

Cells transfected with a CAT reporter plasmid were harvested in 150 µl of Promega reporter lysis buffer per well. Cells were lysed for 10 minutes at room temperature and the debris pelleted at 12000g for 30 seconds. 52 µl of cell lysate was then incubated at 60°C for 10 minutes to deactivate any cellular de-acetylases. CAT reactions were carried out for 5 hours at 37°C using the heat-treated 52 µl of cell lysate together with 20 µl 4mM acetyl co-enzyme A, 70 µl of 0.25 M tris pH 7.8 and 4 µl [^{14}C] chloramphenicol (25 µCi/ml, 50-62 mCi/mmol). The chloramphenicol and its acetylated derivatives were then extracted with 750 µl of ethyl acetate by vortexing. The two phases were separated by centrifugation at 12000g for 1 minute. The upper (organic) layer was then transferred to a new tube and dried down under vacuum in a concentrator at room temperature, until the ethyl acetate had evaporated. The remaining pellet was then resupended in 12 µl of ethyl acetate and spotted onto silica gel-coated thin-layer chromatography (TLC) plates for chromatographic separation. The samples were run on TLC plates for 45 minutes in 95:5 chloroform:methanol in order to separate the ^{14}C chloramphenicol from its acetylated derivatives. Plates were either exposed onto a phosphoimager screen overnight, or onto film for longer periods. In the case of the former, quantitative analysis was done using a Bio-Rad GS-50 phosphoimager and, in the case of the latter, analysis was done using a Bio-Rad imaging densitometer.

$$\% \text{ CAT activity of each sample} = \frac{\text{acetylated chloramphenicol}}{\text{total chloramphenicol}} \times 100\%$$

$$\text{Relative \% CAT activity} = \frac{\text{\% CAT activity of normalised test sample}}{\text{\% CAT activity of control}}$$

$\therefore$ Relative % CAT activity of control = 100%

In all cases, the control samples are cells expressing basal levels of CAT activity without NGF/cAMP/hypoxia stimulation, or the addition of signalling molecule inhibitors.

2.6.2 Luciferase Assays

Assays were carried out using the Promega luciferase assay reagent and reporter lysis buffer according to the protocol provided. PC12 cells were lysed in 100 µl reporter lysis buffer for 10 minutes at room temperature and subsequently centrifuged at 12000g for 30 seconds. 20 µl of lysate was assayed with 100 µl of luciferase assay reagent using luminometer tubes in a Turner luminometer. Luminescence was measured over 45 seconds after a 5 second delay.

2.6.3 Standardisation of Reporter Gene Assays/Beta-Galactosidase Assays

Assays were carried out using the Galacto-Light Plus™ reporter gene assay system. 20 µl of cell lysate was mixed with 20 µl of Galacto Reaction Buffer in luminometer tubes and left for 1 hour at room temperature. 30 µl of Galacton Accelerator II was added to each reaction and the luminescence measured on a Turner luminometer over 30 seconds after a 5 second delay. Values obtained for β-galactosidase activity were then used to normalise results obtained from luciferase/CAT assays.

$$\text{Normalised reporter activity of test sample} = \frac{\text{control sample β-gal activity}}{\text{test sample β-gal activity}} \times \text{relative \% CAT activity of test sample}$$

$$\text{Normalised reporter activity of test sample} = \frac{\text{control sample β-gal activity}}{\text{test sample β-gal activity}} \times \text{luciferase activity of test sample}$$

In all cases the control samples are cells expressing basal levels of luciferase/CAT reporter activity, without NGF/cAMP/hypoxia stimulation or the addition of signalling molecule inhibitors.

2.7 Preparation and Analysis of Protein

2.7.1 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using the Life Technologies Vertical Gel Electrophoresis System, Model V16, according to standard protocol (Sambrook *et al,* 1989), and based on the principles of Laemmli (Laemmli, 1970). With standard electrophoretic techniques protein mobility is determined by both size and charge but, using this method, the presence of SDS allows proteins to be separated on the basis of their molecular weight alone.

Gels were cast between glass plates separated by 1.5 mm plastic spacers and consisted of 2 layers. The upper 5% stacking gel (160 mm x 20 mm) has a slightly acid pH of 6.8 and promotes the formation of sharper protein bands in the lower 12% resolving gel (160 mm x 120 mm). Samples for SDS-PAGE were prepared by growing 1x 10^6 PC12 cells on 10 cm tissue culture plates for 3 days. Some cells were subjected to hypoxia or treatment with cAMP as described in sections 2.5.2 and 2.5.3. Cells were then washed twice in PBS and harvested in 250 µl of 1x SDS gel loading buffer. Samples were heated at 95°C for 3 minutes to denature the protein prior to loading. 40µl of each sample was run on duplicate gels, together with a Rainbow™ coloured protein molecular weight marker. Gels were run for 5 hours at 150V (approx. 150-100 mA) in western running buffer, until the 46 kD yellow marker reached the centre of the resolving gel.

10 ml 5% Stacking gel:	6.8 ml H_2O
	1.7 ml 30% Bis/acrylamide solution
	1.25 ml 1 M Tris pH 6.8
	0.1 ml 10% (w/v) SDS
	0.1 ml 10% (w/v) APS
	0.01 ml TEMED
50 ml 12% Resolving gel:	16.5 ml H_2O
	20 ml 30% Bis/acrylamide solution
	12.5 ml 1.5 M Tris pH 8.8
	0.5 ml 10% (w/v) SDS

0.5 ml 10% (w/v) APS
0.02 ml TEMED

1x SDS Gel loading buffer: 50 mM Tris pH 6.8
0.1% (w/v) Bromophenol blue
2% (w/v) SDS
10% (v/v) Glycerol

1x Western running buffer: 25 mM Tris
250 mM Glycine
0.1% (w/v) SDS
pH 8.8

2.7.2 Western Blot

Proteins were tranferred from the SDS polyacrylamide gels to Hybond C membranes (Sambrook *et al,* 1989). Transfer was carried out overnight in 1x western tranfer buffer at 220mA and 4°C. Once transferred one blot from each gel pair was tested for the presence of phosphorylated CREB and the other for the presence of actin to compare the amounts of protein loaded for each sample.

1x Western transfer buffer: 39 mM Glycine
48 mM Tris
0.037% (w/v) SDS
20% (v/v) MeOH
pH prior to the addition of MeOH = 8.3.

Antibody Staining of Western Blots for Phosphorylated CREB:

Membranes from Western blots were incubated with PBS containing 0.1% Tween-20 (PBS-T) and 5% (w/v) non-fat milk for 1 hour. Primary antibody was added to the milk mixture at 1:1000 and the membranes incubated overnight at 4°C. Membranes were then rinsed and

washed 3x 5 minutes with PBS-T. Membranes were then incubated for 1 hour with HRP-conjugated anti-rabbit secondary antibody dissolved at 1:2000 in PBS-T containing 5% non-fat milk. Membranes were then rinsed and washed for 2x 5 minutes and 1x 20 minutes in PBS-T. All the above procedure was carried out at room temperature, with the exception of the primary antibody step, and incubations/washes were carried out on a rotating shaker. Protein-antibody conjugates were detected using the Enhanced Chemiluminescence System (ECL) according to manufacturer's instructions, and exposure to autoradiographic film.

Antibody Staining of Western Blots for Actin;

This procedure was done as for the antibody staining of phosphorylated CREB with modifications to the antibody steps. Incubation of the membrane with primary antibody was carried out at room temperature for 45 minutes with the primary diluted at 1:2000. The secondary antibody used here was HRP-conjugated anti-goat antibody.

2.8 Electrophoretic Mobility Shift Assays (EMSA)

2.8.1 Preparation of Protein Samples

1x 10^6 cells were grown on 10 cm tissue culture plates for 3 days, then washed in PBS and harvested in 150 µl of ice-cold protein extraction buffer. Samples were then left on ice for 10 minutes, centrifuged at 12000g for 5 minutes and 5 µl of the supernatant used to measure protein concentration. The remaining supernatant was diluted to approx. 1 µg/µl and stored at -70^{0}C until required. Once thawed for EMSA purposes, any un-used sample was discarded.

Protein extraction buffer:
10 mM $MgCl_2$
20 mM HEPES pH 7.0
10 mM KCl
300 mM NaCl
20% Glycerol
0.1% Triton
0.5 mM DTT
0.5 mM PMSF (added fresh)
Stored at -20°C in 1 ml aliquots

2.8.2 Quantification of Protein Concentration

The total protein concentration of cell lysates was determined using the BCA Protein Assay kit according to the manufacturer's instructions. Duplicate 5 µl aliquots of each sample were dispensed onto clear 96-well microtitre plates alongside duplicate BSA standards of 0.2-10 mg/ml. 100 µl of assay buffer was added to each well and the plate incubated for 30 minutes at 37°C. The optical density at 560 nm was then recorded on a microtitre plate reader. The protein content of samples was calculated from the standard curve of BSA.

2.8.3 Oligo-probe Labelling

25 ng double stranded oligo was incubated with 3 μl [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol) and 10 units of T4 polynucleotide kinase in 20 μl of 1x T4 polynucleotide kinase buffer at 37°C for 30 minutes. The probe was purified through a G50 sephadex column by centrifugation for 2 minutes at 400g.

2.8.4 Preparation of DNA:Protein Samples

DNA-protein interaction reactions were carried out on ice in 20 μl volumes of 1x bandshift buffer. 7 μg of PC12 protein were pre-incubated with 2 μg poly-dIdC, and 20-100x ng excess of non-radioactive oligonucleotide if using, in bandshift reaction buffer for 10 minutes. 2.5 ng of radioactive CRE-oligonucleotide were then added to the reaction mixture and incubated for a further 10 minutes. Antibody was then added as appropriate, and the reaction mixtures incubated for a further 30 minutes. For each CRE-oligonucleotide tested, the following controls were run in parallel:

1) A reaction mixture of radioactive CRE-oligonucleotide, poly-dIdC and bandshift buffer only
2) A reaction mixture of radioactive CRE-oligonucleotide, poly-dIdC, excess non-radioactive specific competitor CRE-oligonucleotide, protein and bandshift buffer
3) A reaction mixture of radioactive CRE-oligonucleotide, poly-dIdC, excess non-radioactive non-specific competitor SP1-oligonucleotide, protein and bandshift buffer
4) Samples containing test antibodies were run in parallel with reaction mixture containing a control anti-Gal4 antibody

5x bandshift reaction buffer:	250 mM Tris-HCl pH 7.4
(Sawada *et al*, 1997)	125 mM Tris-HCl pH 8.0
	25 mM EDTA
	125 mM $MgCl_2$
	250 mM NaCl
	50% Glycerol
	Stored at -20°C

2.8.5 Non-denaturing Polyacrylamide Gel Electrophoresis

Using this technique oligonucleotides and oligo: protein complexes are separated by both their size and charge. The free negatively charged DNA moves rapidly through the gel, whilst more weakly negatively charged protein-bound DNA is retarded.

Non-denaturing gel electrophoresis was carried out at 4°C using the Life Technologies Vertical Gel Electrophoresis System, Model V16. The 160 mm x 140 mm non-denaturing 6% gels were cast in glass plates separated by 3 mm spacers; the 8 mm wide wells were formed using a broad-toothed comb. Once set, gels were pre-run at a constant voltage of 180V for approx. 30 minutes, from 50mA to 30mA per gel, in a 0.5x TBE running buffer. Samples were loaded and run for 2 hours at 180V. Gels were then dried under vacuum on a gel dryer at 80°C for 1-2 hours. The radioactivity on the dried gels was detected by exposure to autoradiographic film for 12-48 hours.

6% Gel:	76.15 ml H_2O
	20 ml 30% Bis/acrylamide solution
	2.5 ml 10x TBE
	750 µl 10% APS
	100 µl TEMED
0.5x TBE:	0.045 M Tris-borate
	0.001 M EDTA
1L 10x TBE:	108 g Tris base
	55 g boric acid
	40 ml 0.5 M EDTA pH 8.0

3 Investigation of the Signalling Pathways Involved in the CRE-mediated Activation of the CT/CGRP Promoter in Response to NGF and cAMP in PC12 Cells

3.1 Introduction

3.1.1 Regulation of CT/CGRP by NGF and cAMP in Neuronal Cells

In neuronal cells the neurotrophin NGF and the second messenger cAMP regulate CT/CGRP transcription (Watson and Latchman, 1995). The plasmid Calgcat 1, containing the CGRP promoter from –1679 to +8 linked to the chloramphenicol acetyl transferase (CAT) gene, is sensitive to both NGF and cAMP in PC12 cells. The cAMP-mediated regulation of CT/CGRP transcription in neuronal cells has been shown to be dependent upon a CRE sequence located at bases –103 to –109 of the CT/CGRP gene (Watson and Latchman, 1995). The CRE site located within the CT/CGRP gene promoter is both necessary and sufficient for the response to cAMP in neuronal cells (Watson and Latchman, 1995). It is likely that this effect is mediated by the CREB transcription factor, which is able to bind to CRE sites and becomes active when phosphorylated at serine 133 following cAMP treatment of neuronal cells.

As with cAMP, NGF-mediated activation of the CT/CGRP promoter is dependent upon the CRE site located at bases –103 to –109. In the case of NGF however, the CRE site within the CT/CGRP promoter is necessary but not sufficient for the response to NGF. It has been shown that NGF is able to induce phosphorylation of CREB at serine 133 in neuronal cells (Xing *et al*, 1996). It is therefore likely that both NGF and cAMP activate transcription from the CT/CGRP promoter by mediating the activation of CREB. However, in the case of the NGF response, it is likely that other factors work together with the CRE-bound CREB to activate transcription. The similarities and differences between the responses to NGF and cAMP of the CT/CGRP promoter would be better understood if the signalling pathways involved in promoter activation by these stimuli were characterised.

3.1.2 The Use of Inhibitor Molecules in the Investigation of Intracellular Signalling Cascades

In recent years intracellular signalling cascades have been the focus of a great many studies and as a result numerous signalling pathways and their component molecules have been identified. Many strategies have been employed to aid in the elucidation of these pathways and an important method used in the study of their role in cellular function has been the use of selective chemical inhibitors. These chemical inhibitors are designed to target specific enzymes within a given signalling pathway in such a way that the enzymes are no longer able to activate their downstream effectors. Thus treatment of cultured cells with these inhibitors reduces their ability to activate the targeted signalling cascades. For instance, the compound PD98059 (New England Biolabs) is a selective inhibitor of MEK-1 and MEK-2 (Barrie *et al*, 1997). These are the MAP kinase kinases that phosphorylate p42 and p44 MAP kinases respectively. Treatment of PC12 cells with this compound inhibits cellular processes that are dependent upon p42/p44 MAP kinase signalling, such as neurite outgrowth, and partially blocks the NGF-mediated phosphorylation of CREB within these cells (Xing *et al*, 1998).

As an alterative to chemical inhibition, mutants of specific signalling molecules can also be used to selectively inhibit distinct intracellular signalling cascades. Plasmids have been designed to encode dominant negative (DN) signalling molecules that, when transfected into cells, are able to compete with their functional cellular counterparts as substrates for their upstream effectors, but are unable to activate their downstream effectors. An example of such a mutant is the p42YF185 (Lu *et al*, 1998). In this mutant of p42 MAP kinase, tyrosine 185 in the activation domain has been converted to a phenylalanine. Thus the TEY motif containing the phosphorylation sites crucial for activation by MEK is mutated to TEF. Although DN p42 cannot be phosphorylated by MEK at tyrosine 185 since this site has been mutated to phenylalanine, DN p42 retains the ability to bind to MEK. Overexpression of the DN p42 leads to competition with intracellular p42 for MEK binding, which prevents MEK from activating functional cellular MAP kinase molecules, thus down-regulating the activity of this signalling cascade. PC12 cells transfected with DN p42 fail to extend processes or differentiate in response to NGF (Lu *et al*, 1998).

As well as dominant negative mutants, many plasmids encoding constitutively active mutants of signalling molecules have been constructed. These molecules are able to activate downstream intracellular signalling cascades in the absence of an extracellular stimulus. For instance, the molecule S218D/S222D is a constitutively active mutant of MEK-1 with a basal

activity higher than that of wild-type serum-stimulated MAPKK (Brunet *et al*, 1994). The serine residues within the Raf-dependent regulatory domain have been converted to aspartic acid residues. The aspartic acid residues mimic the effects of phosphorylation at these sites causing the enzyme to constitutively phosphorylate p42 MAP kinase. Transfection of this mutant into fibroblasts is sufficient to promote growth factor signalling and autonomous cell-cycling in these cells, and leads to the activation of genes such as c-fos, even in the absence of growth factor (Brunet *et al*, 1994). However, when applying these constitutively active mutants to signalling studies such as the effects of signalling cascades on the transcription of specific genes, it is important to consider the fact that they artificially activate signalling cascades within test cells that may not normally be active within those cells. Any results should therefore be corroborated with the use of inhibitors.

In summary, selective inhibitors and mutants of specific signalling molecules have proved to play an important role in identifying the functions of the many different intracellular signalling cascades, both at the cellular level and at the level of gene transcription. In view of this, such signalling mutants and inhibitors would be valuable tools in characterising the signalling cascades involved in the activation of the CT/CGRP gene in response to NGF and cAMP.

3.2 Results

3.2.1 The Effects of PKA upon NGF- and cAMP-mediated Activation of the CGRP Promoter

The cAMP induced phosphorylation of CREB is known to be mediated by the signalling enzyme protein kinase A (Lalli and Sassone-Corsi, 1994), and it is therefore likely that PKA is involved in the cAMP-mediated regulation of the CGRP gene. NGF is also known to activate PKA in these cells (Yao *et al*, 1998). In view of this, the role of PKA in both the cAMP- and NGF-mediated activation of the CGRP promoter was investigated. The NGF- and cAMP-sensitive PC12 cell line was transfected with the plasmid Calgcat 1 and subsequently treated with NGF or dibutyryl cAMP, or left unstimulated, in either the presence or absence of the PKA chemical inhibitor H89. The effectiveness of H89 as a PKA inhibitor in PC12 cells has been demonstrated previously (Liu *et al*, 1998). As shown in figure 3.1, both NGF and cAMP are able to stimulate the promoter and upregulate CAT expression, with a much greater effect being produced by cAMP. When cells were treated with H89, as expected, cAMP-mediated promoter activation was virtually abolished. NGF-mediated promoter activation was also reduced; however this effect was much smaller than that observed with cAMP. Analysed statistically using a two-tailed T-test ($P < 0.05$ if results are significant), the promoter activity observed in the presence of NGF together with the PKA inhibitor H89 was not significantly different to that observed with NGF alone ($P = 0.081$). The decrease observed in cAMP-mediated promoter activation upon treatment with H89 was statistically significant ($P = 0.018$).

To corroborate these results, the role of PKA signalling in the NGF- and cAMP-mediated activation of the CGRP promoter was also tested using the heat-stable inhibitor of PKA known as PKI (Day *et al*, 1989). PKI binds to free catalytic subunits of PKA with a high affinity, so inhibiting the kinase activity. PC12 cells were co-transfected with Calgcat 1 together with either a vector encoding PKI or an equivalent amount of empty vector prior to NGF or cAMP stimulation. The results can be seen in figure 3.2. As with H89 treatment, promoter activity in the presence of PKI and NGF was not significantly different to that of NGF alone ($P = 0.885$). However the presence of PKI significantly reduced cAMP-mediated promoter activation when compared with cAMP alone ($P = 0.013$). Co-transfection of PC12 cells with Calgcat 1 and a constitutively active form of PKA increases promoter activity both in the absence of any stimulation and in the presence of either NGF or cAMP (figure 3.2).

Fig. 3.1 – The Effects of the PKA Selective Inhibitor H89 on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when transfected into PC12 cells in the presence or absence of the PKA inhibitor H89. In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated and uninhibited cells.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

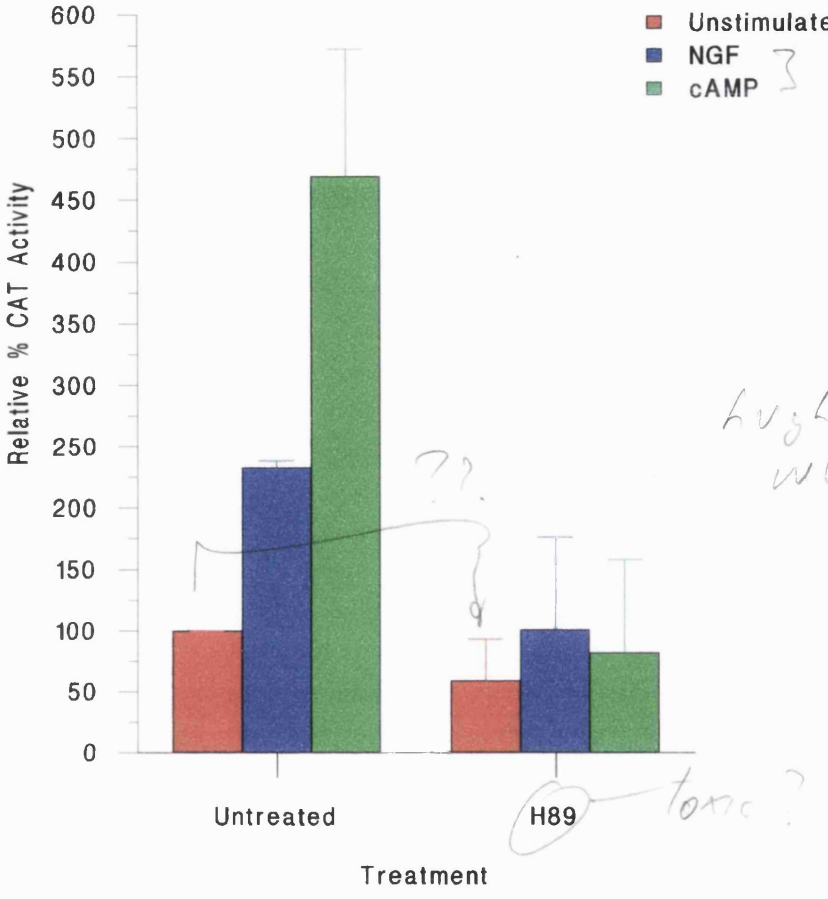
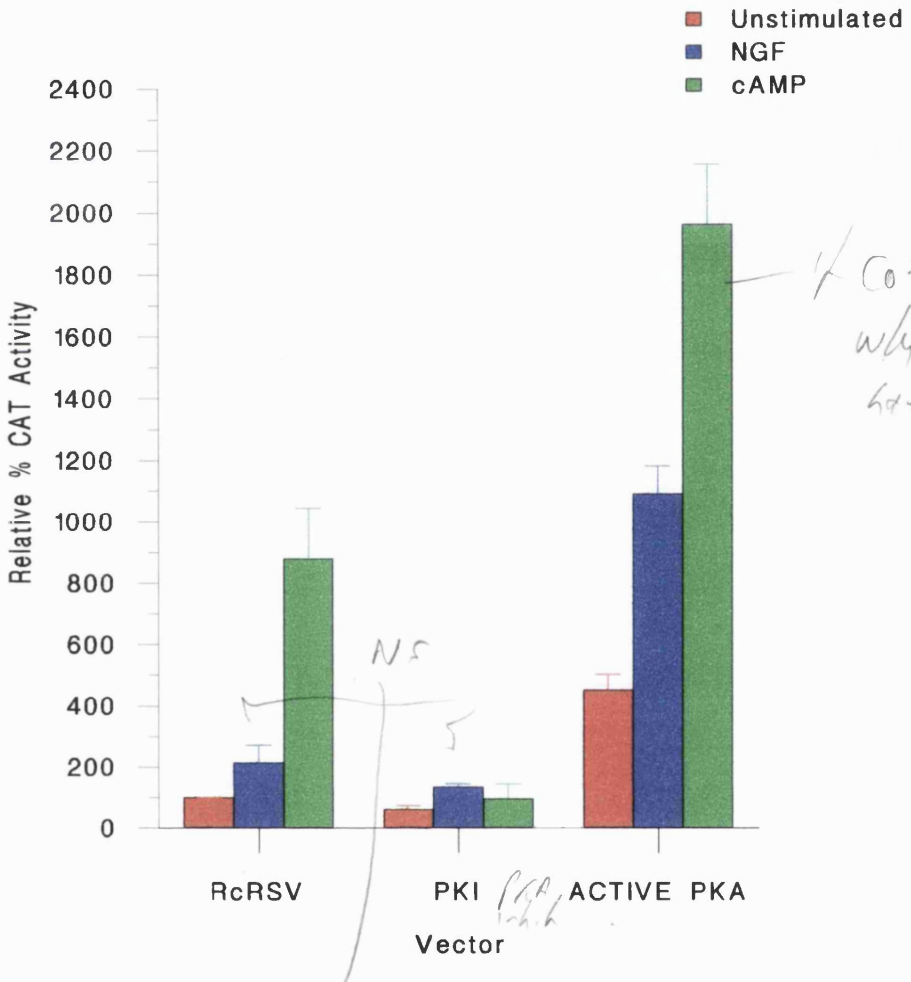

Unstimulated
NGF
cAMP
Relative % CAT Activity
600
550
500
450
400
350
300
250
200
150
100
50
0
Untreated
H89
Treatment

Fig. 3.2 – The Effects of PKI on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV, a vector encoding the PKA inhibitor PKI, or a vector encoding a constitutively active mutant of PKA (Active PKA). In all cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


Unstimulated
NGF
cAMP
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Relative % CAT Activity
RcRSV
PKI
ACTIVE PKA
Vector

As expected, these results suggest that the PKA-mediated activation of CREB is likely to be responsible for virtually all of the stimulatory effect of cAMP upon the CGRP promoter. This is not the case for NGF, however. Inhibition of PKA does not abolish NGF-mediated activation of the promoter, so it is likely that other NGF-activated signalling pathways are involved in promoter stimulation. I therefore investigated the role of PKC and the MAP kinases in promoter activation in response to NGF and cAMP, since these enzymes have been previously implicated in NGF signalling in PC12 cells.

3.2.2 The Effects of PKC and the MAP Kinases upon NGF- and cAMP-mediated Activation of the CGRP Promoter

NGF stimulation of PC12 cells activates PKC-ε (Ohmichi *et al*, 1993), which in turn enhances the NGF-induced phosphorylation of MAP kinases and neurite outgrowth in these cells (Hundle *et al*, 1995). Since MAP kinases contribute to the phosphorylation of CREB in PC12 cells, it is possible that PKC may mediate NGF-induced activation of the CGRP promoter. To test this a selective inhibitor of protein kinase C, H7 dihydrochloride (Chernov *et al*, 1998), was used. PC12 cells were transfected with Calgcat 1 and treated with H7, or left untreated, either in the presence or absence of NGF (see figure 3.3). NGF-induced promoter activation in the presence of H7 was not significantly different to that of NGF alone ($P = 0.275$), suggesting that PKC is not likely to be involved in the regulation of the CGRP promoter in response to NGF in PC12 cells.

As well as activating PKC, NGF is also known to induce the phosphorylation of numerous MAP kinases, which are able to phosphorylate CREB and/or other CREB family members. In the case of PC12 cells, NGF activates p42, p44 and p38 MAP kinases (Xing *et al*, 1998), although it does not activate JNK. Therefore it is possible that MAP kinase signalling may contribute to the CREB-mediated activation of the CGRP promoter. The compound SB203580, a selective inhibitor of p38 MAP kinase, was used to test the specific involvement of p38 MAP kinase signalling in the NGF-mediated activation of the CGRP promoter. Treatment of Calgcat 1-transfected PC12 cells with SB203580 had only a minimal, but significant, inhibitory effect upon the inducibility of the promoter in response to NGF when compared to cells treated with NGF alone ($P = 0.01$), and had no inhibitory effect upon the cAMP-inducibility of the promoter ($P = 0.628$) (see figure 3.4). In parallel experiments, the p42/p44 MAPK signalling inhibitor PD98059 virtually abolished the activation of the

Fig. 3.3 – The Effects of the PKC Selective Inhibitor H7 on the Activation of the CGRP Promoter in Response to NGF

Activity of the CGRP promoter in the plasmid Calgcat 1 when transfected into PC12 cells in the presence or absence of the PKC inhibitor H7. In both cases, the cells were either left unstimulated or treated with NGF. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated and uninhibited cells.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

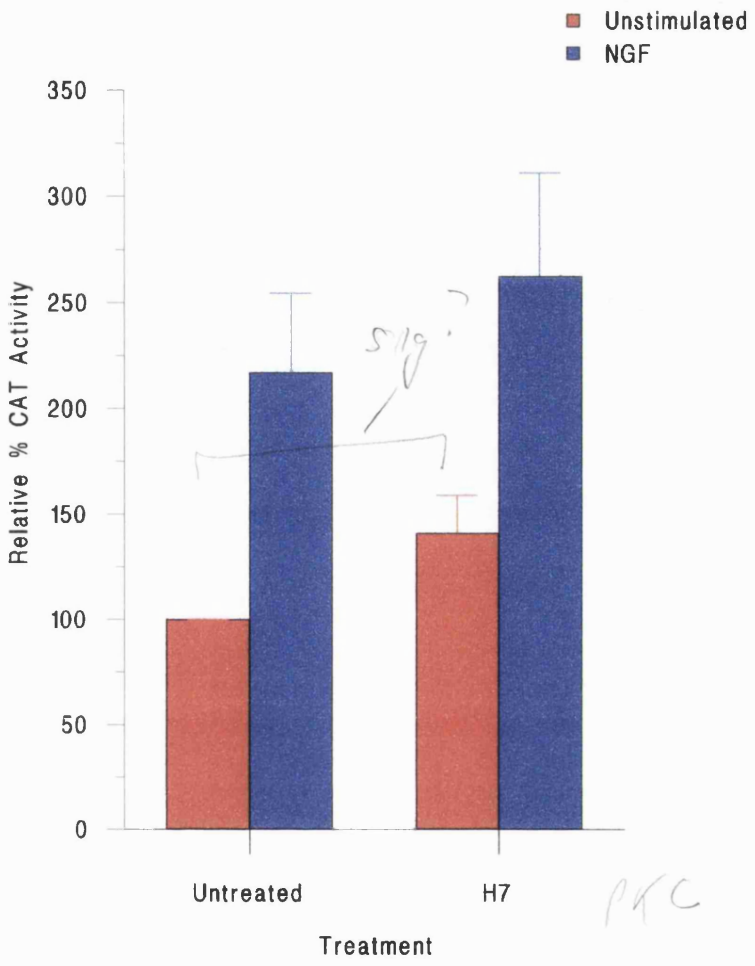
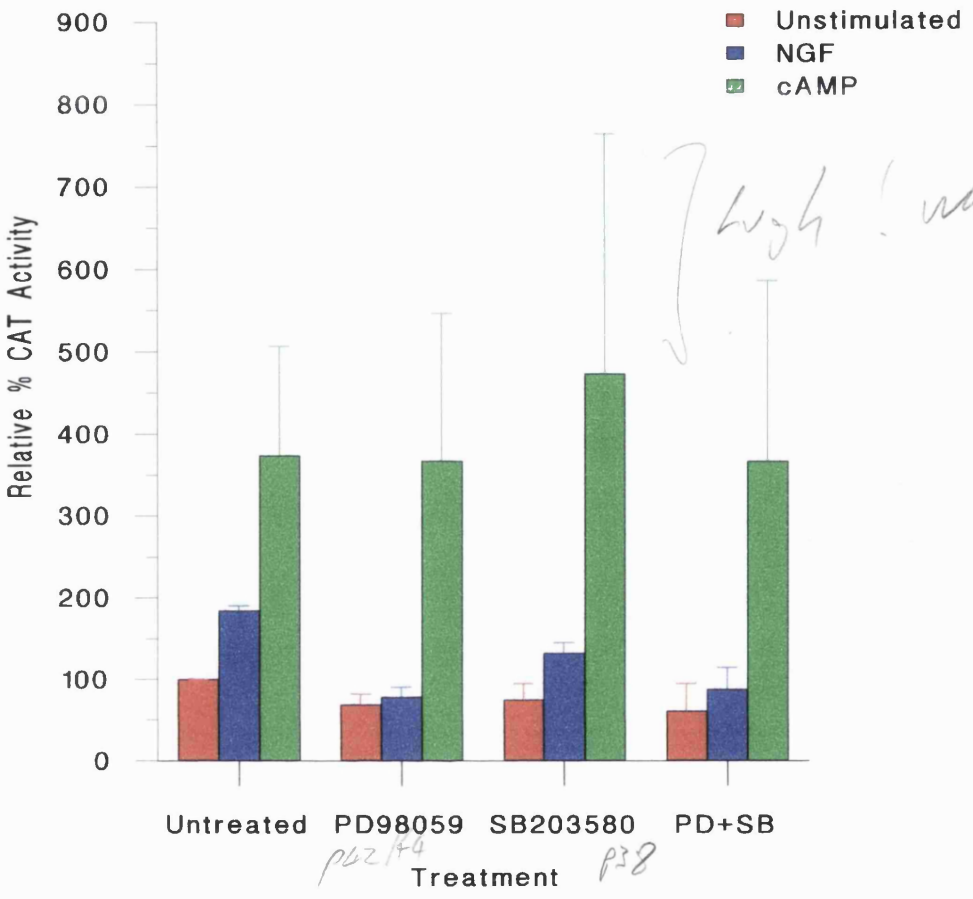

Unstimulated
NGF
350
300
250
200
150
100
50
0
Relative % CAT Activity
Untreated
H7
Treatment

Fig. 3.4 – The Effects of the MAP Kinase Selective Inhibitors on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when transfected into PC12 cells in the presence or absence of the MEK-1/MEK2 inhibitor PD98059 and/or the p38 inhibitor SB203580. Inhibitors were added in DMSO at 1:1000 v/v with an equivalent volume of DMSO being added to control cells. In all cases, the cells were either left unstimulated, treated with NGF, or treated with cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated and uninhibited cells.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


Unstimulated
NGF
cAMP
900
800
700
600
500
400
300
200
100
0
Relative % CAT Activity
Untreated
PD98059
SB203580
PD+SB
Treatment

promoter by NGF; a significant reduction in the NGF-inducibility of the promoter was observed in the presence of the inhibitor when compared to cells treated with NGF alone (P = 0.001), and no significant difference in reporter activity was observed between cells treated with PD98059 alone and those treated with both the inhibitor and NGF (P = 0.404). PD98059 had no effect upon cAMP-mediated activation of the promoter (P = 0.967) (figure 3.4). Treatment of cells with PD98059 together with SB203580 does not further enhance the inhibitory effects of PD98059 on NGF-mediated promoter activation, indicating that p38 MAP kinase contributes little, if at all, to the activation of the CGRP promoter in response to NGF (figure 3.4). In contrast, these results suggest that the p42/p44 MAP kinase signalling cascade is essential for NGF-mediated promoter activation in PC12 cells. In view of this, the role of MAP kinase signalling in CGRP promoter activation was investigated further by using dominant negative mutants of various members of the different MAP kinase signalling cascades.

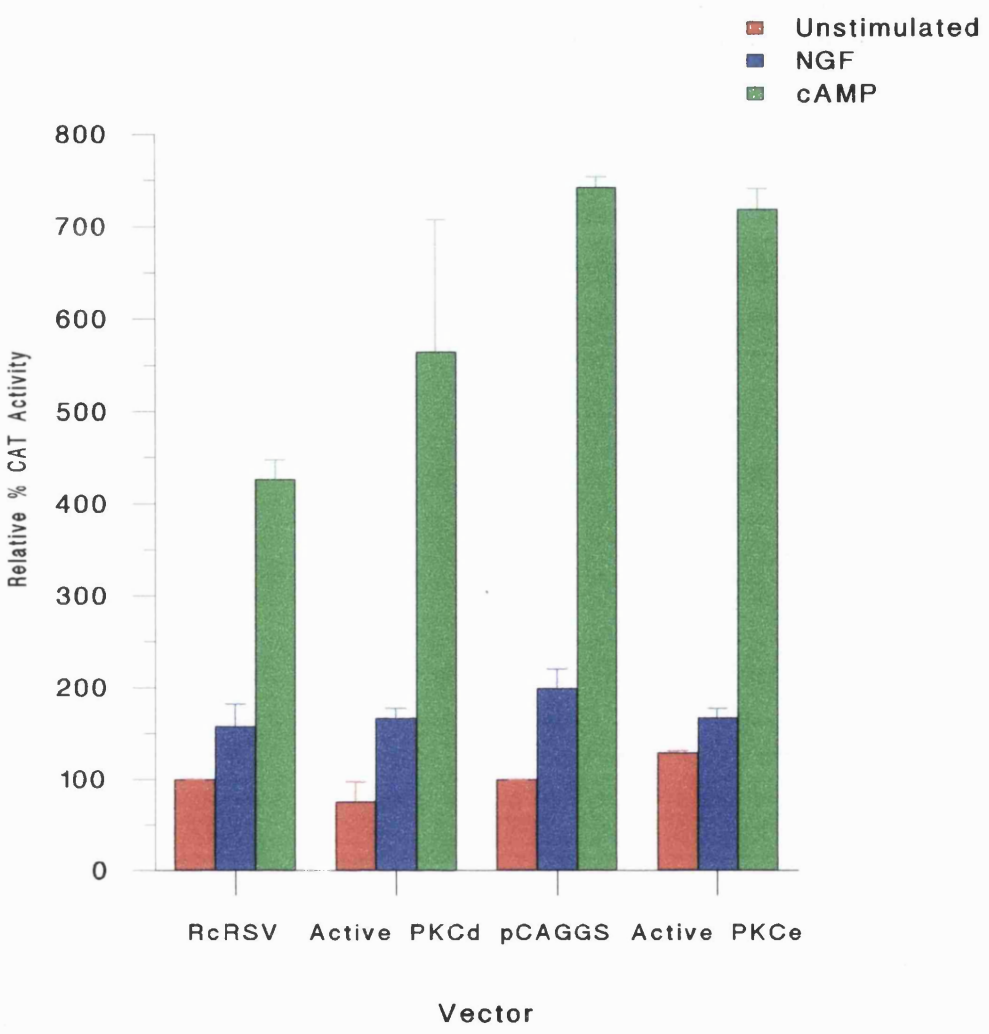
3.2.3 The Effects of PKC and MAP Kinase Signalling Mutants upon NGF- and cAMP-mediated Activation of the CGRP Promoter

Since the enzyme PKC-ε is an activator of p42/p44 MAP kinase in PC12 cells (Hundle *et al*, 1995), and p42/p44 MAP kinase signalling may be involved in NGF-mediated CGRP promoter activation, it is possible that a constitutively active mutant of PKC-ε may enhance promoter activation both at basal levels and in response to NGF. When co-transfected into PC12 cells with Calgcat 1, the constitutively active PKC-ε was unable to enhance promoter activation in response to NGF or cAMP, but did partially increase basal levels of CAT transcription (P = 0.033) (figure 3.5). A constitutively active mutant of PKC-δ affected neither basal levels nor NGF-induced levels of promoter activation (figure 3.5). PKC-δ, although naturally expressed in PC12 cells, is unable to activate p42/p44 MAP kinase signalling (Hundle *et al*, 1995). This again suggests a potential role for p42/p44 MAP kinase signalling in NGF-induced promoter activation, as artificial stimulation of this pathway with PKC-ε is accompanied by an increase in basal transcription of CAT. The role of p42/p44 MAP kinases in PKC-ε-mediated Calgcat 1 promoter up-regulation was not investigated, but could be determined by the addition of the p42/p44 inhibitor PD98059 to the experiment. However, since PKC-ε is unable to enhance NGF-stimulated transcription and inhibition of PKC with H7 does not affect NGF-mediated promoter activation (figure 3.3), this suggests

Fig. 3.5 – The Effects of Constitutively Active Mutant PKC Isoforms on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV or a plasmid encoding a constitutively active mutant of PKC-δ (Active PKCd). Also represented is the activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector pCAGGS or a pCAGGS plasmid encoding a constitutively active mutant of PKC-ε (Active PKCe). In all cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene. In the case of PKC-δ, promoter activity is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the empty vector RcRSV. In the case of PKC-ε, promoter activity is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the empty vector pCAGGS.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


Unstimulated
NGF
cAMP
800
700
600
500
400
300
200
100
0
Relative % CAT Activity
RcRSV
Active PKCd
pCAGGS
Active PKCe
Vector

that PKC does not actually contribute to CRE-mediated CGRP promoter activation in response to NGF.

To confirm the findings that p42/p44 signalling is important in the activation of the CGRP promoter in response to NGF but not cAMP, the effect of the dominant negative p42 MAP kinase p42YF185 (Lu *et al*, 1998) on CGRP promoter activation was investigated in transfection experiments. For comparison, similar transfection experiments were carried out using dominant negative mutants of the p38 activator MEK-6 (MKK6) and JNK.

The results of these experiments are displayed in figures 3.6-3.8. As expected, DN p42 MAP kinase strongly repressed promoter activation in response to NGF when compared to promoter activation in the presence of NGF alone ($P = 0.023$) (figure 3.6). In contrast, DN p42 had no effect upon cAMP-induced promoter activation ($P = 0.745$). This confirms the results seen with the MEK-1 chemical inhibitor PD98059 (figure 3.4), that p42/p44 signalling is involved in NGF-induced, but not cAMP-induced, promoter activation.

In the case of DN MEK-6, no significant inhibitory effect was observed for either NGF-mediated ($P = 0.093$) or cAMP-mediated ($P = 0.665$) promoter activation (figure 3.7). Together with the data obtained from experiments using the p38 inhibitor SB203580 (figure 3.4), this confirms that p38 MAP kinase signalling is not involved in the CRE-mediated activation of the CGRP promoter in response to either stimulus.

Transfection experiments using DN p54b, a dominant negative mutant of JNK, showed similar results to DN MEK-6 (figure 3.8). Neither NGF- nor cAMP-stimulated promoter activation was significantly affected by the presence of DN p54b ($P = 0.675$ and $P = 0.898$ respectively). This result was expected since NGF does not activate JNK in PC12 cells. However, to confirm that the lack of effect seen with the DN p54b was not due to inactivity of the construct, the effects of DN p54b upon the basal activity and anisomycin activation of the tyrosine hydroxylase promoter, which are known to be sensitive to JNK (Hazzalin *et al*, 1998) were investigated. In PC12 cells transfected with DN p54b and TH272CAT, a plasmid containing the TH promoter linked to CAT, the mutant JNK repressed both basal level and anisomycin-induced activation of the TH promoter (figure 3.9). This shows that the construct is functional in PC12 cells.

Fig. 3.6 – The Effects of a Dominant Negative Mutant of p42 MAP Kinase on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcCMV or a vector encoding the dominant negative p42YF185 (DN p42). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcCMV vector.

The results represent the mean values of four independent experiments and the error bars show the standard deviation.

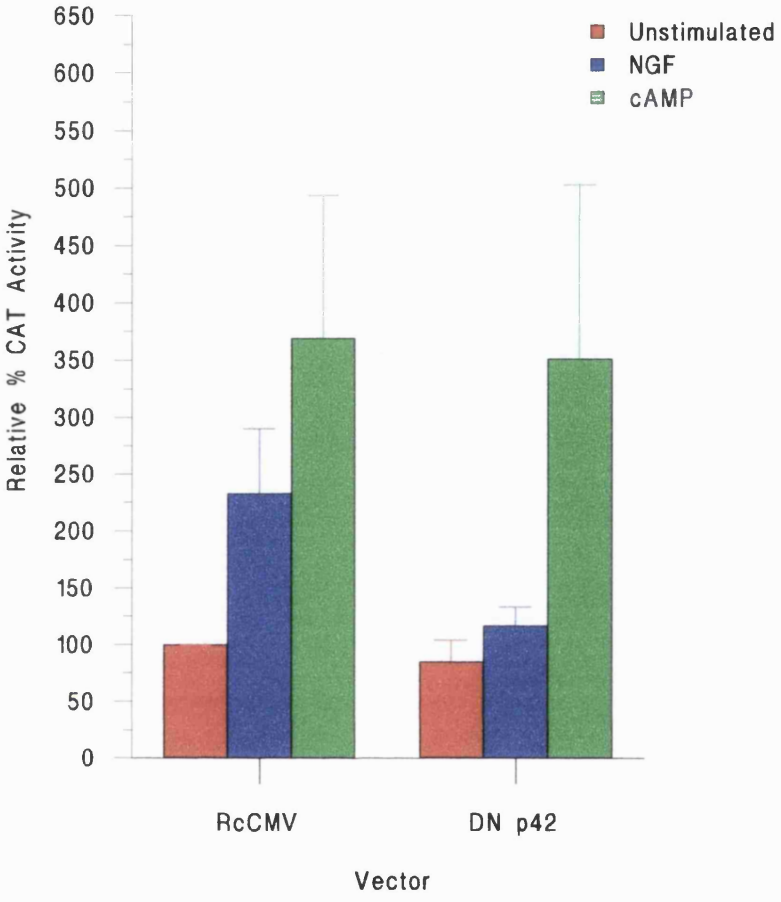

650
600
550
500
450
400
350
300
250
200
150
100
50
0
Relative % CAT Activity
Unstimulated
NGF
cAMP
RcCMV
DN p42
Vector

Fig. 3.7 – The Effects of a Dominant Negative Mutant of MEK-6 on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcCMV or a vector encoding a dominant negative mutant of the p38 activator MEK-6 (DN MEK-6). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcCMV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

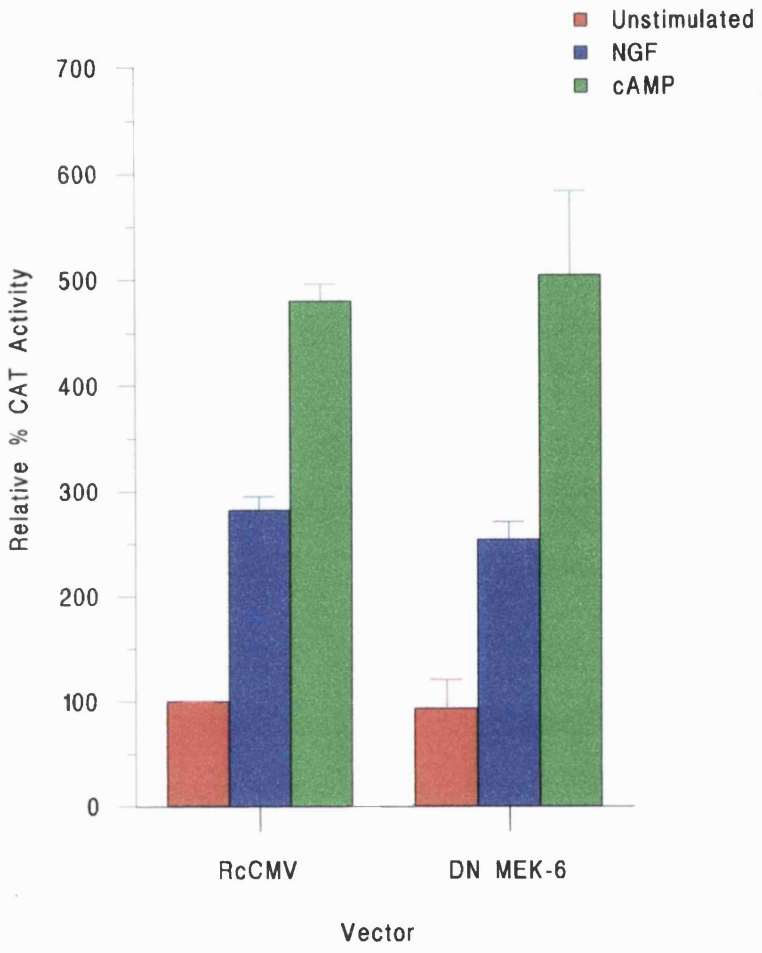

Unstimulated
NGF
cAMP
700
600
500
400
300
200
100
0
Relative % CAT Activity
RcCMV
DN MEK-6
Vector

Fig. 3.8 – The Effects of a Dominant Negative Mutant of JNK on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV or a vector encoding a dominant negative mutant of the c-Jun N-terminal kinase p54b (DN JNK). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

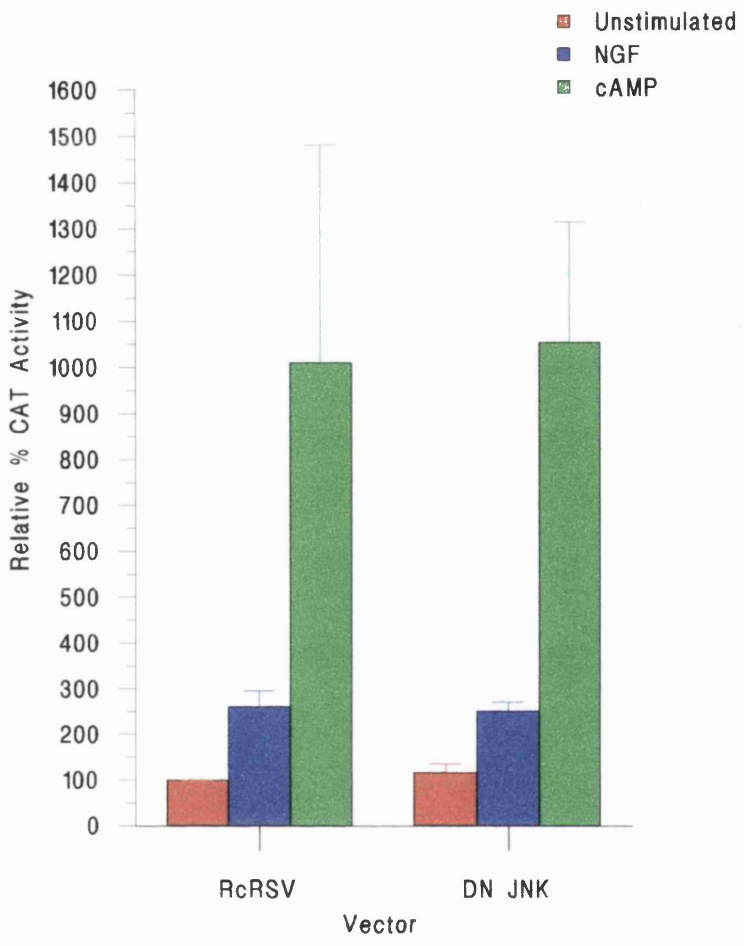
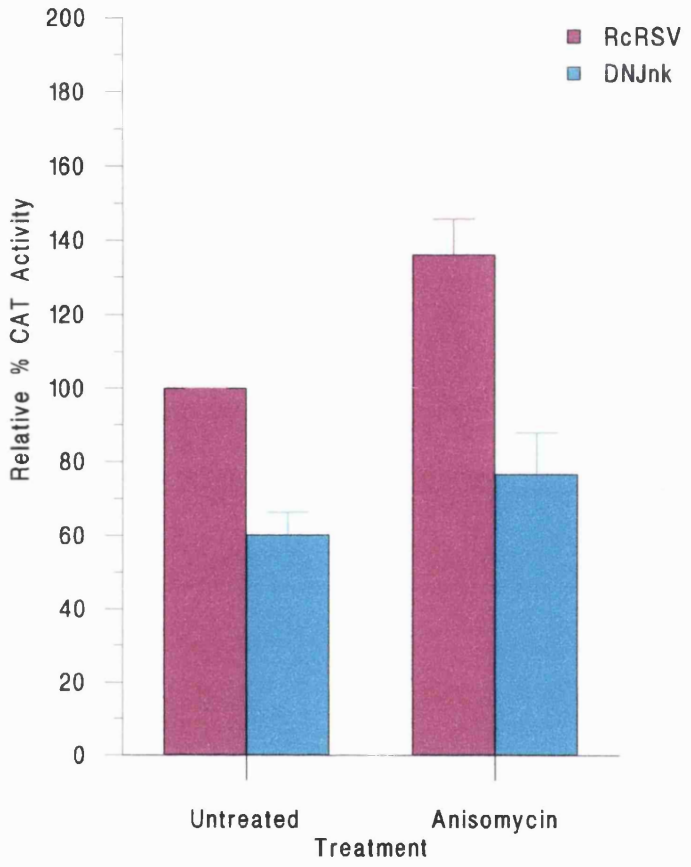

Unstimulated
NGF
cAMP
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Relative % CAT Activity
RcRSV
DN JNK
Vector

Fig. 3.9 – The Effects of the Dominant Negative Mutant of JNK on the Activation of the TH Promoter in Response to Anisomycin

Activity of the tyrosine hydroxylase promoter in the plasmid TH272 when transfected into PC12 cells. TH272 was co-transfected into cells with either the empty expression vector RcRSV or the vector encoding DN JNK. In both cases, the cells were either left unstimulated or were treated with anisomycin. Anisomycin was added in MeOH at 1:1000 v/v with an equivalent volume of MeOH being added to control cells. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


200
180
160
140
120
100
80
60
40
20
0
Relative % CAT Activity
RcRSV
DNJnk
Untreated
Anisomycin
Treatment

3.2.4 The p42/p44 MAP Kinase Signalling Cascade and CGRP Promoter Activation

NGF activation of the p42/p44 MAP kinases involves activation of the GTPase Ras, which activates Raf, which in turn activates MEK-1 and MEK-2. MEK-1 phosphorylates p42 and MEK-2 phosphorylates p44. The MAP kinases then phosphorylate RSKs, which phosphorylate transcription factors such as CREB (Froedin and Gammeltoft, 1999). Having established the importance of the p42/p44 MAP kinases in NGF-mediated activation of the CGRP promoter, the roles of other members of this signal transduction pathway in promoter activation were investigated further. PC12 cells were co-transfected with Calgcat 1 and plasmids encoding dominant negative mutants of Ras, MEK-1 or p44 (figure 3.10). With the addition of DN RasN17, there is a significant difference in the inducibility of the promoter by NGF ($P = 0.012$), and no significant difference between basal level and NGF-induced promoter activation ($P = 0.903$) as NGF-inducibility of the promoter was virtually abolished. The presence of DN MEK-1 also dramatically reduced NGF-stimulated activation of the promoter ($P < 0.001$) leaving no significant difference between basal and NGF-stimulated expression of CAT ($P = 0.108$). As expected, the two mutants of p44 (p44T192A and p44Y194F) also reduced NGF-mediated activation of the promoter ($P < 0.001$ and $P = 0.001$ respectively), although there was still a significant increase in the NGF response when compared to basal expression ($P = 0.006$ and $P = 0.046$ respectively). As with the p42YF mutant, each of these DN p44 MAP kinases lacks a single phosphorylation site in the TEY motif of the activation domain, therefore cannot be activated by MEK (Pages *et al*, 1993). The residual NGF-inducibility of the promoter in the presence of DN p44 is likely to be due to the presence of active p42, suggesting that p42 and p44 are able to stimulate promoter activation independently of one another.

Whilst the presence of DN Ras, DN MEK-1 or DN p44 strongly represses NGF-mediated promoter activation, the promoter remains inducible by cAMP. This suggests that cAMP-mediated activation of the CGRP promoter can occur independently of Ras signalling. However, in certain cases some repression of cAMP induction did occur, indicating that this pathway may partially contribute towards cAMP-mediated CGRP promoter activation.

Once p42 and p44 have been activated by Ras, their activation can be sustained by the GTPase Rap in a Ras-independent manner (York *et al*, 1998). In view of this I tested the role of Rap in NGF-mediated CGRP promoter activation using a dominant negative mutant of the GTPase, RapN17 (figure 3.11). Cells transfected with Calgcat 1 and RapN17 showed only a partial, but significant, repression of NGF-induced promoter activity when compared to NGF-

Fig. 3.10 – The Effects of Various Dominant Negative Mutants of Components of the p42/p44 MAP Kinase Signalling Cascade on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV, a plasmid encoding dominant negative Ras N17 (DN Ras), a plasmid encoding a dominant negative mutant of MEK-1 (DN MEK-1), a vector encoding the dominant negative mutant p44Y194F (DN p44YF), or a vector encoding the dominant negative mutant p44T192A (DN p44TA). In all cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

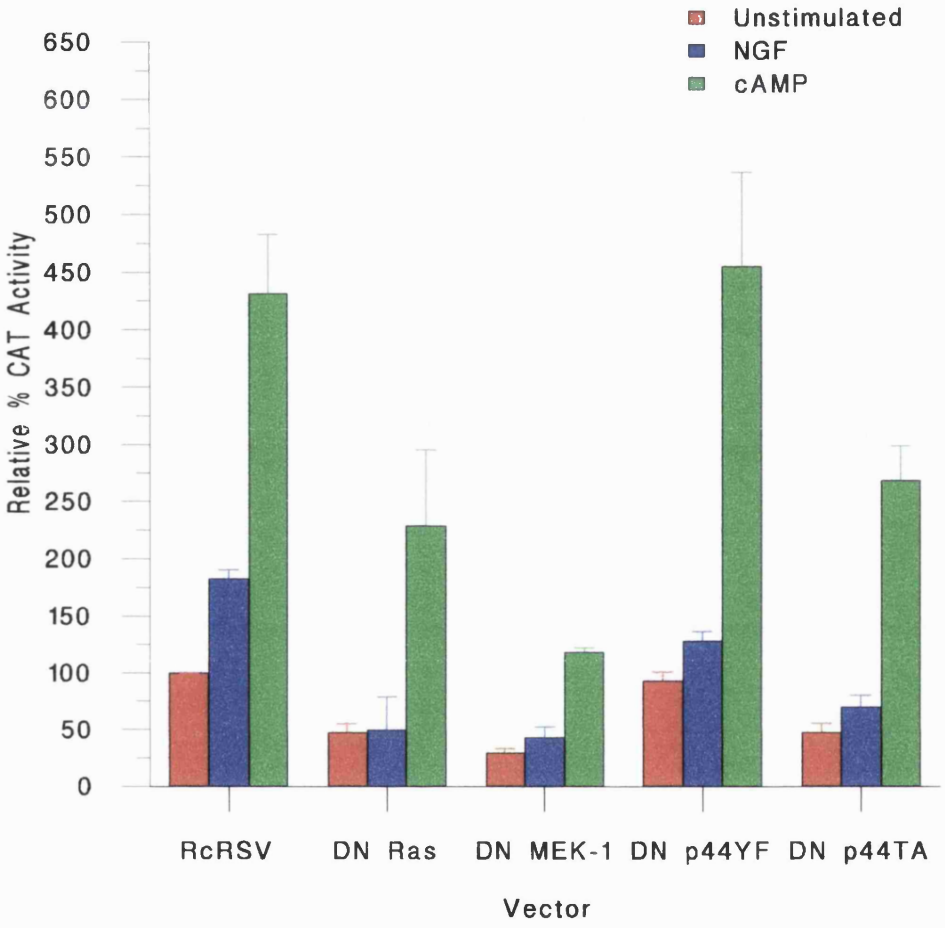
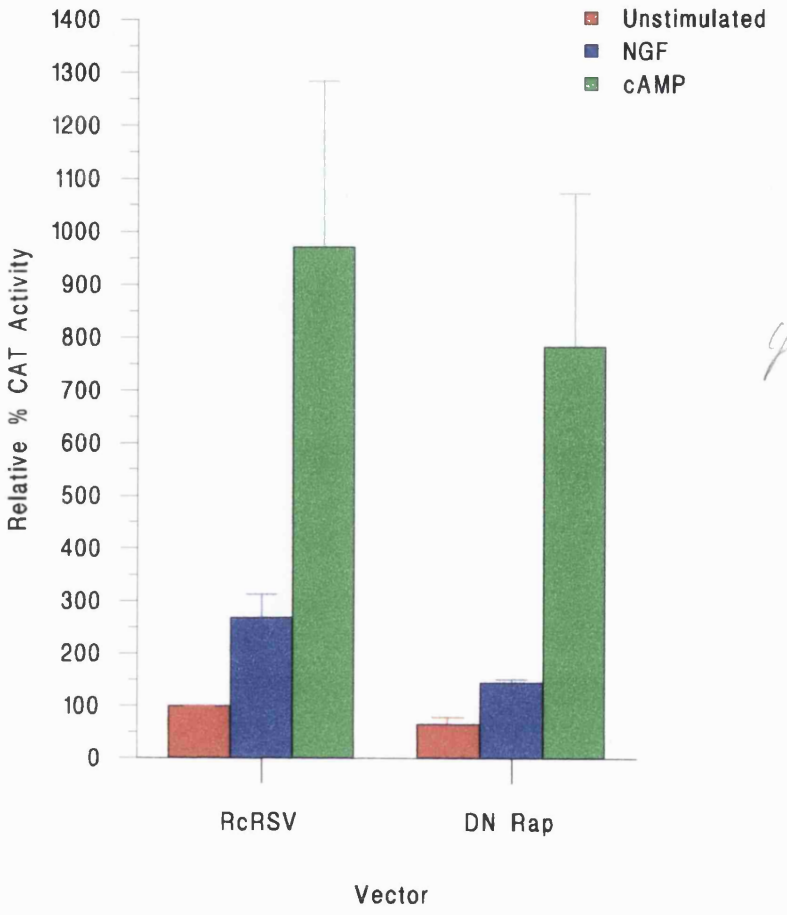

Unstimulated
NGF
cAMP
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Relative % CAT Activity
RcRSV
DN Ras
DN MEK-1
DN p44YF
DN p44TA
Vector

Fig. 3.11 – The Effects of a Dominant Negative Mutant of Rap on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV or a vector encoding the dominant negative mutant RapN17 (DN Rap). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


Unstimulated
NGF
cAMP
Relative % CAT Activity
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
RcRSV
DN Rap
Vector

stimulated cells transfected with Calgcat 1 and an empty expression vector ($P = 0.039$); no repression of cAMP-induced promoter activity was observed ($P = 0.487$). This suggests that, whilst Rap may contribute to promoter activation in response to NGF, promoter activation can occur independently of Rap in response to this stimulus.

RSK proteins, and to a lesser extent the MSK proteins, are the downstream effectors of p42 and p44 MAP kinases. Although the MAP kinases bind preferentially to RSK-3, RSK-2 appears to have functions specific to the nervous and skeletal systems (Froedin and Gammeltoft, 1999). Since CGRP is expressed in neuronal tissues, the role of RSK-2 in CGRP promoter activation was investigated. PC12 cells were transfected with Calgcat 1 and stimulated with NGF, cAMP or left unstimulated either in the presence or absence of the inhibitor Ro-318220. Interestingly this inhibitor is sold as an inhibitor of PKC; however it has been demonstrated that Ro-318220 is also an effective inhibitor of RSK-2 at the concentration used (Alessi, 1997). Although there appears to be a partial reduction in the response to cAMP, inhibition of RSK-2 and PKC does not repress NGF-mediated promoter activation (figure 3.12). This confirms the results obtained with the selective PKC inhibitor H7 (figure 3.3). Parallel transfections were also carried out using Calgcat 1 and a dominant negative inhibitor of RSK-2, KR100 (figure 3.13). Neither cAMP- nor NGF-stimulated promoter activity altered significantly in the presence of DN RSK-2 ($P = 0.501$ and $P = 0.566$ respectively). These results suggest that RSK-2 is not involved in the activation of the CGRP promoter in response to these stimuli and RSK-3 or RSK-1, or even the MSK proteins, are the predominant enzymes required. It is also possible that other RSK enzymes compensated for the loss of RSK-2. However mutant forms of other RSKs/MSKs were not available to test this.

Downstream transcription factor targets of p42 and p44 signalling include the transcription factor CREB. It is also known that both the NGF- and cAMP-responsiveness of the CGRP promoter are dependent upon a non-consensus CREB binding site (CRE) within that promoter (Watson and Latchman, 1995). To test the role of CREB in CGRP promoter activation, further experiments were carried out in PC12 cells in which Calgcat 1 was co-transfected into cells with either killer CREB (KCREB) or ACREB expression vectors. KCREB is a dominant negative mutant of CREB, which is capable of forming heterodimers with its associated proteins, including c-Jun, ATF-2 and CREB, but has poor DNA-binding affinity (Walton *et al*, 1992; Yang *et al*, 1996). In this way KCREB quenches CREB-associated factors within the transfected cell and reduces their ability to activate transcription by reducing their affinity for DNA. ACREB is a dominant negative CREB capable of forming homodimers and heterodimers with ATF-1, and has an acidic amino acid extension that interacts with the basic domain to prevent DNA-binding (Ahn *et al*, 1998). Thus ACREB is able to quench cellular

Fig. 3.12 – The Effects of the RSK-2 Inhibitor Ro-318220 on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when transfected into PC12 cells in the presence or absence of the RSK-2 inhibitor Ro-318220. The inhibitor was added in DMSO at 1:1000 v/v with an equivalent volume of DMSO being added to control cells. In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated and uninhibited cells.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

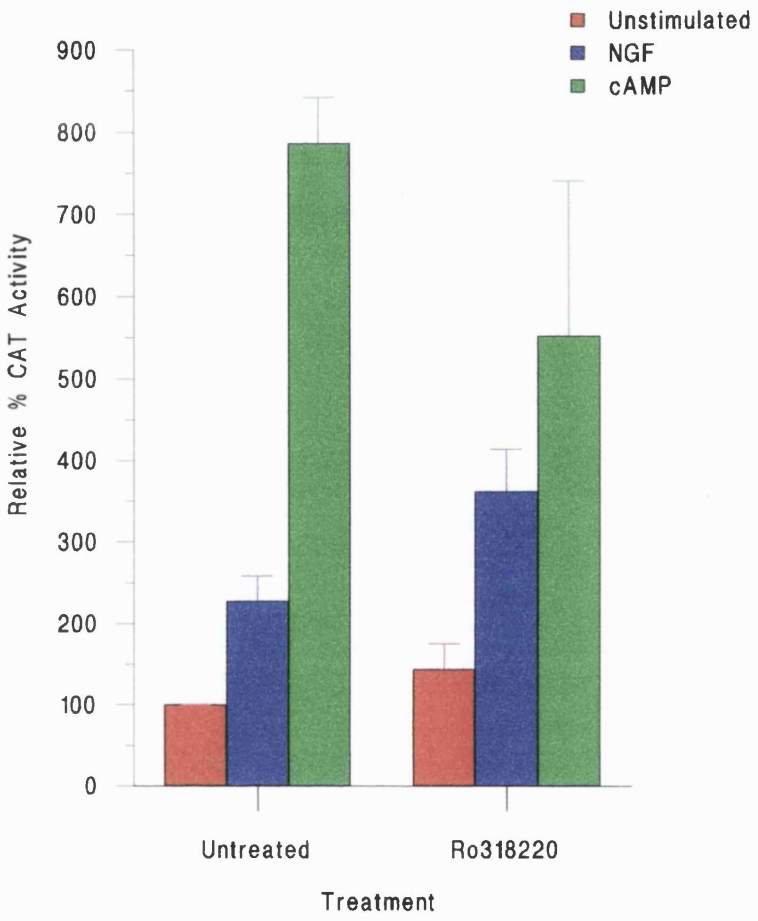
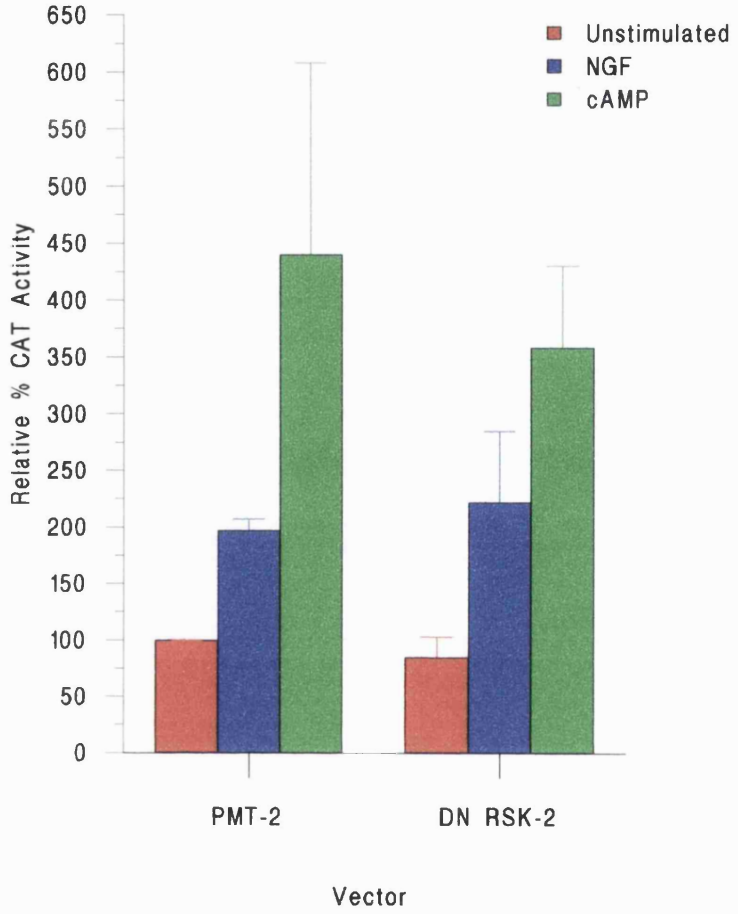

Unstimulated
NGF
cAMP
900
800
700
600
500
400
300
200
100
0
Relative % CAT Activity
Untreated
Ro318220
Treatment

Fig. 3.13 – The Effect of a Dominant Negative Mutant of RSK-2 on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector PMT-2 or a vector encoding the dominant negative mutant RSK-2KR100 (DN RSK-2). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the PMT-2 vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


650
600
550
500
450
400
350
300
250
200
150
100
50
0
Relative % CAT Activity
Unstimulated
NGF
cAMP
PMT-2
DN RSK-2
Vector

CREB and abolish its ability to activate transcription. It has been reported that ACREB is able to completely inhibit cAMP-mediated activation of a partial c-fos promoter as well as reduce NGF-mediated activation of that promoter (Ahn *et al*, 1998).

PC12 cells transfected with either ACREB or KCREB (figure 3.14) exhibited significantly reduced NGF-mediated activation of the CGRP promoter when compared to cells treated with NGF alone ($P = 0.018$ and $P = 0.011$ respectively). As expected, ACREB also dramatically reduced cAMP-mediated promoter activation ($P = 0.005$). Although KCREB appeared to inhibit the response to cAMP, the difference between promoter activation in the presence and absence of KCREB was not significant ($P = 0.102$). This might be explained by the fact that KCREB is a much weaker inhibitor of CREB than ACREB as it is still able to bind to DNA and activate transcription, albeit with a lower affinity than normal CREB. To confirm that CREB activates transcription from the CGRP promoter after activation by the p42/p44 signalling pathway, PC12 cells were co-transfected with Calgcat 1 and a plasmid encoding constitutively active MEK-1, S218D/S222D (Brunet *et al*, 1994), either in the presence or the absence of ACREB or KCREB. As expected, the active MEK-1 was sufficient to significantly induce promoter activation in the absence of NGF ($P = 0.037$) (figure 15). Furthermore this induction was almost totally abolished by ACREB ($P = 0.031$) and partially repressed by KCREB ($P = 0.049$). Taken together, these observations suggest that CREB is the CRE-binding factor involved in CGRP promoter activation by cAMP/PKA and NGF/p42/p44 MAP kinases.

Fig. 3.14 – The Effects of Dominant Negative CREB on the Activation of the CGRP Promoter in Response to NGF and cAMP

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV, a vector encoding the dominant negative ACREB, or a vector encoding the dominant negative mutant killer CREB (KCREB). In both cases, the cells were either left unstimulated, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of four independent experiments and the error bars show the standard deviation.

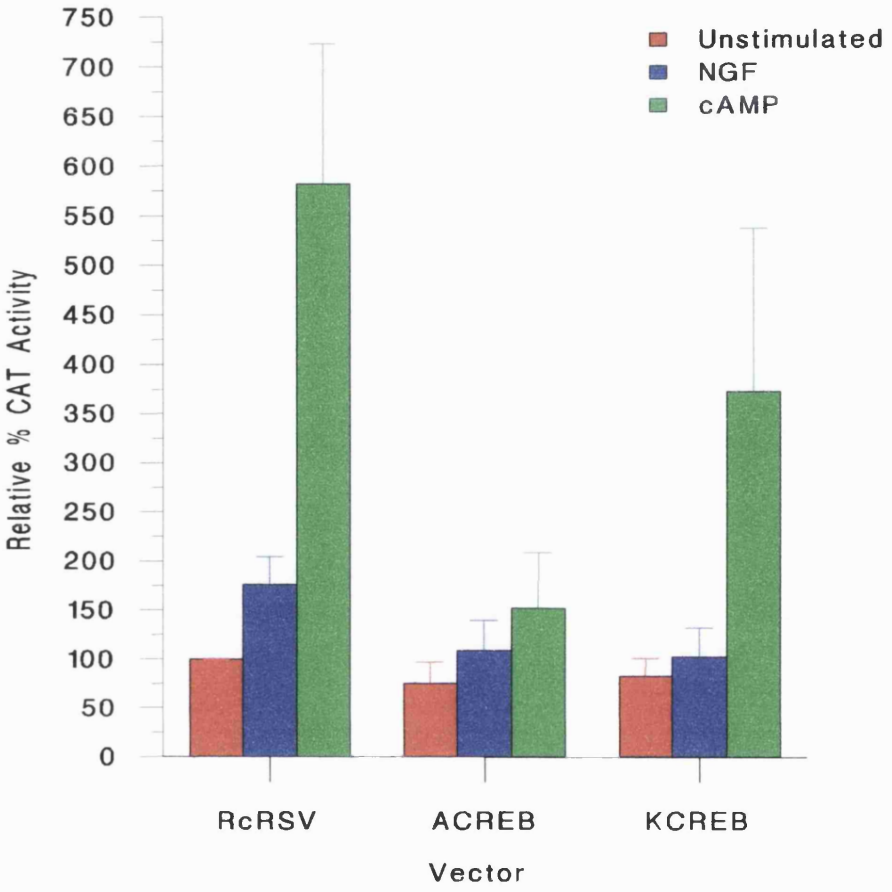
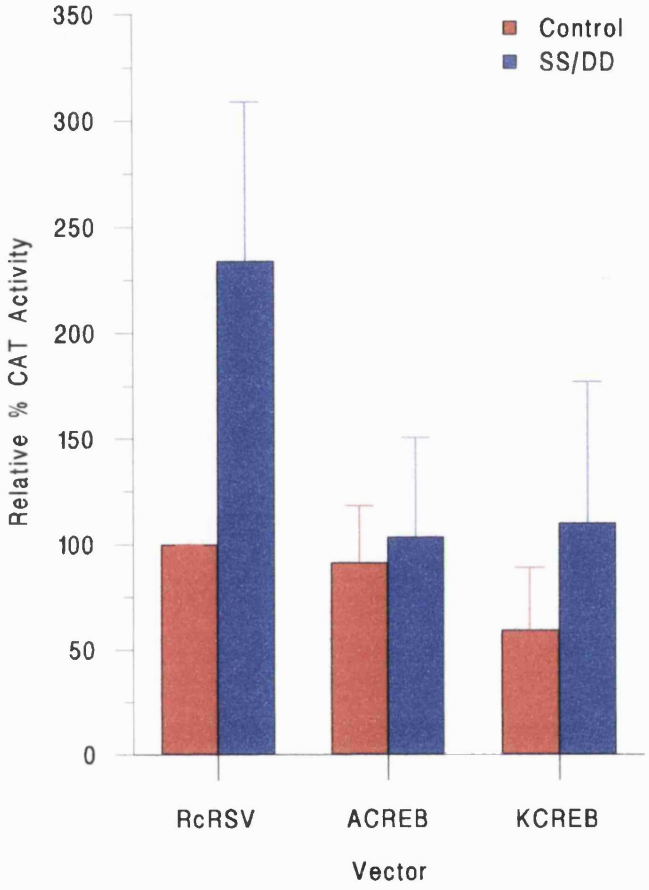

750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Relative % CAT Activity
Unstimulated
NGF
cAMP
RcRSV
ACREB
KCREB
Vector

Fig. 3.15 – The Effects of Dominant Negative CREB on the MEK-1-mediated Activation of the CGRP Promoter

Activity of the CGRP promoter in the plasmid Calgcat 1 when co-transfected into PC12 cells with either the empty expression vector RcRSV or the constitutively active MEK-1 mutant S218D/S222D (SS/DD). In both cases the cells were also transfected with either an additional amount of RcRSV, the vector encoding ACREB, or the vector KCREB. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the RcRSV vector.

The results represent the mean values of four independent experiments and the error bars show the standard deviation.


350
300
250
200
150
100
50
0
Relative % CAT Activity
Control
SS/DD
RcRSV
ACREB
KCREB
Vector

3.3 Discussion

PC12 cells differentiate in the presence of both NGF and cAMP. The second messenger cAMP generally mediates its effects via the enzyme protein kinase A (Lalli and Sassone-Corsi, 1994). The neurotrophin NGF triggers several distinct intracellular signalling cascades when it binds to Trk-A receptors on the surface of PC12 cells. Trk-A activation of the Ras GTPase stimulates the MAP kinases p38, p42 and p44 (Xing *et al*, 1996; Xing *et al*, 1998), whilst Trk-A activation of protein kinase A and the Rap GTPase activate p42 and p44 MAP kinases (Yao *et al*, 1998). NGF is also able to trigger the activation of protein kinase C (Kaplan and Stephens, 1994). By the inhibition of each of these individual signalling pathways in PC12 cells, the different signalling cascades involved in the activation of the CGRP promoter in response to the stimuli NGF and cAMP have been identified.

Firstly, the expectation that the cAMP-mediated activation of the CGRP promoter requires PKA has been confirmed. In contrast, although NGF is also able to activate PKA, this enzyme is only partially responsible for the NGF-mediated activation of this promoter. These studies have demonstrated that the critical signalling pathway involved in the response to NGF is the p42/p44 MAP kinase signalling cascade. Both PKA and p42/p44 MAP kinases are able to mediate the phosphorylation of the transcription factor CREB; the active catalytic subunit of PKA can phosphorylate CREB at serine 133 directly (Lalli and Sassone-Corsi, 1994), and p42/p44 MAP kinases phosphorylate CREB at this site via the RSK proteins (Xing *et al*, 1996). Since both NGF- and cAMP-mediated activation of the CGRP promoter is dependent upon a CREB-binding site within the promoter (Watson and Latchman, 1995), it is likely that CREB is responsible for activating transcription in response to both stimuli, but via two distinct signalling pathways.

It has previously been reported that the p38 MAP kinase signalling cascade can also phosphorylate CREB at serine 133 in reponse to NGF via MSK family proteins (Deak *et al*, 1998). Indeed inhibition of both p38 signalling and p42/p44 signalling cascades with the chemicals PD98059 and SB203580 is necessary for the total inhibition of CREB serine 133 phosphorylation in NGF-stimulated PC12 cells (Xing *et al*, 1998). However, in the case of CGRP, it has been demonstrated that inhibition of p42 and p44 strongly represses NGF-mediated promoter activation, whilst inhibition of p38 has only a minimal effect. When both inhibitors are added to PC12 cells together, SB203580 does not enhance the effects of PD98059 suggesting that p42/p44 signalling, but not p38 signalling, is essential for promoter activation.

As well as p42/p44 MAP kinase inhibition, chemical and genetic inhibition of PKA also reduced CGRP promoter activation in response to NGF. This effect was weaker than that observed with cAMP-induced promoter activation, where PKA plays an essential role. It is possible that this partial inhibition could be the result of cross-talk between PKA and p42/p44 signalling since PKA has been implicated in mediating the sustained activation of p42 and p44 MAP kinases via the GTPase Rap (Yao *et al*, 1998). Inhibition of Rap signalling also partially reduces the level of NGF-mediated promoter activation. Secondly, it has been demonstrated that PKA is required for the NGF-induced nuclear translocation of p42, since H89 reduces the level of p42 localisation in the nucleus (Yao *et al*, 1998). Since CREB is phosphorylated by MAP kinases within the nucleus, this suggests that H89 would consequently reduce the level of NGF-induced CREB phosphorylation.

In addition to PKA and Rap, the PKC isoform PKC-ε is also able to enhance p42/p44 MAP kinase signalling in PC12 cells (Hundle *et al*, 1995). Here it has been demonstrated that, in the case of the CGRP gene, PKC signalling is not involved in NGF-mediated promoter activation. However, a constitutively active mutant of PKC-ε causes a slight up-regulation of the CGRP promoter in the absence of any stimulus. This is probably due to the persistent activation of p42/p44 MAP kinases, although this has not been confirmed, providing further indirect evidence that p42/p44 MAP kinase signalling is pivotal in the NGF response.

Despite the differences in the signalling mechanisms involved, both NGF and cAMP are able to mediate the phosphorylation of CREB at serine 133, and I have demonstrated that inhibition of CREB represses CGRP promoter activation in response to both stimuli. However, previous studies have shown that, whilst active CRE-bound CREB is necessary and sufficient for cAMP-stimulated promoter activation, it is necessary but not sufficient for the response to NGF (Watson and Latchman, 1995). In the case of NGF, CREB co-operates with at least one other DNA-binding transcription factor to increase levels of transcription. This factor may be required due to the differences in kinetics of CREB phosphorylation by the different kinases (Nguyen *et al*, 1993; Impey *et al*, 1998; Yao *et al*, 1998). It is also possible that NGF and cAMP may differentially modify DNA-bound CREB at residues other than serine 133 and that, as a result, NGF may inhibit the ability of CREB to activate transcription in the absence of the second factor. Such differential post-translational modification of CREB has been observed with the calcium/calmodulin-dependent protein kinases CAMKII and CAMKIV (see section 1.6.3); CAMKII inhibits CREB activation by phosphorylating both serine 133 and the inhibitory site serine 142, whilst CAMKIV is only able to phosphorylate CREB at serine 133 and stimulates CREB-mediated transcription (Sun *et al*, 1994).

The mechanisms involved in the NGF-mediated regulation of CGRP demonstrated here share many similarities with those involved in the regulation of c-fos transcription. NGF-stimulation of the c-fos pomoter is also known to be regulated in a CRE-dependent manner. The CRE is necessary but not sufficient for the response to NGF; a larger promoter containing the CRE and a second transcription factor binding-site is required for NGF-mediated transcription (Ginty *et al*, 1994; Bonni *et al*, 1995). The factor, which binds to the second non-CRE binding site necessary for the NGF response of the c-fos promoter, has already been identified as a serum-response factor (SRF). The SRF is phosphorylated in response to NGF via the p42/p44 MAP kinase signalling pathway. The SRF binds to a serum response element (SRE) within the c-fos promoter located adjacent to the CRE approximately 300bp from the start site (Ginty *et al*, 1994; Bonni *et al*, 1995). Whilst the additional factor required for CREB-mediated activation of the CGRP promoter in response to NGF has not yet been identified, it is possible that NGF induces the phosphorylation of this factor, as is the case with the SRF and c-fos promoter up-regulation. Furthermore, having identified the pivotal role of p42/p44 MAP kinase signalling in NGF-mediated CGRP promoter activation, p42/p44 MAP kinase may induce phosphorylation of both the factor co-operating with CREB as well as CREB itself. However, as yet there is no evidence to support this theory; it is also possible that the unidentified nuclear factor may be constitutively active even in the absence of the NGF stimulus. If this were so, then p42/p44-induced CREB phosphorylation alone would be the trigger for NGF-mediated CGRP promoter activation. The identification of this unknown factor and its DNA-binding site may greatly improve our understanding of the mechanisms behind CGRP promoter regulation.

In addition to its role in the activation of CREB and other DNA-binding factors, p42/p44 MAP kinase signalling is also known to regulate the activity of the CREB-binding protein (CBP) (Lui *et al*, 1998). This protein will only bind to CREB phosphorylated at serine 133 (Parker *et al*, 1996), and acts to stimulate transcription as a CREB co-activator (Kwok *et al*, 1994). However, recent studies have suggested that CBP can modulate transcription even when recruited to DNA by a heterologous DNA-binding domain, and that CBP activity is repressed by inhibition of the p42/p44 MAP kinase sinalling pathway (Lui *et al*, 1998). Thus activation of the CGRP promoter by NGF may be dependent upon the p42/p44-mediated stimulation of CBP activity, as well as the p42/p44-dependent phosphorylation of CREB. It would be interesting to investigate the importance of CBP and MAP kinase signalling in CGRP promoter activation.

In conclusion, several factors including CREB, CBP and at least one other DNA-binding factor may be regulated by p42/p44 MAP kinases to increase the level of transcription from

the CGRP promoter in response to NGF. However, the experiments described within this chapter have clearly demonstrated that, whilst the p42/p44 MAP kinase signalling pathway is essential for the activation of this promoter in response to NGF, it is not required for promoter activation in response to cAMP. Rather, the effects of cAMP are mediated by the enzyme PKA. Thus, although both the cAMP and NGF responses require the transcription factor CREB and an intact CRE within the promoter, distinct signalling pathways are involved in mediating the effects of these stimuli upon CGRP promoter activation, paralleling the differences in the role of the CRE in the two responses.

4 The Effects of Hypoxia on the Bcl-2 Promoter in PC12 Cells

4.1 Introduction

4.1.1 Bcl-2 and Hypoxia-induced Cell Death

Bcl-2 belongs to a family of cytoplasmic proteins expressed in many different tissues. These proteins function as regulators of programmed cell death; certain family members, such as Bax and Bcl-xs, induce apoptosis whilst others, such as Bcl-2 and Bcl-xl, inhibit apoptotic cell death. As described previously, Bcl-2 has been shown to reduce levels of apoptosis in both the immune system (Nakayama *et al*, 1993; Veis *et al*, 1993) and the nervous system (Allsopp *et al*, 1993; Martinou *et al*, 1994; Greenlund *et al*, 1995; Pinon *et al*, 1997). In addition, an increase in Bcl-2 expression enabling Bcl-2-mediated rescue of neuronal cells from programmed cell death has been observed in response to many different apoptotic stimuli, including hypoxia, growth factor withdrawal and serum withdrawal in many cell types (Jacobson and Raff, 1995; Wilson *et al*, 1996; Bossenmeyer-Pourie and Daval, 1998).

In cultured neurons from the foetal rat forebrain, Bcl-2 expression is increased following hypoxia; the cells are able to up-regulate the survival factor during a 24-hour re-oxygenation period following 6 hours of hypoxic injury. This up-regulation can be further increased if the cells are pre-conditioned with a one-hour exposure to hypoxia 24 hours prior to the 6-hour treatment. The increase in Bcl-2 expression reduces the level of hypoxia-induced cell death (Bossenmeyer-Pourie and Daval, 1998). PC12 cells are able to express Bcl-2 in response to the neurotrophin NGF (Riccio *et al*, 1999). It would be interesting to see whether PC12 cells, like the neurons of the foetal rat brain, could also increase their level of Bcl-2 expression during re-oxygenation in response to hypoxia. If so, PC12 cells could be used as a model system to investigate the mechanisms behind the up-regulation of Bcl-2 expression observed in response to hypoxia in neuronal cells, since they are more easily transfectable than cultured neurons.

The tyrosine hydroxylase (TH) gene is also sensitive to hypoxia; reduced oxygen levels can increase the rate of TH transcription and increase the stability of TH mRNA in PC12 cells

(Czyzyk-Krzeska *et al*, 1994). As described in section 1.9.2, hypoxia induces the rapid and persistent phosphorylation of CREB at serine 133 in these cells. Active CREB bound to the CRE site located at –38 to –45 of the TH promoter then activates transcription of the tyrosine hydroxylase gene. The Bcl-2 promoter contains a CRE site located at –1546 to –1537, therefore it is possible that CREB may be involved in the activation of Bcl-2 transcription in response to hypoxia. It would be beneficial to identify those promoter sequences necessary for the hypoxia-induced transcription of Bcl-2 in order to provide a better understanding of the mechanisms involved in the up-regulation of Bcl-2 in response to reduced oxygen levels.

4.1.2 The Bcl-2 Promoter

As stated previously in section 1.9.3, Bcl-2 has a protective effect in the immune system where it is able to rescue immature B cells from apoptosis, and Bcl-2 also plays a role in the activation of mature B cells (Wilson *et al*, 1996). Consequently the regulatory regions of the Bcl-2 promoter have been extensively studied in B cells and many important sequences have been identified. Bcl-2 gene transcription is regulated by two promoters; an upstream promoter known as P1 and a downstream promoter located entirely downstream of –1280 known as P2 (see figure 4.1). The predominant promoter in non-neuronal cells, such as B cells, is P1 (Chen and Boxer, 1995; Wilson *et al*, 1996). The Bcl-2 CRE site located at –1546 to –1537 (Wilson *et al*, 1996) lies in P1 and was the first positive regulatory element to be identified (Wilson *et al*, 1996). Interestingly, transfection studies showed that although elevated levels of cAMP were unable to activate the CRE-dependent Bcl-2 promoter, PKC is able to phosphorylate CREB and activate transcription in these cells (Xie and Rothstein, 1995). Thus, as well as being a mediator of basal expression, CRE-bound CREB is also responsible for activation of the Bcl-2 promoter upon activation of the PKC signalling pathway in B cells.

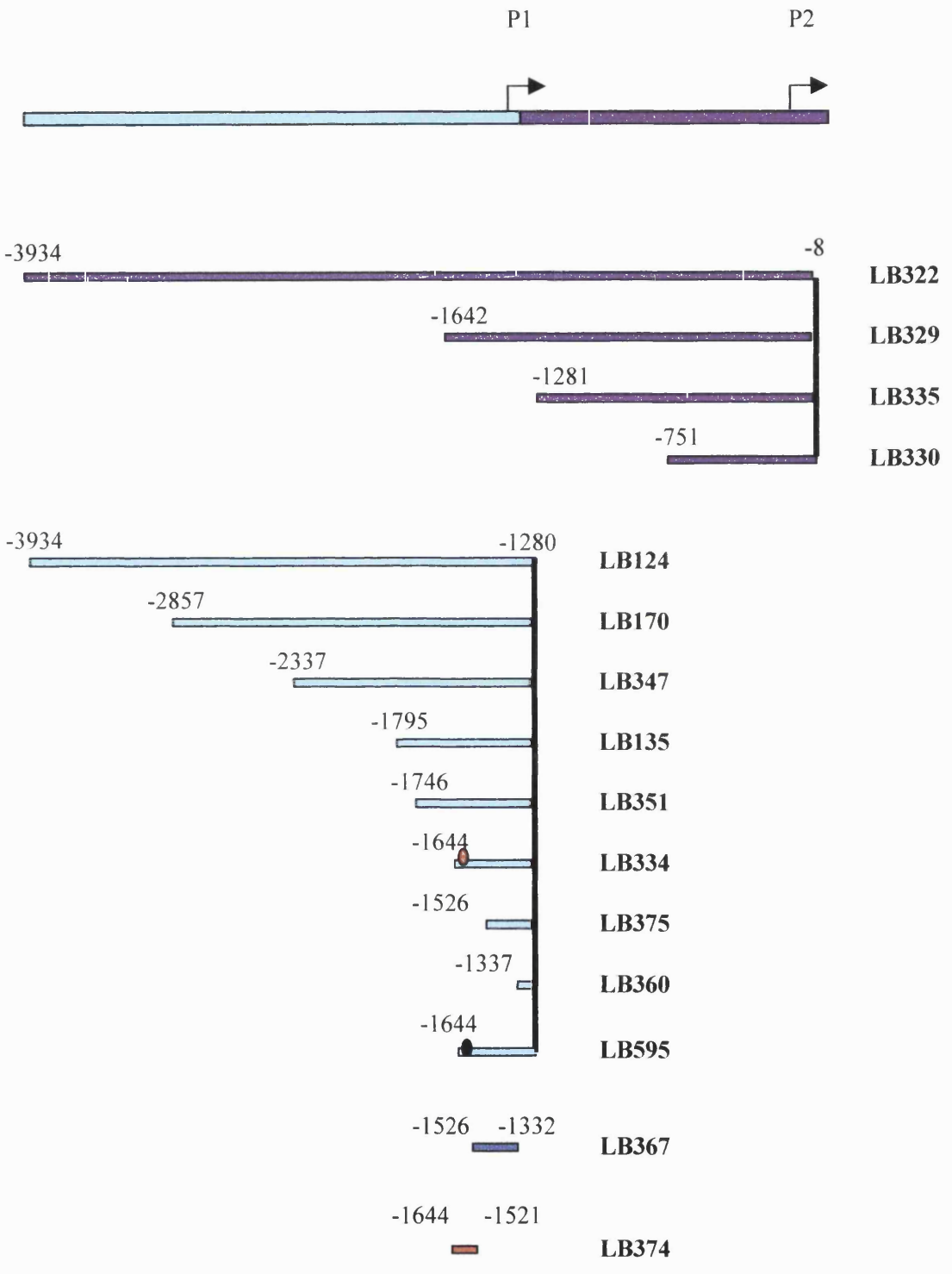
Negative regulatory elements responsible for the down-regulation of Bcl-2 in B cells have also been described. Bcl-2 expression in pre-B cells is much lower than that observed in mature B cells, indicating that Bcl-2 may be involved in the control of apoptosis during development and B cell differentiation. Three π1-binding sites located within the regions –1795 to –1775, –1675 to –1755 and –1047 to –1027 act as negative regulators in pre-B cells that are non-functional in mature B cells (Chen and Boxer, 1995).

As discussed in section 1.9.3, studies in neuronal cells have shown that the P2 promoter is the major transcriptional promoter in neuronal cells (Smith *et al*, 1998a; Smith *et al*, 1998b). The neuronally expressed transcription factor Brn-3a mediates Bcl-2 P2 promoter activation via a sequence located between –584 and –594 base pairs relative to the start site (Smith *et al*, 1998a; Smith *et al*, 1998b). However, regions of the isolated P1 promoter are also important for neuronal transcription regulation of Bcl-2. For example, deletion-mapping experiments showed that the NGF-sensitivity of P1 in neuronal cells is located at a site downstream of the CRE at –1472 to –1414 (Liu *et al*, 1999). Despite the importance of CREB in the transcription of a number of neuronally expressed genes in response to NGF (e.g., c-fos and CT/CGRP), mutational analysis suggested that the CRE site was not involved in the NGF-mediated activation of Bcl-2 (Liu *et al*, 1999). However, more recent conflicting evidence suggests that NGF-mediated regulation of Bcl-2 in PC12 cells may actually require the CRE (Riccio *et al*, 1999); therefore the role of CREB in Bcl-2 promoter activation in response to NGF is still unclear. Interestingly, the Bcl-2 CRE is also thought to be insensitive to cAMP (Liu *et al*, 1999).

In order to carry out promoter studies in B cells and neuronal cells a series of plasmids containing various regions of the Bcl-2 promoter was created with progressive 5' and 3' truncations of the Bcl-2 promoters linked a luciferase reporter gene (see figure 4.1). These plasmids enabled the identification of the regulatory elements described above, and the stimuli to which they respond (Liu *et al*, 1999; Smith *et al*, 1998a; Smith *et al*, 1998b; Wilson *et al*, 1996). In view of this, these plasmids would be useful tools for the identification of hypoxia-sensitive sequences within the Bcl-2 promoter regions when transfected into PC12 cells subjected to hypoxia.

Fig. 4.1 – Bcl-2 Promoter Fragments Tested for Hypoxia-responsiveness in this Study

A schematic representation of the human Bcl-2 5' regulatory sequences showing the relative positions of P1 and P2, together with the various LB Bcl-2 promoter constructs used in this study. All promoter sequences are linked to a luciferase reporter gene in the vector pGL2. The position of the Bcl-2 CRE site is indicated by the red circle in LB334, and the mutated CRE site in LB595 is represented by a black circle.


P1
P2
-3934
-8
LB322
-1642
LB329
-1281
LB335
-751
LB330
-3934
-1280
LB124
-2857
LB170
-2337
LB347
-1795
LB135
-1746
LB351
-1644
LB334
-1526
LB375
-1337
LB360
-1644
LB595
-1526
-1332
LB367
-1644
-1521
LB374

4.2 Results

4.2.1 Identification of Hypoxia-sensitive Sites within the Bcl-2 Promoter

In order to identify sequences of the Bcl-2 promoter sensitive to hypoxia, plasmids containing progressively truncated sequences of the Bcl-2 promoter regions P1 and P2 linked to a luciferase reporter gene were transfected into PC12 cells. The truncated promoter sequences tested are illustrated in figure 4.1. Transfected cells were deprived of oxygen for a period of 4 hours and subsequently allowed to re-oxygenate for a period of 24 hours before harvesting and testing for luciferase activity. The first promoter sequences to be tested for hypoxia sensitivity included the full-length promoter (P1 and P2) and progressive 5' truncations of the downstream sequence. As displayed in figure 4.2, neither the Bcl-2 promoter sequences in LB322 (–3934 to –8), LB329 (–1642 to –8) or LB335 (–1281 to –8) significantly modified the expression of the luciferase reporter gene in response to hypoxia when compared to basal levels of expression ($P = 0.052$, $P = 0.666$ and $P = 0.251$ respectively). In the case of LB330 (–751 to –8), reporter gene levels were almost halved following hypoxia when compared to basal expression ($P = 0.010$).

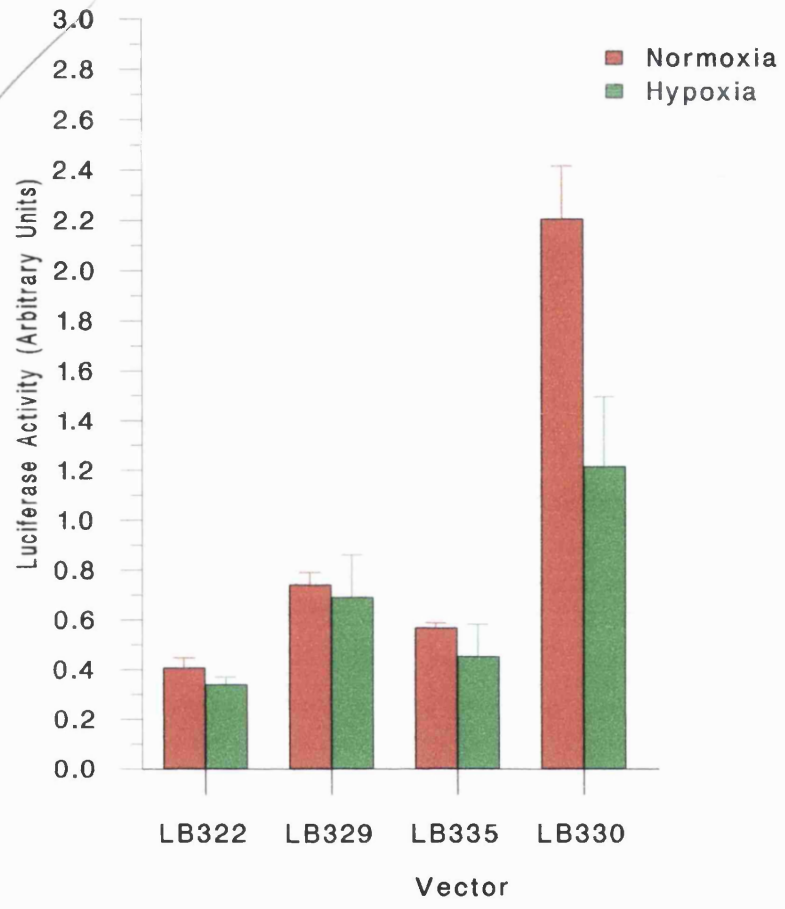
The next Bcl-2 promoter sequences to be tested for hypoxia sensitivity were 5' truncations of the Bcl-2 CRE-containing P1 promoter terminating at –1280 (see figure 4.3). P2 is located entirely downstream of –1280 (see section 1.9.3); therefore truncations of the Bcl-2 promoter with their 3' end at –1280 contain upstream sequences of the isolated P1 promoter only. Plasmids containing various truncations of the P1 promoter linked to the luciferase gene were transfected into PC12 cells, and subsequent luciferase assays revealed that the longest P1-containing promoter sequence tested, LB124 (–3934 to –1280), showed no sensitivity to hypoxia when compared to basal levels of expression ($P = 0.974$). The promoter sequences within LB170 (–2857 to –1280) and LB347 (–2337 to –1280) are down regulated following hypoxia treatment ($P = 0.021$ and $P = 0.020$ respectively), as are the sequences within LB375 (–1526 to –1280) and LB360 (–1337 to –1280) ($P = 0.015$ and $P = 0.023$ respectively). However, the promoter sequence spanning the region –1644 to –1280 in LB334 showed a small, but significant, increase in reporter gene expression in response to hypoxia. This suggests the presence of a hypoxia-sensitive sequence within this region that is either suppressed or absent in the other constructs tested thus far. Since the Bcl-2 CRE site is located within the region –1644 to –1280, it is possible that this element is responsible for the low-level response to hypoxia ($P = 0.008$).

Fig. 4.2 – The Effects of Hypoxia on various Bcl-2 Promoter Constructs

Activity of various Bcl-2 promoter constructs when transfected into PC12 cells. Cells were either left unstimulated, or subjected to four hours of hypoxia treatment followed by a 24-hour re-oxygenation period. Promoter activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

why not relative to control

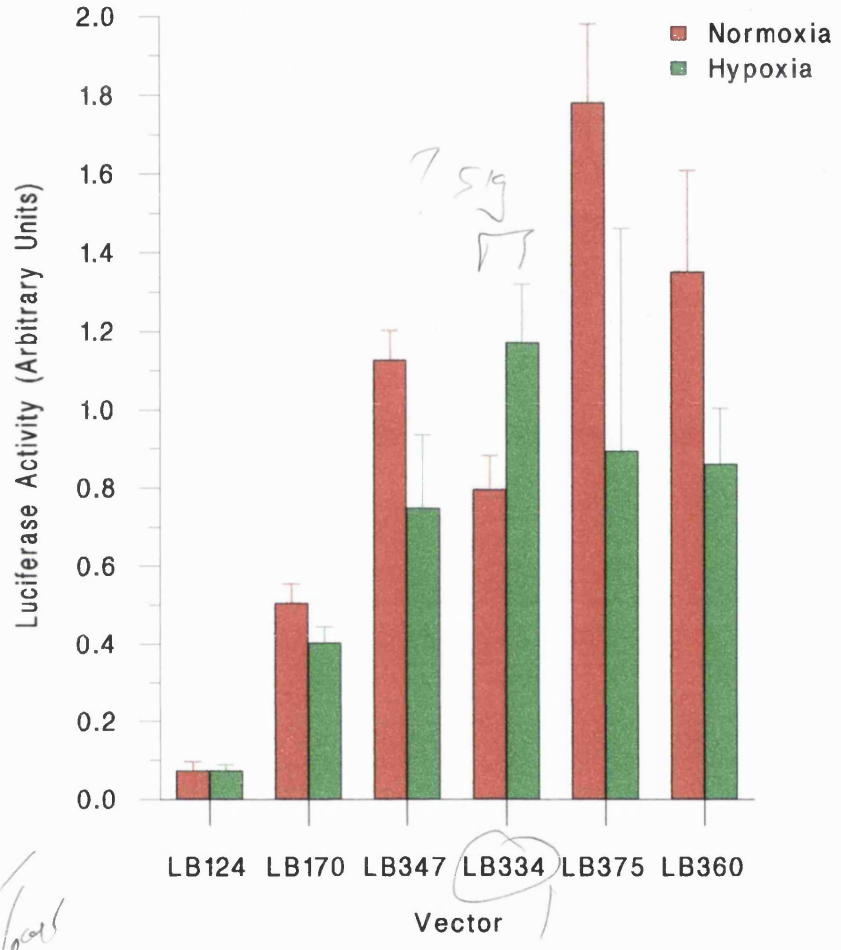


time courses of hypoxia } ? done
recovery

Fig. 4.3 – The Effects of Hypoxia on the Region –3934 to –1280 of the Isolated Bcl-2 P1 Promoter

Activity of various Bcl-2 promoter constructs when transfected into PC12 cells. Cells were either left unstimulated, or subjected to four hours of hypoxia treatment followed by a 24-hour re-oxygenation period. Promoter activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of four independent experiments and the error bars show the standard deviation.


Normoxia
Hypoxia
Luciferase Activity (Arbitrary Units)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LB124
LB170
LB347
LB334
LB375
LB360
Vector

To analyse the region –2337 to –1280 in finer detail, PC12 cells were transfected with plasmids containing progressive 5' and 3' truncations within this region (see figure 4.4). In the case of LB135 (–1795 to –1280) and LB351 (–1746 to –1280) it was observed that neither promoter construct is sensitive to hypoxia when luciferase activity of treated cells is compared with that of untreated cells ($P = 0.844$ and $P = 0.724$ respectively), whereas transfections with LB334 showed a two-fold increase in luciferase expression in response to hypoxia ($P = 0.008$). Since the constructs LB135 and LB351 both contain the hypoxia-responsive element identified in the promoter region –1644 to –1280 of LB334, this suggests that the hypoxia-sensitivity of these constructs is suppressed, possibly by the presence of an inhibitory element located upstream of –1644.

The constructs LB367 and LB374 were also tested for their responsiveness to hypoxia (figure 4.4). These plasmids contain Bcl-2 regulatory sequences between –1644 and –1332 linked to a heterologous promoter. The Bcl-2 regulatory element found in LB374 (–1644 to –1522) is able to confer hypoxia-responsiveness to the heterologous promoter ($P = 0.0002$); therefore this region is likely to be responsible for the response to hypoxia observed with LB334. LB367 (–1526 to –1337) is inhibited following hypoxia ($P = 0.011$), suggesting that the hypoxia-responsive element is not present in this construct. Interestingly, LB374 contains the Bcl-2 CRE site whereas LB367 does not, confirming that the CRE site may be responsible for any response to hypoxia observed.

In summary, although the full-length Bcl-2 promoter is not activated by hypoxia in PC12 cells, these data strongly suggest that a 122bp region of the Bcl-2 promoter containing the CRE site is indeed hypoxia-responsive.

4.2.2 The Role of the CRE Site in Bcl-2 Promoter Activation

To investigate the role of the CRE site in hypoxia-induced activation of LB334, PC12 cells were transfected with either LB334 or LB595 prior to hypoxia treatment. LB595 carries a mutation at the CRE site, but is otherwise identical to LB334. As shown in figure 4.5, in hypoxia treated cells the mutation of the CRE site in LB595 causes a significant reduction in promoter activity when compared the intact CRE in LB334 ($P = 0.002$). LB334 promoter activity significantly increased following hypoxia when compared to basal levels of expression ($P = 0.001$), but no such rise was observed in the case of LB595 ($P = 0.981$). This confirms that the CRE site is essential for the response to hypoxia observed with LB334.

Fig. 4.4 – The Effects of Hypoxia on the Region –1795 to –1280 of the Bcl-2 Promoter

Activity of various Bcl-2 promoter constructs when transfected into PC12 cells. Cells were either left unstimulated, or subjected to four hours of hypoxia treatment followed by a 24-hour re-oxygenation period. Promoter activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

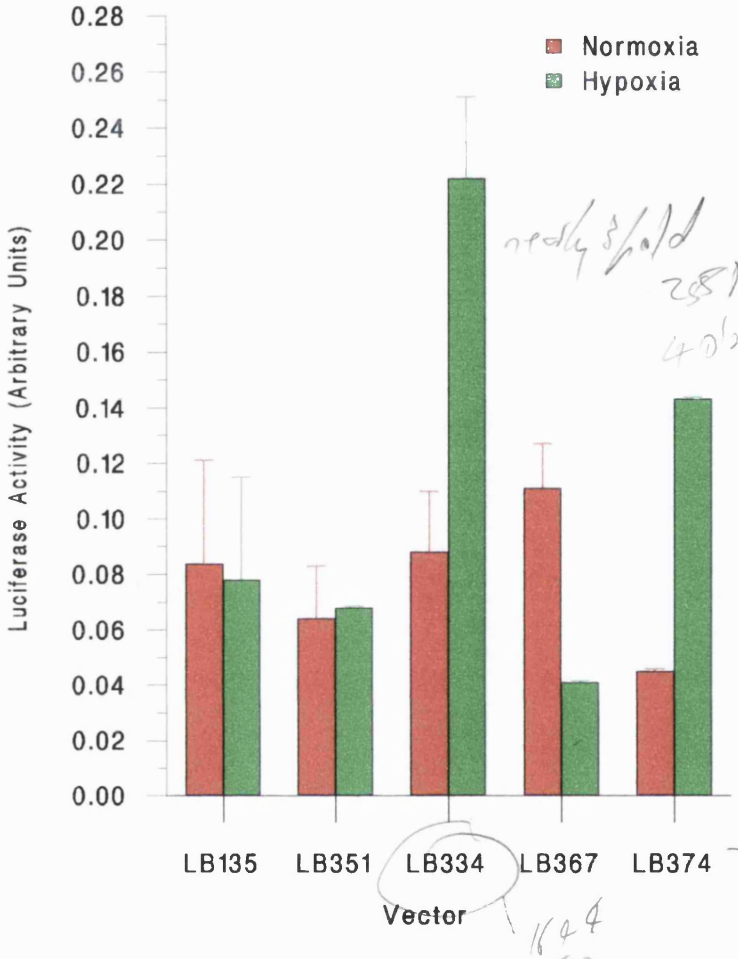
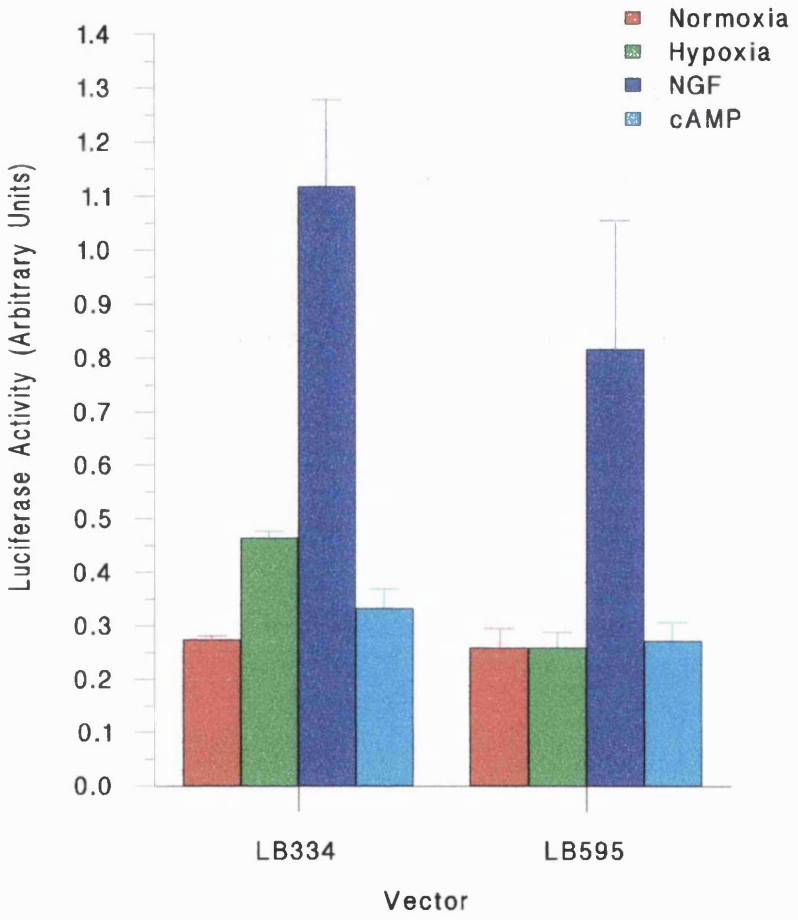

Normoxia
Hypoxia
Luciferase Activity (Arbitrary Units)
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
LB135
LB351
LB334
LB367
LB374
Vector

Fig. 4.5 – The Effects of a CRE Mutation on the Activation of LB334 in Response to Hypoxia, NGF and cAMP

Promoter activity of the Bcl-2 promoter constructs LB334 and LB595 when transfected into PC12 cells. In both cases, the cells were either left unstimulated, subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period, treated with NGF, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.



N=3
yet SD have
so much wobbles

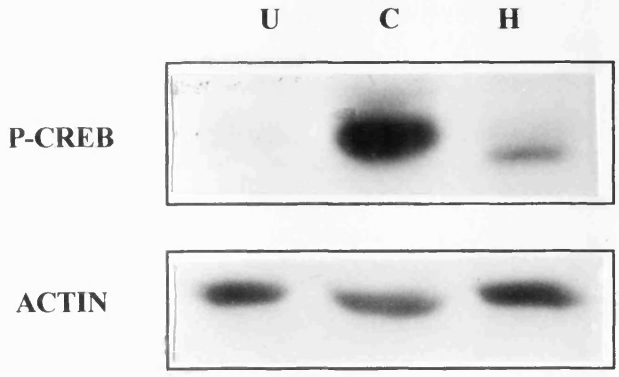
As described in section 1.9.3, the Bcl-2 promoter is thought to be insensitive to cAMP despite the presence of a CRE site within its sequence (Liu, unpublished data). The above experiments have indicated that, although the full-length Bcl-2 promoter is insensitive to hypoxia, the Bcl-2 CRE site within LB334 is able to mediate promoter activation in response to hypoxia. In view of this, the sensitivity of LB334 and LB595 to cAMP was tested. As shown in figure 4.5, no significant difference in either LB334 or LB595 promoter activity in the presence or absence of the stimulus was observed ($P = 0.106$ and $P = 0.663$), indicating that the hypoxia-sensitive sequence –1644 to –1280 is not sensitive to cAMP despite the presence of a CRE site.

In parallel experiments the effects of NGF upon LB334 and LB595 were also tested since conflicting data exists regarding the sensitivity of the CRE to NGF; recent data has shown that the NGF-mediated regulation of Bcl-2 requires the CRE site (Riccio *et al*, 1999), whilst a previous report showed that the Bcl-2 CRE is not involved in the NGF response (Liu *et al*, 1999). In the case of both LB334 and LB595 there is a significant up-regulation of promoter activity in response to NGF treatment when compared to basal activity ($P = 0.012$ and $P = 0.049$ respectively), confirming that the Bcl-2 promoter fragment –1644 to –1280 is inducible by NGF even in the absence of an intact CRE site (see figure 4.5). Mutation of the CRE site in LB595 did appear to partially inhibit the response to NGF seen with the intact CRE in LB334; however the difference between the NGF-mediated activation of LB334 and the NGF-mediated activation of LB595 was not significant ($P = 0.150$), suggesting that the CRE site is not necessary for the NGF response. Thus the Bcl-2 CRE is able to mediate the LB334 response to hypoxia, but cannot mediate a response to NGF or cAMP.

It is known that the hypoxia-induced activation of the tyrosine hydroxylase promoter is CRE-dependent and that this is accompanied by the rapid and persistent phosphorylation of the transcription factor CREB (Beitner-Johnson and Millhorn, 1998). Having shown that hypoxia-sensitivity of LB334 is also dependent upon the CRE, it is probable that CREB is involved in transcriptional activation of this construct following hypoxia. To investigate the potential role of CREB in hypoxia-induced gene expression, hypoxia-treated cells were tested for the presence of phosphorylated CREB. PC12 cells treated with cAMP, a known inducer of CREB phosphorylation, were also tested for comparison. Western blot analysis of PC12 cell extract using an antibody specific for CREB phosphorylated at serine 133 (see figure 4.6), revealed that untreated PC12 cells contain undetectable levels of phosphorylated CREB. Hypoxia-treated cells, as well as those treated with cAMP, have detectable levels of phosphorylated CREB. Therefore it is possible that phosphorylated CREB mediates at least

Fig. 4.6 – The Effects of Hypoxia and cAMP on the Phosphorylation of CREB in PC12 Cells

Western blot analysis of the level of CREB phosphorylation in PC12 cell extracts using the anti-phospho CREB (serine 133) antibody. PC12 cells were either left unstimulated (U), treated with cAMP (C), or subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period (H). The results of immunoblotting with the anti-actin antibody are also shown to indicate the level of protein within each sample.


U
C
H
P-CREB
ACTIN

some of the transcriptional effects of oxygen deprivation in these cells. The high level of phosphorylated CREB in cAMP-treated cells confirms that cAMP is a strong mediator of CREB phosphorylation. In contrast, the level of phosphorylated CREB is relatively low in hypoxia-treated cells suggesting that hypoxia induces CREB phosphorylation more weakly. This could explain the low level of CRE-dependent LB334 promoter activation observed in the response to hypoxia.

To investigate the role of CREB in hypoxia-induced Bcl-2 promoter activation, further experiments were carried out in PC12 cells in which LB334 was co-transfected into cells with either KCREB (Walton *et al*, 1992; Yang *et al*, 1996) or ACREB (Ahn *et al*, 1998) expression vectors encoding dominant negative forms of CREB. PC12 cells transfected with either ACREB or KCREB (see figure 4.7) exhibited significantly reduced hypoxia-induced activation of the LB334 promoter sequence when compared to cells treated with hypoxia alone ($P = 0.008$ and $P = 0.014$ respectively). As expected, the inhibitory effects of KCREB are lower than those of ACREB, since KCREB is a much weaker inhibitor of CREB than ACREB.

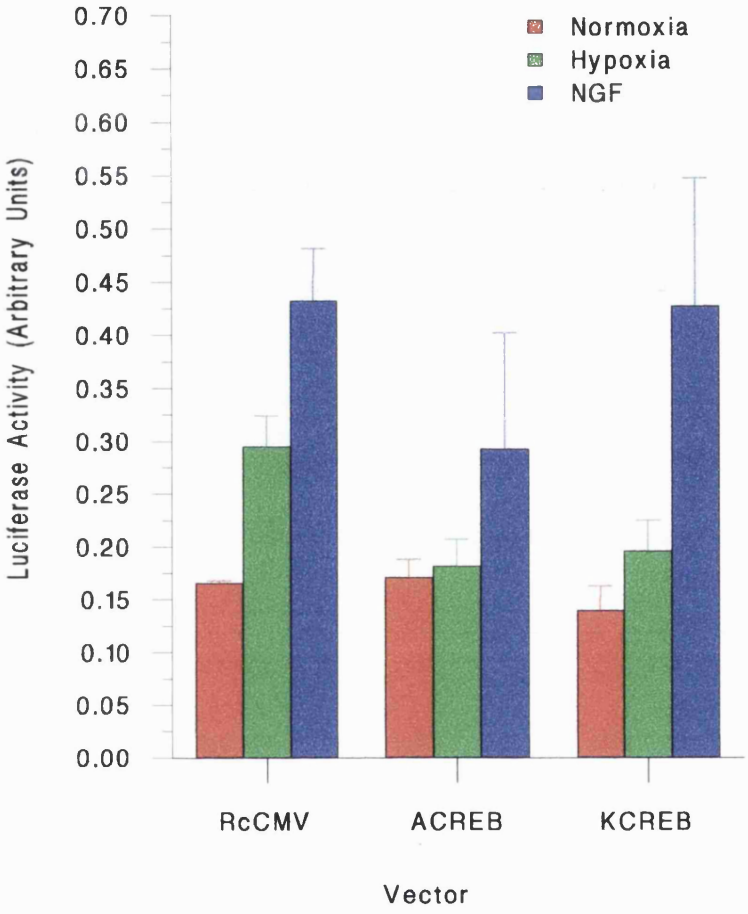
The role of CREB in NGF-mediated Bcl-2 promoter activation was also tested. The presence of ACREB did appear to partially reduce NGF-stimulated promoter activation, however this effect was not statistically significant ($P = 0.143$). KCREB had no effect on NGF-mediated promoter activation when compared to NGF-mediated promoter activation in those cells transfected with the RcCMV empty vector ($P = 0.955$).

Having confirmed that CREB transcription factor is phosphorylated following hypoxia and that it mediates the hypoxia-induced activation of the Bcl-2 promoter in LB334, the effects of hypoxia upon the CREB co-activator CREB-binding protein (CBP) were investigated. This protein will only bind to phosphorylated CREB (Parker *et al*, 1996) and acts to stimulate transcription. However, as stated previously CBP can also modulate transcription when recruited to DNA by a heterologous DNA-binding domain and stimulated by NGF (Lui *et al*, 1998). Using this to advantage, PC12 cells were transfected with two plasmids; one encoding the C-terminal transcription activation domain of CBP linked to the Gal4 DNA-binding domain and the second carrying five Gal4 DNA-binding sites linked to a luciferase gene (5xGal4-E1B-Luciferase). Control experiments using the Gal4 DNA-binding domain expression vector lacking CBP (RpxG4) were also carried out. When translated the chimeric Gal4/CBP protein is able to bind to the Gal4 DNA-binding sites of the second plasmid and activate transcription of the luciferase gene. As shown in figure 4.8, activity of the CBP C-terminal domain is trebled by NGF stimulation when compared to basal CBP activity ($P =$

Fig. 4.7 – The Effects of Dominant Negative CREB on the Activation of the Bcl-2 Promoter in Response to Hypoxia and NGF

Activity of the Bcl-2 promoter in the plasmid LB334 when co-transfected into PC12 cells with either the empty expression vector RcRSV, a vector encoding the dominant negative ACREB, or a vector encoding the dominant negative mutant killer CREB (KCREB). In all cases, the cells were either left unstimulated, subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period, or treated with NGF. Promoter activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

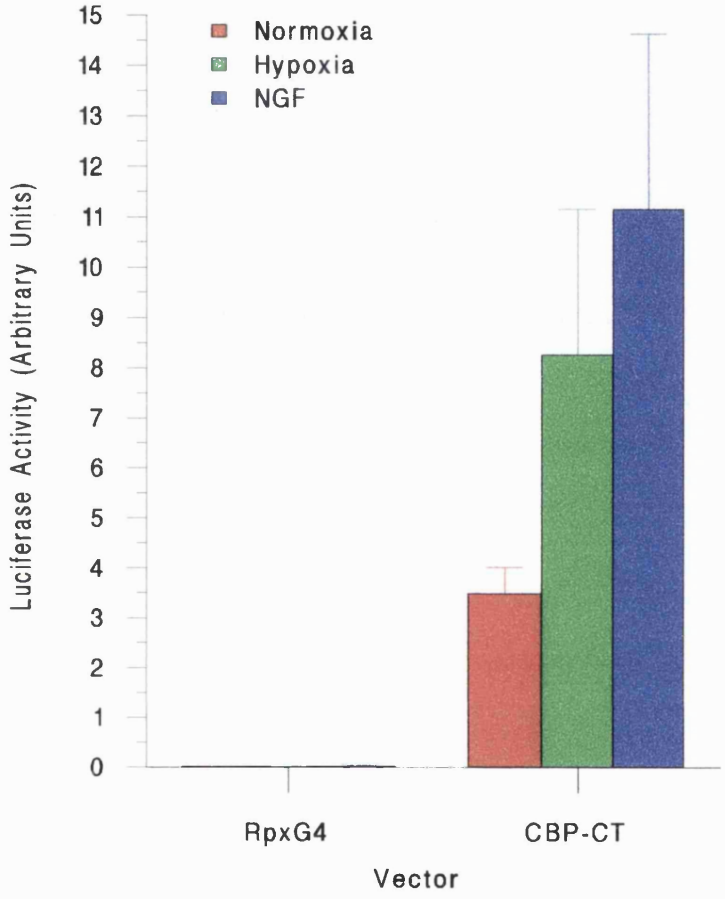


NGF - completely creb + crp independent!!

Fig. 4.8 – The Effects of Hypoxia and NGF on the Activation of CBP

CBP activity in PC12 cells co-transfected with the plasmid Gal4-E1B-Luciferase and either the Gal4 DNA-binding domain expression plasmid (RpxG4), or the expression plasmid encoding the CBP C-terminal domain linked to the Gal4 DNA-binding domain. In both cases, the cells were either left unstimulated, subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period, or treated with NGF. CBP activity was determined by assays for the luciferase reporter gene.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


Normoxia
Hypoxia
NGF
Luciferase Activity (Arbitrary Units)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
RpxG4
CBP-CT
Vector

0.020), as expected (Liu *et al*, 1998). Interestingly, CBP activity is also more than doubled following hypoxia treatment ($P = 0.043$), suggesting that the CBP co-activator is probably activated by hypoxia in PC12 cells.

4.3 Discussion

Extended periods of oxygen depletion induce programmed cell death, which can be suppressed by the expression of the protein Bcl-2 (Jacobson and Raff, 1995; Bossenmeyer-Pourie and Daval, 1998). Several control elements within the Bcl-2 promoter have been identified, which can mediate its response to a number of different stimuli in B cells (Young and Korsmeyer, 1993; Chen and Boxer, 1995; Wilson *et al*, 1996), ND7 cells (Smith *et al*, 1998a; Smith *et al*, 1998b) and in PC12 cells (Liu *et al*, 1999; Riccio *et al*, 1999), activating Bcl-2 expression and protecting those cells from apoptosis. The data described here have shown that, whilst the region –1644 to –1280 of the isolated Bcl-2 P1 promoter can be induced by hypoxia in PC12 cells, neither P2 nor the full-length P1 promoter is responsive to hypoxia in these cells. This suggests that other anti-apoptotic proteins, such as Bcl-xl, are likely to be involved in PC12 cell rescue following hypoxia-induced apoptosis rather than Bcl-2. PC12 cells cannot, therefore, be used as system for studying the effects of hypoxia on Bcl-2 transcription, nor should they be used to investigate the protective role of Bcl-2 in rescue from apoptosis following hypoxia.

No region of the Bcl-2 promoter other than sequences located between –1644 to –1280 is able to confer hypoxia-responsiveness to a heterologous promoter and induce activation of transcription. Indeed, certain sequences upstream of –1644 appear to repress hypoxia-induced gene expression in PC12 cells since neither the full-length Bcl-2 promoter nor the sequence spanning –1746 to –1280 are responsive to hypoxia, despite the presence of a hypoxia-responsive element.

The region of the Bcl-2 promoter responsive to hypoxia in LB334 contains a non-consensus CRE site (Wilson *et al*, 1996; Liu *et al*, 1999; Riccio *et al*, 1999). I have shown that the CRE-binding protein CREB is phosphorylated in PC12 cells that have been subjected to four hours of hypoxia, and that CREB is able to mediate the activation of the LB344 promoter during a subsequent period of re-oxygenation. Further transfection experiments have confirmed that the CRE site is essential for the hypoxia-induced activation of LB334, since a mutation at this site completely inhibited the response. This correlates with previous findings that CREB and the CRE site within the tyrosine hydroxylase promoter are required for the up-regulation of TH expression in PC12 cells that have been subjected to low oxygen levels (Beitner-Johnson and Millhorn, 1998).

As stated previously, the co-activator CBP interacts with phosphorylated CREB and is able to activate transcription of CREB-regulated genes. Both CREB and CBP can be activated by NGF via the same signalling pathway (Liu *et al*, 1998). My observations indicate that the transcriptional activity of CREB and CBP is also increased in PC12 cells that have been subjected to hypoxia. Although not conclusive evidence, this suggests a potential role for CBP in mediating the effects of hypoxia in PC12 cells, and possibly even a role in the CRE-dependent activation of the LB334 promoter. It would be interesting to investigate the effects of CBP inhibition on hypoxia-induced LB334 and tyrosine hydroxylase promoter activation.

In addition to studying the hypoxia-responsiveness of the Bcl-2 CRE, the effects of NGF and cAMP on the Bcl-2 CRE have been re-examined. In previous studies it was observed that the Bcl-2 promoter is not sensitive to cAMP (Liu, unpublished data), despite the presence of a CRE site. The results shown here have confirmed that the short promoter sequence in LB334 containing the CRE site is not sensitive to cAMP. In contrast, the role of the CRE site in NGF-mediated Bcl-2 transcription has not been fully elucidated. CREB is known to be an important mediator of NGF-dependent gene expression, yet one report suggests that the CRE site is not involved in NGF-mediated Bcl-2 expression; in transfection studies a mutation in the CRE site had no effect upon the activity of the promoter in response to NGF. Rather, a second site located within the region –1472 to –1414 is thought to be responsible (Liu *et al*, 1999). My own data was obtained using constructs similar to those in this study and confirms that the CRE is not required for NGF-mediated transcription.

However, more recent conflicting data suggests that the CRE site is in fact essential for NGF-induced transcription from the Bcl-2 promoter. Here, a different mutation in the CRE site strongly down-regulated the promoter in the presence of NGF. The reason behind such conflicting data is unclear, although it is unlikely that the nature of the mutation in the CRE site is the cause, since neither mutant is capable of binding CREB (Wilson *et al*, 1996; Riccio *et al*, 1999). The predominant difference between the two studies is the length of time cells were exposed to NGF. In the initial study, as well as in the experiments described in the previous section, cells were stimulated with NGF for a period of at least 27 hours, whereas in the later study cells were exposed to NGF for just six hours. The sustained NGF-mediated activation of the Bcl-2 promoter requires the GTP-binding protein Rap1 and the p42/p44 MAP kinase signalling pathway, which act on an as yet unidentified protein that binds to a regulatory element within the region –1472 to –1414 of the Bcl-2 promoter (Liu *et al*, 1999). As described in section 1.4.8, it is believed that Rap is involved in maintaining sustained NGF signalling, whilst Ras is responsible for the initial NGF response (York *et al*, 1998). It is therefore possible that the initial activation of the Bcl-2 promoter in PC12 cells is partially

dependent upon CREB (and possibly the second, unidentified factor), yet sustained NGF-mediated activation of the promoter is dependent upon the second transcription factor alone, perhaps due to differences in the signalling pathways involved. It would be interesting to carry out a time-course experiment, monitoring both the CREB-dependence of NGF-induced Bcl-2 promoter activation and the roles played by Ras and Rap signalling pathways.

5 A Comparison of the Bcl-2, CT/CGRP and Tyrosine Hydroxylase Promoter CRE Sites

5.1 Introduction

5.1.1 CRE-mediated Regulation of Gene Expression

Promoter studies on cAMP-responsive genes, such as those encoding somatostatin and c-fos, have led to the identification of several promoter elements that modulate gene transcription in response to elevated levels of intracellular cAMP, the best characterised of which is the cAMP response element, or CRE (Andrisani *et al*, 1987; Ziff, 1990; Lalli and Sassone-Corsi, 1994; Sassone-Corsi, 1995). The numerous CRE sites that have been identified often vary slightly in sequence, but the consensus CRE, found in the promoters of genes such as tyrosine hydroxylase and somatostatin, is the 8bp palindrome 5'-TGACGTCA-3'. The CRE site is recognised by the DNA-binding domains of CREB/ATF family transcription factors, many of which are regulated by cAMP. As discussed in section 1.6, CRE-bound CREB activates transcription when phosphorylated at serine 133, an event mediated by protein kinase A (PKA) in response to increases in intracellular cAMP (Gonzalez and Montminy, 1989). However, some CRE-binding factors, such as ATF-1, are only weakly sensitive to cAMP and others, such as ATF-2, are totally insensitive to changes in intracellular cAMP (Flint and Jones, 1991). ATF-1 is regulated by p38 MAP kinase and ATF-2 is regulated by c-Jun N-terminal kinase (JNK). CREB regulation is also not limited to phosphorylation by changes in cAMP levels. Both p38 and p42/p44 MAP kinases are able to phosphorylate CREB at serine 133 (Xing *et al*, 1996; Froedin and Gammeltoft, 1999). Therefore, although the CRE site was identified because of its sensitivity to cAMP, the CRE site is in fact sensitive to the many different stimuli that activate CREB/ATF family transcription factors. Thus the CRE-mediated sensitivity of any promoter is dependent upon the nature of the transcription factor bound to the CRE, and the signals to which that transcription factor responds.

As stated above, the tyrosine hydroxylase (TH) promoter has a consensus CRE site, which is located at –38 to –45 in the rat gene. In PC12 cells both the CREB activating transcription factor and the CREM repressor are able to bind to the TH CRE (Tinti *et al*, 1996; Piech-Dumas and Tank, 1999). The CRE site is required for basal levels of transcription and is

sensitive to cAMP (Fung *et al*, 1992; Kim *et al*, 1993; Lazaroff *et al*, 1995). Furthermore the CRE site is also sensitive to hypoxia by an unknown signalling pathway (Beitner-Johnson and Millhorn, 1998). Finally, the TH promoter is sensitive to NGF, an activator of MAP kinase signalling. The role of the CRE, if any, in this effect has not been established. However, it is known that NGF-mediated TH transcription activation is dependent upon an AP1 site and c-Fos/c-Jun heterodimers rather than CREB in certain tissues (Gizang-Ginsberg and Ziff, 1990; Ghee *et al*, 1998).

The CT/CGRP promoter has a CRE site located at –103 to –109 with the sequence 5'-TGACGCA-3' (Watson and Latchman, 1995). This site is sensitive to both NGF and cAMP in PC12 cells (Watson and Latchman, 1995) and, as described in Chapter 3, both responses require the CREB transcription factor. The CT/CGRP promoter has not yet been tested for its sensitivity to hypoxia, therefore it would be interesting to discover if the CT/CGRP promoter is inducible by hypoxia and, if so, whether the CRE site mediates that hypoxia sensitivity.

The Bcl-2 gene promoter also has a CRE site located at –1546 to –1537 with the sequence 5'-TGACGTTA-3' (Wilson *et al*, 1996). As described in Chapter 4, the Bcl-2 CRE appears to be insensitive to hypoxia in the context of the full-length promoter, and only weakly sensitive to hypoxia in the 364bp sequence of LB334 in PC12 cells. This response to hypoxia requires CREB. Recent data has also described a role for the CRE site in short-term NGF-mediated Bcl-2 promoter activation (Riccio *et al*, 1999) although my own and other data (Liu *et al*, 1999) suggest that the CRE site is only partially, if at all, involved in a sustained response to NGF. Finally, the Bcl-2 CRE is insensitive to cAMP.

Thus CREB regulates transcription mediated by the TH, CT/CGRP and Bcl-2 CRE sites in PC12 cells, yet these sites do not respond to the same stimuli. The effects of NGF, cAMP and hypoxia on the TH, CT/CGRP and Bcl-2 promoters and their respective CRE sites in PC12 cells are summarised in the table below ('+' represents a positive response, 'X' represents no response and '?' represents response unknown).

Gene	**Stimulus Acting on Full-length Promoter**			**Stimulus Acting on CRE Element**		
	NGF	**cAMP**	**Hypoxia**	**NGF**	**cAMP**	**Hypoxia**
TH	+	+	+	?	+	+
CT/CGRP	+	+	?	+	+	?
Bcl-2	+	X	X	?	?	+

The differences in CRE-sensitivity observed could be explained by the CRE sites themselves; all three CRE sites share the sequence TGACG, but vary at their 3' sequence. They may, therefore, bind to different CREB homo- or heterodimers with different affinities. The sequence directly flanking the CRE site in each promoter may also influence CREB-binding, or the response to it, in some way. Thirdly, the position of the CRE site within the promoter may affect the ability of CREB to activate transcription. Usually the CRE site is located within 200bp of the transcription initiation site (Delmas *et al*, 1994), as in the case of TH and CT/CGRP. In the case of Bcl-2, the CRE is located upstream of the P2 promoter, over 1500bp away from the start site, which may affect the ability of CREB to activate transcription in the context of the full-length promoter. Finally, other regulatory elements, or even CRE-binding repressors, may inhibit CRE-mediated activation in certain promoters. The mechanisms behind the differential CRE-dependent regulation of Bcl-2, TH and CT/CGRP transcription in response to NGF, cAMP and hypoxia would be better understood if the sensitivity of the three different CRE sites to these stimuli was investigated when isolated from their respective promoter sequences, i.e., when linked to a heterologous promoter.

5.2 Results

5.2.1 The Effects of Hypoxia on Calgcat 1

To determine whether the CT/CGRP promoter is sensitive to hypoxia, PC12 cells were transfected with the Calgcat 1 plasmid described in Chapter 3. The following day test cells were either left untreated, subjected to four hours of hypoxia followed by a 24 hour re-oxygenation period, or stimulated with NGF (see figure 5.1). As expected, NGF approximately doubled basal levels of CAT expression ($P = 0.028$). However, hypoxia did not increase CAT expression beyond basal levels ($P = 0.876$), suggesting that the CT/CGRP promoter is not hypoxia sensitive. Since the Bcl-2 CRE, but not the Bcl-2 promoter, is sensitive to hypoxia, the effects of hypoxia upon the isolated CT/CGRP CRE site was investigated. Similar studies using the CRE sites from the TH and Bcl-2 promoters were also carried out. In addition, since the CT/CGRP CRE is sensitive to NGF, but the role of the CRE site in the NGF-mediated activation of Bcl-2 is unclear, the effects of NGF upon all three CRE sites when linked to a heterologous promoter were also tested.

5.2.2 The Effects of NGF and Hypoxia upon the CRE Sites of the TH, Bcl-2 and CT/CGRP Promoters when Linked to a Heterologous Promoter

To test the inducibility of the CT/CGRP CRE site when linked to a heterologous promoter, an oligonucleotide containing the CT/CGRP CRE was obtained and cloned into the BamHI site of the pBLCAT2 expression vector multiple cloning region. A single clone containing one copy of the CRE site in the correct orientation was then selected and amplified. Similarly, oligonucleotides containing the Bcl-2 and TH CRE sites were also generated, cloned into pBLCAT2 and clones carrying a single copy of each CRE were selected. For the purposes of this study these clones have been designated CGRPCrecat, Bcl-2Crecat and THCrecat. PC12 cells were then transfected with either pBLCAT2, CGRPCrecat, Bcl-2Crecat or THCrecat and were either left untreated, subjected to 4 hours of hypoxia followed by 24 hours of re-oxygenation, or stimulated with NGF. The CAT activity of the PC12 cell extract was used as a measure of promoter activity.

As shown in figure 5.2, neither NGF nor hypoxia significantly increases pBLCAT2 basal levels of CAT expression ($P = 0.757$ and $P = 0.755$ respectively), indicating that the intrinsic

Fig. 5.1 – The Effects of Hypoxia on the CGRP Promoter

Activation of the CT/CGRP promoter in the plasmid Calgcat 1 when transfected into PC12 cells that were either left unstimulated, subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period, or treated with NGF. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.

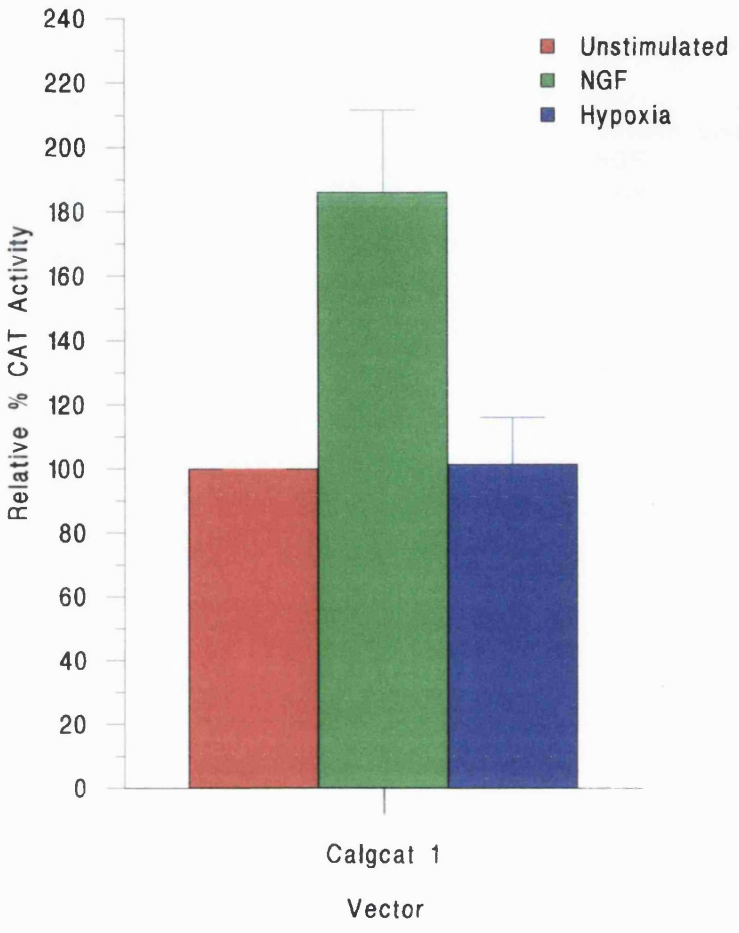
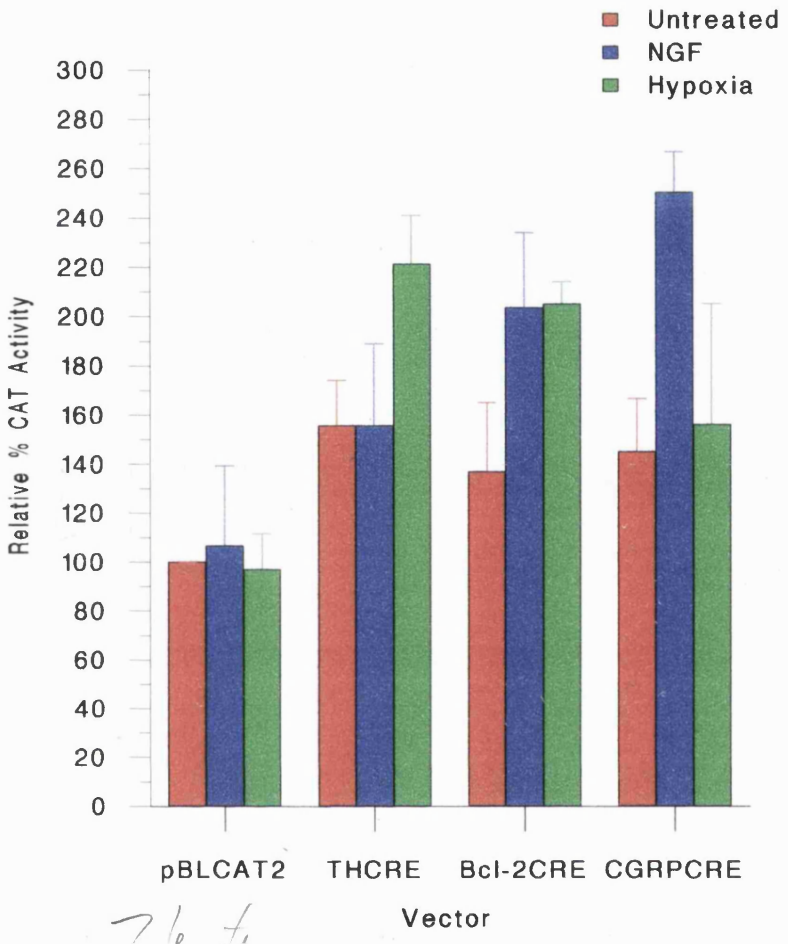

240
220
200
180
160
140
120
100
80
60
40
20
0
Relative % CAT Activity
Unstimulated
NGF
Hypoxia
Calgcat 1
Vector

Fig. 5.2 – The Effects of Hypoxia and NGF on the TH, Bcl-2 and CT/CGRP CRE Sites when Linked to a Heterologous Promoter

Promoter activity of the constructs pBLCAT2, CGRPCrecat, Bcl-2Crecat and THCrecat when transfected into PC12 cells. In all cases, the cells were either left unstimulated, subjected to four hours of hypoxia followed by a 24-hour re-oxygenation period or treated with NGF. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the pBLCAT2 vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.



? empty
no a
mutated or
other oligo of
same length

promoter activity is not sensitive to these stimuli. Interestingly, the presence of the TH CRE in pBLCAT2 significantly increased basal levels of CAT expression by 156% ($P = 0.035$), which correlates with the fact that the CRE site is essential for basal TH promoter activity in PC12 cells (Kim *et al*, 1993). Cells that were transfected with THCrecat and subjected to hypoxia showed elevated levels of CAT expression when compared to the basal CAT activity in unstimulated cells ($P = 0.013$), suggesting that the isolated TH CRE is sensitive to hypoxia when linked to a heterologous promoter. This is not surprising since the TH CRE is sensitive to hypoxia even in the context of the TH promoter (Beitner-Johnson and Millhorn, 1998). Interestingly, NGF-stimulated THCrecat promoter activity was identical to basal THCrecat activity, indicating that the TH CRE is not sensitive to NGF. This is consistent with the idea that AP1 rather than CREB is responsible for the NGF-mediated activation of the TH gene.

The presence of the Bcl-2 CRE in pBLCAT2 did not significantly affect basal levels of CAT expression ($P = 0.149$). However, as expected, in cells transfected with Bcl-2Crecat a significant increase in CAT expression was observed in hypoxia-stimulated cells when compared to unstimulated cells ($P = 0.042$). The results also suggest that the Bcl-2 CRE is similarly up-regulated by the presence of NGF ($P = 0.049$). The sensitivity of the Bcl-2 CRE to NGF could explain the partial (though statistically insignificant) decrease in the NGF-inducibility of LB334 when the CRE is mutated (see figure 4.5) or in the presence of ACREB (see figure 4.7).

The insertion of the CT/CGRP CRE into pBLCAT2 does not significantly increase basal CAT activity ($P = 0.067$), and hypoxia also has no effect ($P = 0.755$). However, the CT/CGRP CRE does confer NGF-responsiveness to the pBLCAT2 promoter ($P = 0.003$). In fact, the increase in expression from CGRPCrecat caused by the CT/CGRP CRE in response to NGF is of a similar level to that observed in Calgcat 1 in response to NGF (see figure 3.1).

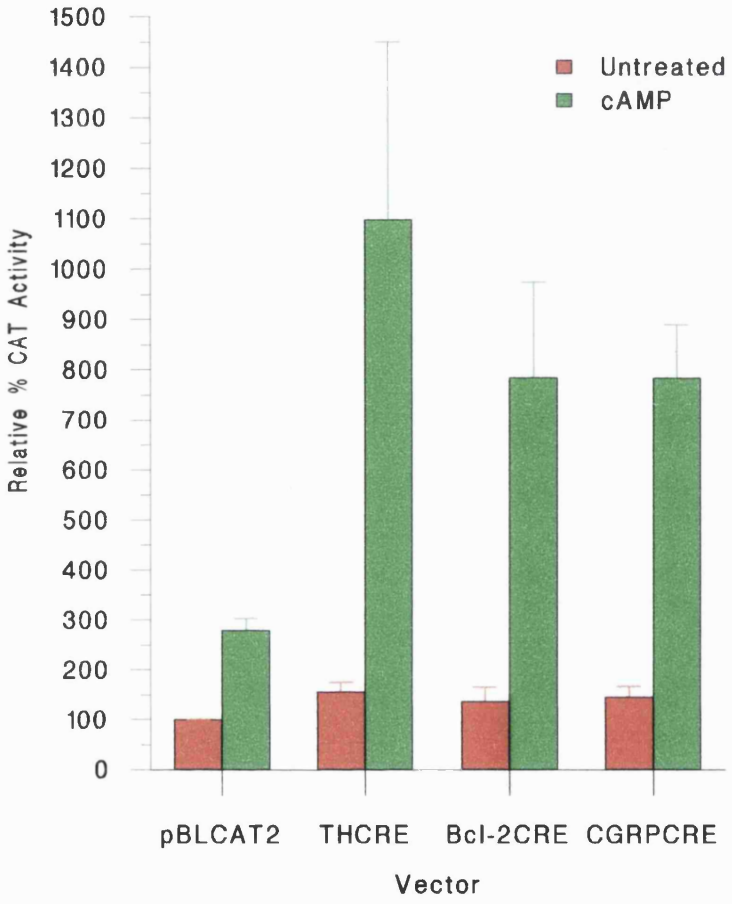
5.2.3 The Effects of cAMP upon the CRE Sites of the TH, Bcl-2 and CT/CGRP Promoters when Linked to a Heterologous Promoter

In further transfection experiments, pBLCAT2, THCrecat, Bcl-2Crecat and CT/CGRPCrecat were tested for their cAMP inducibility. As displayed in figure 5.3, the promoter activity in pBLCAT2 increases two fold in the presence of cAMP, possibly due to the presence of a non-CRE cAMP-responsive site ($P = 0.006$). When the TH CRE is inserted into pBLCAT2,

Fig. 5.3 – The Effects of cAMP on the TH, Bcl-2 and CT/CGRP CRE Sites when Linked to a Heterologous Promoter

Promoter activity of the constructs pBLCAT2, CGRPCrecat, Bcl-2Crecat and THCrecat when transfected into PC12 cells. In all cases, the cells were either left unstimulated, or treated with dibutyryl cAMP. Promoter activity was determined by assays for the CAT reporter gene and is expressed as percentages of CAT activity relative to the basal activity observed in unstimulated cells transfected with the pBLCAT2 vector.

The results represent the mean values of three independent experiments and the error bars show the standard deviation.


1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Relative % CAT Activity
Untreated
cAMP
pBLCAT2
THCRE
Bcl-2CRE
CGRPCRE
Vector

cAMP-induced promoter activity is increased nearly seven fold ($P = 0.043$), a far greater increase than could be explained by the intrinsic pBLCAT2 cAMP response. This suggests that the TH CRE is strongly inducible by cAMP when linked to the heterologous promoter. This is expected since the TH promoter is inducible by cAMP in a CRE-dependent manner (Piech-Dumas and Tank, 1999).

The Bcl-2 CRE and CT/CGRP CRE are also responsive to cAMP when linked to a heterologous promoter. CAT activity in PC12 cells transfected with either Bcl-2Crecat or CGRPCrecat increases nearly six fold over basal levels in the presence of cAMP ($P = 0.025$ and $P = 0.007$ respectively). Surprisingly this indicates that the Bcl-2 CRE is sensitive to cAMP, despite the fact that neither the full-length Bcl-2 promoter nor the shorter LB334 are inducible by cAMP in PC12 cells. In the case of the CT/CGRP CRE, the increase in promoter activity induced by cAMP is similar to the increase observed in the cAMP-induced CRE-dependent activation of the CGRP promoter in Calgcat 1 (see figure 3.1).

In summary, all three CRE sites are inducible by cAMP when linked to a heterologous promoter. However, only the TH and Bcl-2 CREs are responsive to hypoxia, and only the CT/CGRP and Bcl-2 CREs are sensitive to NGF. To find a potential explanation for this, the protein binding capabilities of the three different CRE oligonucleotides were tested in an electrophoretic mobility shift assay (EMSA) using PC12 cell extracts. It is possible that the oligonucleotides may interact with other proteins in addition to CREB, or indeed with different isoforms of CREB, in order to confer such differing responses.

5.2.4 The Protein-binding Capabilities of the TH, Bcl-2 and CT/CGRP CRE Sites

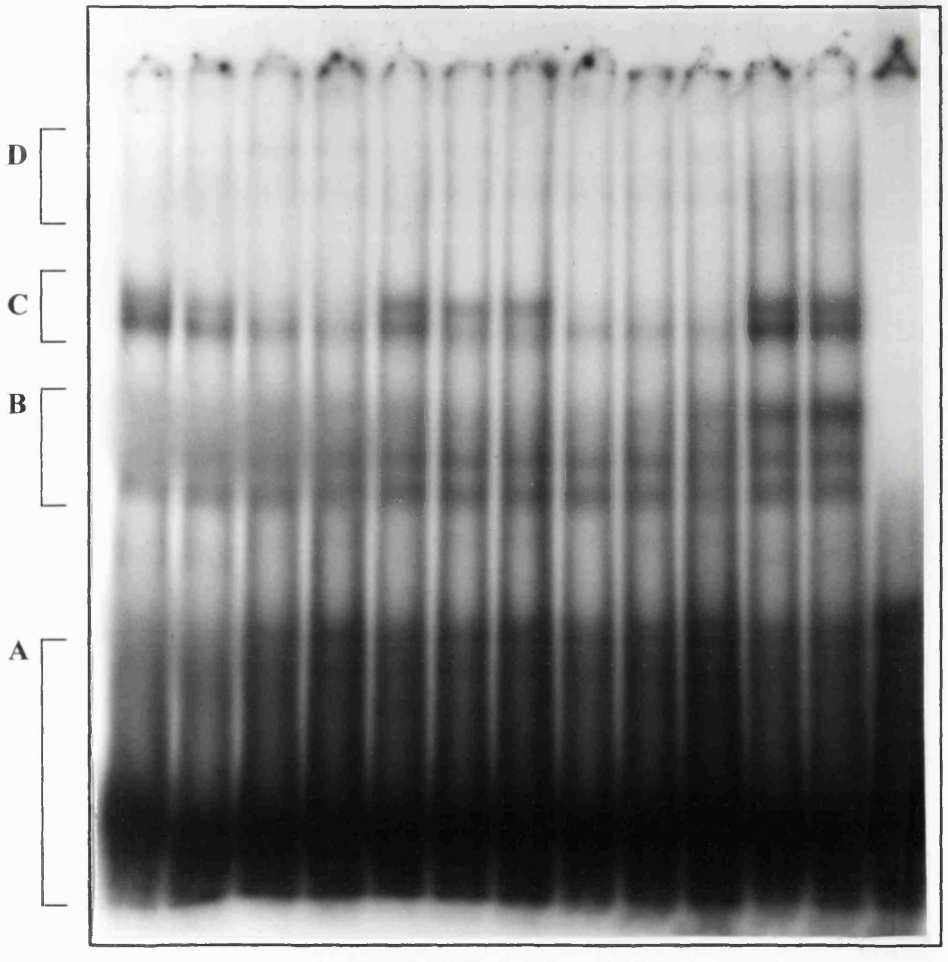
For each CRE oligonucleotide, two different electrophoretic mobility shift assays were carried out; the first to determine whether the three different CREs could compete with each other for the same cellular proteins, and the second to identify the protein(s) bound. Figure 5.4, lane 1, shows the effects of incubating a radiolabelled TH CRE oligonucleotide probe with PC12 cell extract. Lane 13 contains radiolabelled probe alone, with no protein present; the lower band, A, can therefore be identified as free probe. The bands in lane 1 above this region represent the different proteins that have interacted with, and consequently retarded, the oligonucleotide probe. These include a triplet, B, and a doublet, C, as well as some less distinct bands in region D. The presence of increasing excess amounts of unlabelled Bcl-2 CRE oligonucleotide competitor in samples 2-4 does not affect the intensity of the triplet B. This

Fig. 5.4 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive TH CRE Oligonucleotide Probe

A representative gel of an EMSA using a [^{32}P] end-labelled TH CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of increasing concentrations of unlabelled specific (CRE) or non-specific (SP1 site) competitor oligonucleotides as described below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 10x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 3: probe + cell extract + 50x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 5: probe + cell extract + 10x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 6: probe + cell extract + 50x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 7: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 8: probe + cell extract + 10x excess of unlabelled TH CRE oligonucleotide
Lane 9: probe + cell extract + 50x excess of unlabelled TH CRE oligonucleotide
Lane 10: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 11: probe + cell extract + 50x excess of unlabelled SP1 site oligonucleotide
Lane 12: probe + cell extract + 100x excess of unlabelled SP1 site oligonucleotide
Lane 13: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.


Bcl2
CLNP
TH
SPI
1 2 3 4 5 6 7 8 9 10 11 12 13
D
C
B
A

suggests that the Bcl-2 CRE is unable to compete with the labelled TH CRE for these proteins. In fact, excess amounts of unlabelled CT/CGRP CRE (lanes 5-7), TH CRE (lanes 8-10) and an oligonucleotide containing an SP1 site (lanes 11 and 12) are also unable to compete for these proteins. The inability of the unlabelled TH CRE to compete for the proteins suggests that these bands represent non-specific DNA-binding proteins, rather than proteins specific for the CRE site itself.

The unlabelled TH CRE oligonucleotide is, however, able to effectively compete for the upper band and, albeit less effectively, for the lower protein band in doublet C. In addition, the SP1 site oligonucleotide cannot compete for this protein or proteins, suggesting that this protein (or proteins) bind specifically to the TH CRE oligonucleotide and are not general DNA-binding proteins. The presence of unlabelled Bcl-2 CRE oligonucleotide also reduces the intensity of the upper band more than the lower band in doublet C, but it is generally a less effective competitor than the TH CRE. Interestingly, the CT/CGRP CRE is the least effective competitor for the upper protein band, but competes for the lower band to a similar degree as the Bcl-2 CRE. This suggests that the protein/DNA complex of greater mobility binds to all three CRE sites, but has a higher affinity for the TH CRE. The more retarded protein/DNA complex binds to the Bcl-2 and TH CRE sites with a much higher affinity than for the CT/CGRP CRE site. Thus clear differences in the protein binding capabilities of the three CRE sites are detectable.

Figure 5.5 shows the effects of incubating various antibodies with PC12 cell extract and the radiolabelled TH CRE oligonucleotide. Although the upper band of the doublet C is less clear and intense here owing to poor separation, the four bands in region D are more distinguishable than in figure 5.4. Excess amounts of unlabelled TH, Bcl-2 and CT/CGRP CRE oligonucleotides (lanes 2,3 and 4), but not the SP1 oligonucleotide (lane 5), are able to effectively compete for these proteins, suggesting that these are also CRE-specific. The presence of an anti-ATF-1 antibody in the sample (lane 6) does not cause a shift in any of the CRE-specific protein bands. This suggests that ATF-1 does not bind to the TH CRE in PC12 cells. However, the presence of anti-CREB antibody in the sample (lane 7) does cause a shift in the proteins of region D, and forms a single band, E. A control anti-Gal4 antibody was also used to show that the effects of the test antibodies were specific (lane 8). No shift was observed in the presence of this antibody. This indicates that CREB binds to the TH CRE site in PC12 cells. The CREB antibody was unable to shift the protein/DNA complexes in doublet C, however, suggesting that this CRE-specific protein is not recognised by the antibody.

Fig. 5.5 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive TH CRE Oligonucleotide Probe in the Presence of Antibodies

A representative gel of an EMSA using a [^{32}P] end-labelled TH CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of unlabelled specific (CRE) or non-specific (SP1 site) competitor oligonucleotides, or in the presence of antibodies against ATF-1, CREB or the yeast protein Gal4, as described below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 3: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 5: probe + cell extract + 100x excess of unlabelled SP1 site oligonucleotide
Lane 6: probe + cell extract + anti-ATF-1
Lane 7: probe + cell extract + anti-CREB
Lane 8: probe + cell extract + anti-Gal4
Lane 9: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.

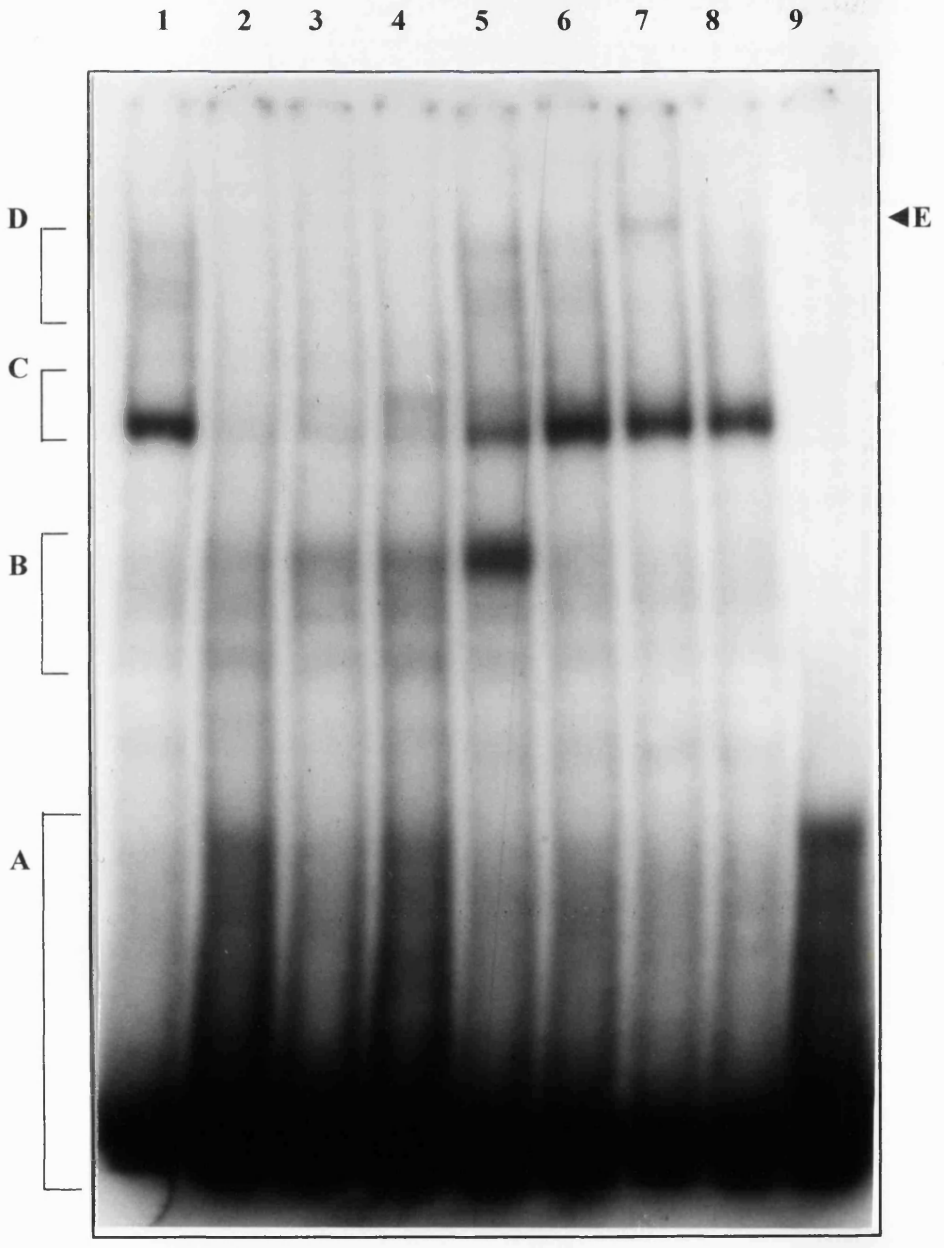

1
2
3
4
5
6
7
8
9
D
C
B
A
◀E

Fig. 5.6 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive Bcl-2 CRE Oligonucleotide Probe

A representative gel of an EMSA using a [^{32}P] end-labelled Bcl-2 CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of increasing concentrations of unlabelled competitor oligonucleotides as specified below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 10x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 3: probe + cell extract + 50x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 5: probe + cell extract + 10x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 6: probe + cell extract + 50x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 7: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 8: probe + cell extract + 10x excess of unlabelled TH CRE oligonucleotide
Lane 9: probe + cell extract + 50x excess of unlabelled TH CRE oligonucleotide
Lane 10: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 11: probe + cell extract + 50x excess of unlabelled SP1 oligonucleotide
Lane 12: probe + cell extract + 100x excess of unlabelled SP1 oligonucleotide
Lane 13: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.

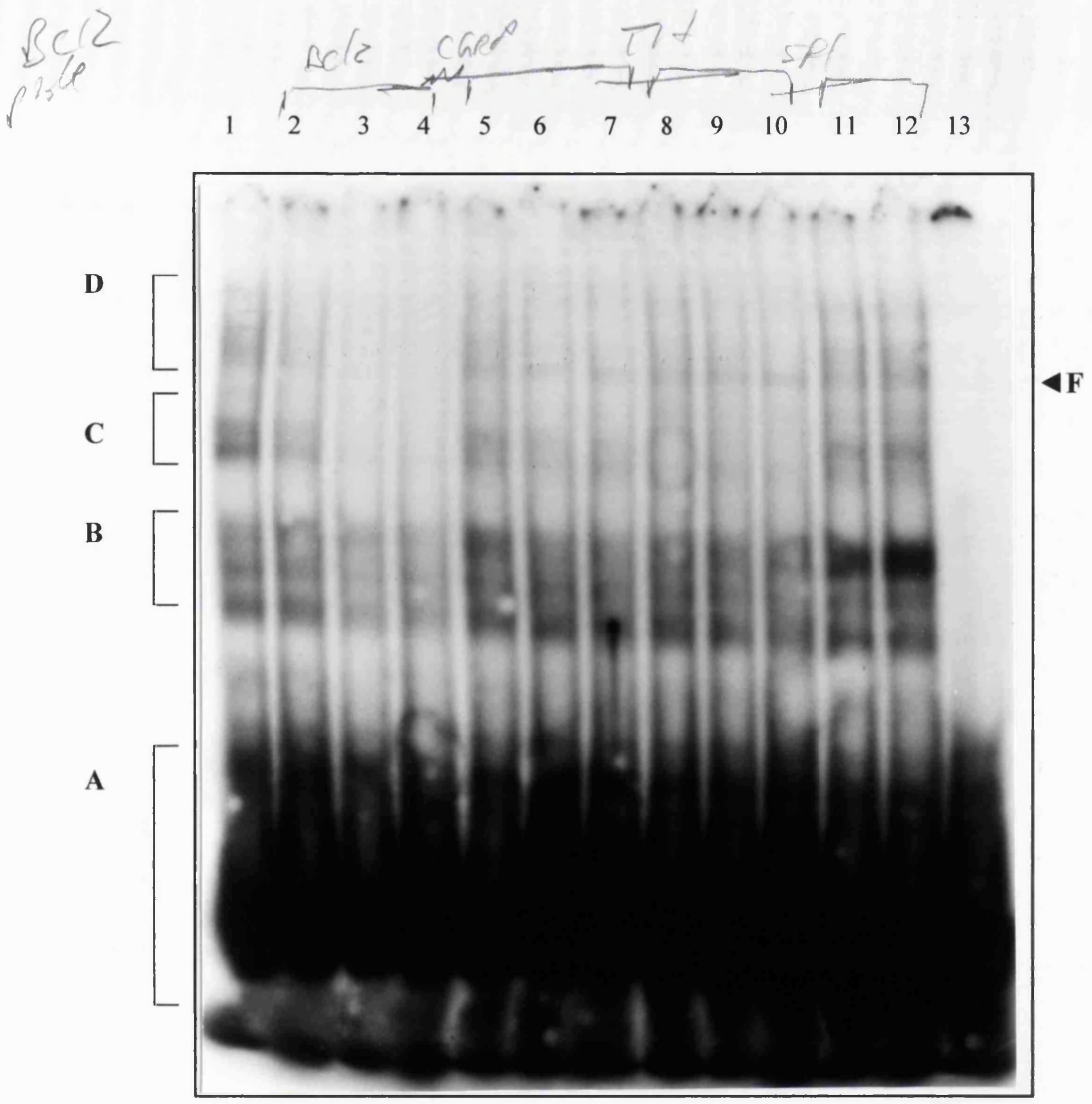

1 2 3 4 5 6 7 8 9 10 11 12 13
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Figure 5.6 shows the effects of incubating a radiolabelled Bcl-2 CRE oligonucleotide with PC12 cell extract. As in figure 5.4, region A represents free oligonucleotide probe. The Bcl-2 CRE probe has a similar protein-binding pattern to that of the TH CRE probe; a lower triplet, B, consists of non-specific DNA-binding proteins that are not competed for by an excess of unlabelled oligonucleotides (lanes 2-12). The CRE-binding protein bands of doublet C are less intense than for the TH CRE, possibly due to a lower affinity for the proteins. The Bcl-2 and, perhaps more so, the TH CRE unlabelled oligonucleotides successfully compete the bands away (lanes 2-4 and 8-10 respectively), but the CT/CGRP CRE oligonucleotide is a less effective competitor for the proteins (lanes 5-7). The SP1 oligonucleotide is not a competitor for these proteins. These observations again suggest that the protein bands in doublet C bind specifically to the Bcl-2 and TH CRE sites and with a lower affinity to the CT/CGRP CRE site. Of the four bands in region D, all are competed out in the presence of excess unlabelled Bcl-2 CRE oligonucleotide (lanes 2-4), but not in the presence of SP1 oligonucleotide (lanes 11 and 12), suggesting that these too are Bcl-2 CRE-specific proteins. All but band F can also be competed away by the other CRE oligonucleotides. It seems, therefore, that band F is specific to the Bcl-2 CRE, whilst the other bands in region D bind to all three CRE sites.

Figure 5.7 shows the effects of incubating anti-Gal4, ATF-1 and CREB antibodies with the radiolabelled Bcl-2 oligonucleotide and PC12 cell extract. Again the gel clearly shows that the doublet, C, is competed away by the presence of excess unlabelled Bcl-2 and TH CRE sites (lanes 2 and 3), whilst the CT/CGRP CRE site is a less effective competitor, particularly for the upper band (lane 4). The antibodies against ATF-1 and Gal4 have no effect on oligonucleotide mobility (lanes 6 and 8 respectively); however, the anti-CREB antibody causes a shift and creates a single band E (lane 7). All of the bands in region D are retarded by their interaction with the antibody except for the band F, indicating that these proteins consist, at least in part, of CREB monomers.

The PC12 protein-binding pattern of a radiolabelled CT/CGRP CRE oligonucleotide is very different to that of the TH and Bcl-2 CRE sites. As shown in figure 5.8, with the CT/CGRP CRE there is no lower triplet or doublet of bands, but rather three distinct bands designated G, H and I. The lower band G is too faint to be analysed, but band H appears to be a non-specific DNA-binding protein that is not affected by the presence of any excess unlabelled oligonucleotide (lanes 2-12). All three unlabelled CRE oligonucleotides (lanes 2-10), but not the SP1 oligonucleotide (lanes 11 and 12), are able to compete for band I, although the CT/CGRP CRE is a more effective competitor than the Bcl-2 and TH CRE sites. This suggests that protein I has a higher affinity for the CT/CGRP CRE than the other two CRE

Fig. 5.7 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive Bcl-2 CRE Oligonucleotide Probe in the Presence of Antibodies

A representative gel of an EMSA using a [^{32}P] end-labelled Bcl-2 CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of unlabelled competitor oligonucleotides, or in the presence of antibodies against ATF-1, CREB or the yeast protein Gal4, as described below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 3: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 5: probe + cell extract + 100x excess of unlabelled SP1 site oligonucleotide
Lane 6: probe + cell extract + anti-ATF-1
Lane 7: probe + cell extract + anti-CREB
Lane 8: probe + cell extract + anti-Gal4
Lane 9: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.

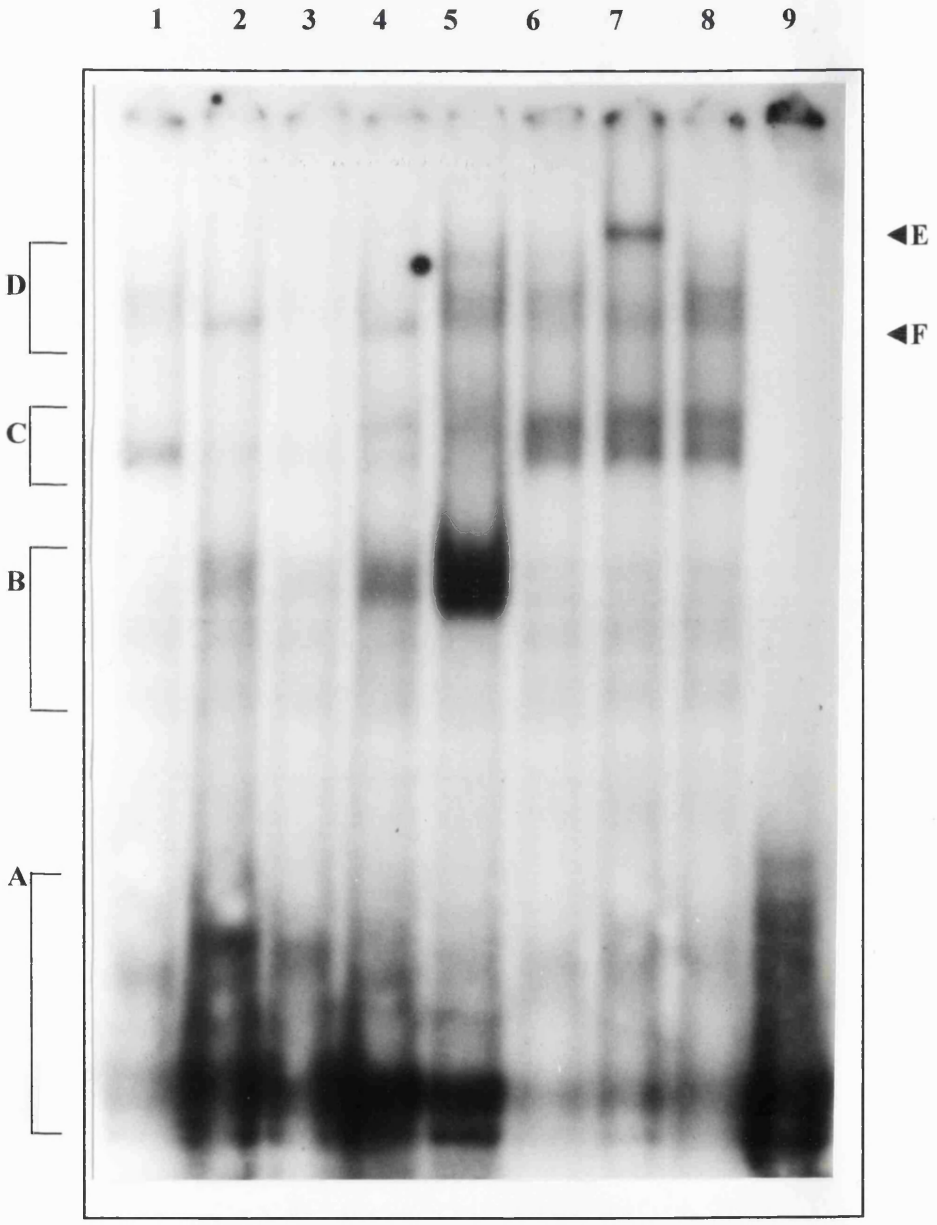
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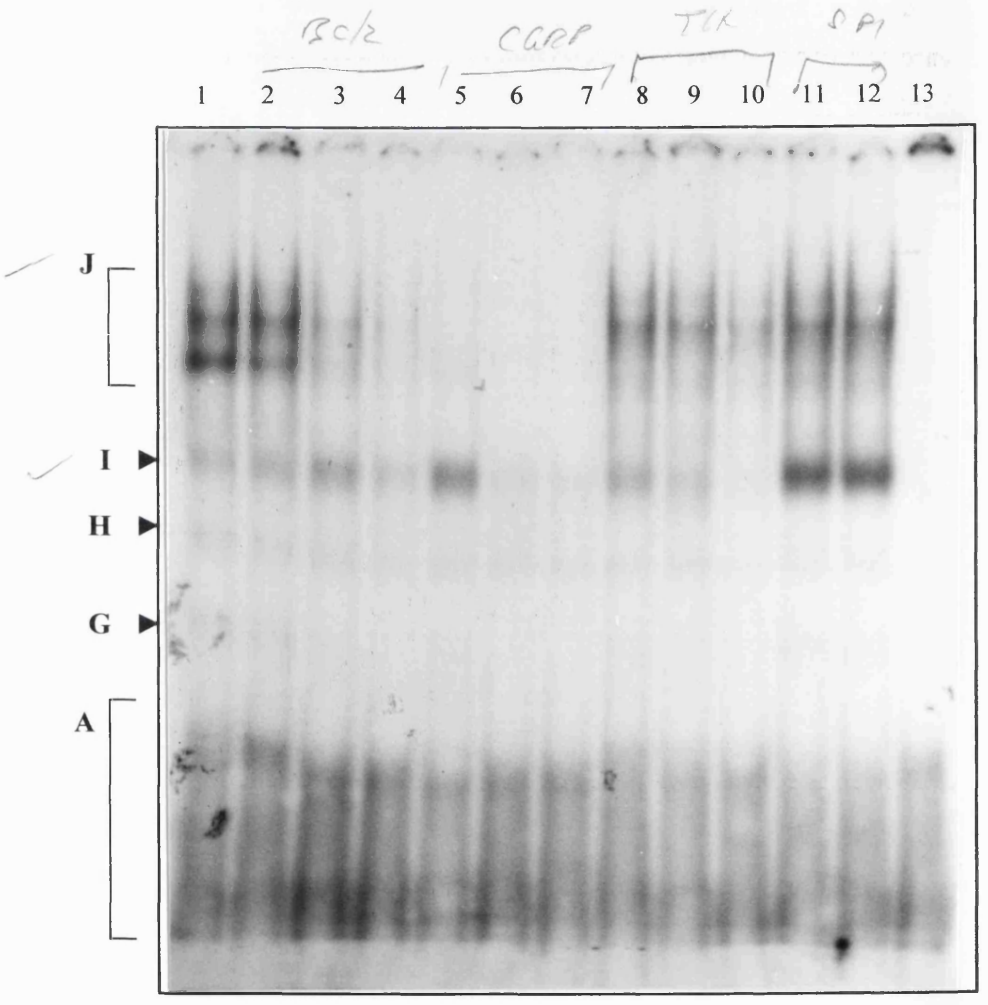
Fig. 5.8 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive CT/CGRP CRE Oligonucleotide Probe

A representative gel of an EMSA using a [^{32}P] end-labelled CT/CGRP CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of increasing concentrations of unlabelled competitor oligonucleotides as specified below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 10x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 3: probe + cell extract + 50x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 5: probe + cell extract + 10x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 6: probe + cell extract + 50x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 7: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 8: probe + cell extract + 10x excess of unlabelled TH CRE oligonucleotide
Lane 9: probe + cell extract + 50x excess of unlabelled TH CRE oligonucleotide
Lane 10: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 11: probe + cell extract + 50x excess of unlabelled SP1 oligonucleotide
Lane 12: probe + cell extract + 100x excess of unlabelled SP1 oligonucleotide
Lane 13: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.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sites. In addition to these bands, the CT/CGRP CRE also retards a group of four proteins (region J) in and identical pattern to those in region D of figures 5.4 and 5.6. However these proteins have a much higher affinity for the CT/CGRP CRE, and to a lesser extent the Bcl-2 CRE, than for the consensus TH CRE. The gel shows that the unlabelled TH oligonucleotide (lanes 8-10) competes for the proteins in region J much less effectively than either excess Bcl-2 CRE or CT/CGRP CRE oligonucleotides (lanes 2-4 and 5-7 respectively).

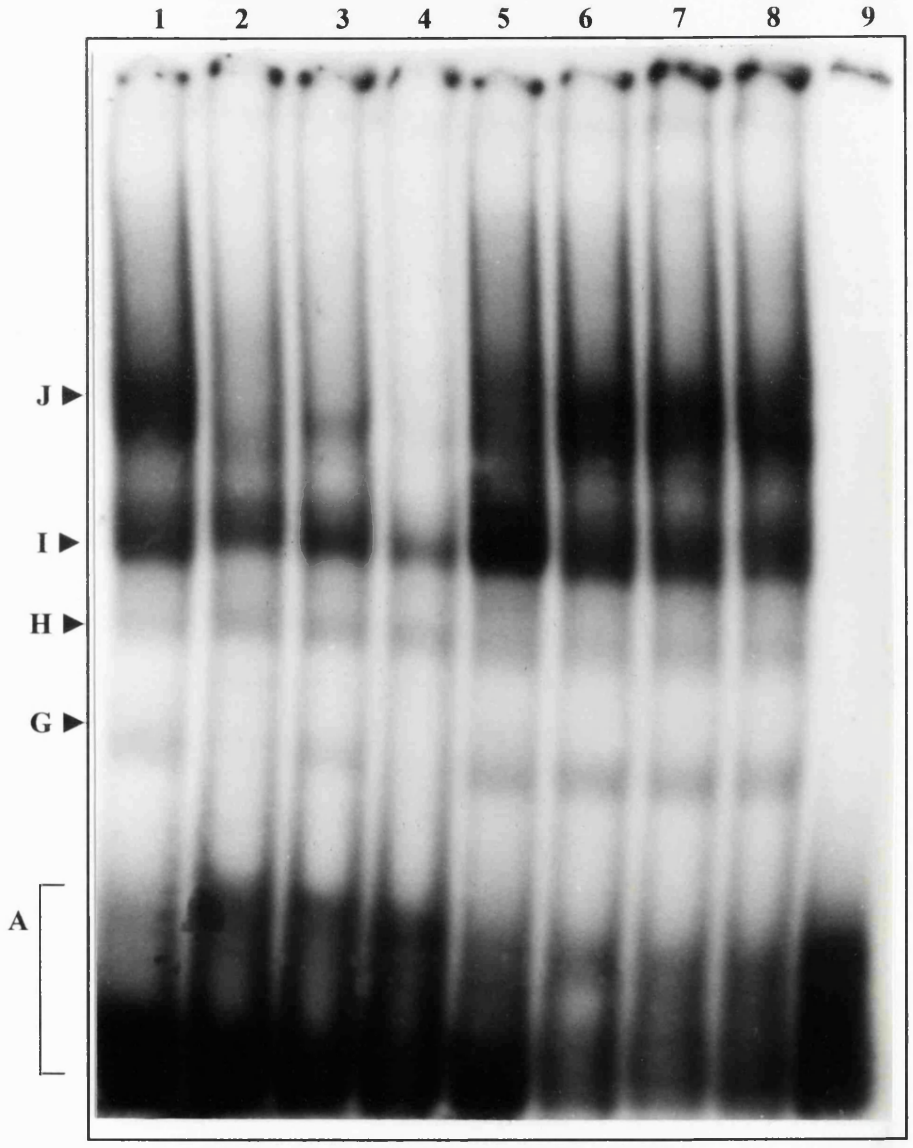
Figure 5.9 shows the effects of adding anti-Gal4, ATF-1 and CREB to a radiolabelled CT/CGRP CRE probe and PC12 cell extract. Although the bands are more diffuse, they are still clearly distinguishable. This gel-shift indicates that the band G described in figure 5.8 is in fact a CRE-specific band that is competed away in the presence of excess unlabelled TH and CT/CGRP oligonucleotide (lanes 3 and 5). The gel also shows that none of the antibodies tested cause a shift in the protein bands. As described in chapter 3, CRE-mediated CT/CGRP promoter activation can be reduced by the action of mutant proteins ACREB and KCREB on cellular CREB-related proteins. The former quenches cellular CREB and ATF-1, and the latter is able to quench cellular CREB, ATF-2 and c-Jun. This suggests that CREB or a closely related protein mediates CRE-dependent CT/CGRP promoter activation in PC12 cells, yet the protein-binding pattern of CT/CGRP CRE is considerably different to that of the consensus TH CRE. It appears that the CT/CGRP CRE sequence binds to an isoform of CREB, or a CREB-related protein, that is not recognised by the anti-CREB antibody used.

Fig. 5.9 – An Electrophoretic Mobility Shift Assay of PC12 Cell Extract using a Radioactive CT/CGRP CRE Oligonucleotide Probe in the Presence of Antibodies

A representative gel of an EMSA using a [^{32}P] end-labelled CT/CGRP CRE oligonucleotide probe incubated with PC12 cell extract. Incubations were carried out either in the absence or presence of unlabelled specific (CRE) or non-specific (SP1 site) competitor oligonucleotides, or in the presence of antibodies against ATF-1, CREB or the yeast protein Gal4, as described below.

Lane 1: probe + cell extract
Lane 2: probe + cell extract + 100x excess of unlabelled TH CRE oligonucleotide
Lane 3: probe + cell extract + 100x excess of unlabelled Bcl-2 CRE oligonucleotide
Lane 4: probe + cell extract + 100x excess of unlabelled CT/CGRP CRE oligonucleotide
Lane 5: probe + cell extract + 100x excess of unlabelled SP1 site oligonucleotide
Lane 6: probe + cell extract + anti-ATF-1
Lane 7: probe + cell extract + anti-CREB
Lane 8: probe + cell extract + anti-Gal4
Lane 9: Free probe

Radiolabelled probe was detected by exposure to autoradiographic film.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5.3 Discussion

The CRE-binding protein CREB has been implicated in mediating the activation of gene expression in response to many different stimuli. However, whilst many genes contain CRE sites, they will not necessarily respond to the same stimuli, even within the same cell type.

The summary table found at the beginning of this chapter describing the effects of NGF, cAMP and hypoxia on the TH, CT/CGRP and Bcl-2 promoters and their respective CRE sites in PC12 cells can now be completed:

Gene	Stimulus Acting on Full-length Promoter			Stimulus Acting on CRE Element		
	NGF	cAMP	Hypoxia	NGF	cAMP	Hypoxia
TH	+	+	+	X	+	+
CT/CGRP	+	+	X	+	+	X
Bcl-2	+	X	X	+	+	+

Novel data described in this chapter is indicated in blue.

The mechanisms behind the differential CRE-dependent regulation of the many different CRE-containing genes are unclear. However the results shown here indicate that, in the case of TH, Bcl-2 and CT/CGRP promoter activation in PC12 cells, certain differences between the promoter sensitivities lie within the CRE sequences themselves, whilst others are dependent upon other factors.

Firstly, neither the Bcl-2 CRE nor the CT/CGRP CRE affect basal transcription when linked to a heterologous promoter, yet the consensus TH CRE is able to increase the basal activity of a heterologous promoter. This is consistent with reports that the CRE site contributes to basal TH activity in PC12 cells (Kim *et al*, 1993; Lazaroff *et al*, 1995), but there is no evidence to suggest that either Bcl-2 or CT/CGRP CRE sites contribute to basal promoter activity in PC12 cells. It is likely that the sequence of the CRE site itself is responsible for this difference, rather than any other factor, since the isolated Bcl-2 and CT/CGRP CRE sites are not capable of conferring an increase in basal activity to the heterologous promoter.

In contrast, the isolated TH CRE is not sensitive to NGF, whereas the CT/CGRP and Bcl-2 sites are weakly responsive to NGF. These observations are consistent with previous reports using intact promoters; the NGF-responsiveness of the TH promoter has been attributed to the AP1 site rather than the CRE site (Ghee *et al*, 1998). In the context of the CT/CGRP promoter, the CT/CGRP CRE is sensitive to NGF (Watson and Latchman, 1995), though interestingly, the isolated CT/CGRP CRE is not sensitive to NGF in DRG neurons, indicating that this effect is cell-type specific. The sensitivity of the Bcl-2 CRE to NGF is consistent with the finding that the CRE site within the isolated Bcl-2 P1 promoter is capable of responding to NGF in PC12 cells (Riccio *et al*, 1999). It could also explain the partial (though statistically insignificant) decrease in the NGF-inducibility of LB334 when the CRE site is mutated or CREB is inhibited (figures 4.5 and 4.7 respectively). However, there is no evidence to suggest that the full-length Bcl-2 promoter is responsive to NGF in a CRE-dependent manner.

The differences in sequence of the three CRE sites also appear to affect their ability to respond to hypoxia treatment. Neither the CT/CGRP promoter nor the CT/CGRP CRE are responsive to hypoxia, yet both the Bcl-2 and TH CRE sites are able to confer hypoxia-responsiveness to a heterologous promoter. This latter observation is unsurprising since both the Bcl-2 promoter in LB334 (see chapter 3) and the TH promoter (Beitner-Johnson and Millhorn, 1998) are also responsive to hypoxia in a CRE-dependent manner. The sequence differences in the three CRE sites do not affect their ability to respond to cAMP; despite the fact that the Bcl-2 promoter insensitive to cAMP (see chapter 4), the Bcl-2 CRE is able to confer cAMP-responsiveness to a heterologous promoter, as are the TH and CT/CGRP CREs.

The differences in promoter sensitivity caused by the CRE sequences themselves may in part be due to the proteins that bind to these sites. Gel shift assays have shown that the three different CRE sites each have their own distinct pattern of protein binding. The binding patterns of the TH and Bcl-2 CRE oligonucleotides are relatively similar, but the CT/CGRP CRE binding pattern is very different. This could explain why the Bcl-2 and TH CREs are able to confer a response to hypoxia to a heterologous promoter, whereas the CT/CGRP CRE cannot. Although most of these proteins have not been identified, it is likely that many of them are CREB homo- or heterodimers. The data does suggest, however, that the CT/CGRP CRE site interacts with an isoform of CREB, or a related protein, not recognised by the TH and Bcl-2 CRE sites. In addition, although the protein-binding patterns of the Bcl-2 and TH CRE are very similar, their affinities for those proteins are often different. Previous studies have shown that the affinity of CREB for its binding site affects the level of CREB activity, and that symmetric CRE sites have a higher affinity for CREB than asymmetric sites (Lee and

Masson, 1993). This is consistent with the observation here, that the palindromic TH CRE has a higher affinity for the oligonucleotide-bound proteins than the asymmetric Bcl-2 CRE, and therefore could contribute to the differences in sensitivity of these two CRE elements.

However, the differences in TH, Bcl-2 and CT/CGRP promoter sensitivity are not limited to the CRE site. As stated previously, the consensus TH CRE, the Bcl-2 CRE and the CT/CGRP CRE are all responsive to cAMP when linked to a heterologous promoter; therefore all three CRE sites are sufficient to confer a response to cAMP. The cAMP-induced activation of the TH and CT/CGRP promoters is indeed CRE-dependent (Watson and Latchman, 1995; Lazaroff *et al*, 1995; Ghee *et al*, 1998). In contrast the Bcl-2 promoter is not sensitive to cAMP (Liu, unpublished data) suggesting that the Bcl-2 CRE is inhibited in some way within the context of the Bcl-2 promoter, possibly due to the presence of an inhibitory element within the promoter region LB334. If this were the case, regulatory proteins other than CREB, or even CRE-binding repressors, may inhibit CRE-mediated activation of Bcl-2. Similarly, the Bcl-2 CRE is responsive to hypoxia in LB334, but not in the context of the full-length promoter. An upstream repressor element may be inhibiting the effects of the CRE on the full-length promoter.

In addition to the presence of repressor elements, the DNA structure and position of the CRE in the Bcl-2 promoter may also affect its ability regulate transcription. When cloned in to pBLCAT2 the CRE sites are all positioned approximately 150bp upstream of the start site. Experiments on the TH promoter have shown that the position of the CRE with respect to the start site is crucial to its function (Tinti *et al*, 1997). The TH CRE is located less than 50bp from the transcription initiation site, but when its position is altered by the insertion of 5 or 10bp, the mutation causes a three-fold increase in promoter activity. In fact, the CRE was able to stimulate promoter activity in any position within a boundary of approximately 200bp upstream of the transcription initiation site. Outside of this window, the CRE was unable to stimulate promoter activity, indicating that the CRE affects TH transcription in a distance-dependent manner (Tinti *et al*, 1997). The CT/CGRP gene CRE is located at just over 100bp from the start site (Watson an Latchman, 1995) and indeed, most CRE sequences are located within the first 200bp of the gene promoter (Delmas *et al*, 1994). In contrast, the Bcl-2 gene CRE (which is probably not involved in the up-regulation of Bcl-2 in response to cAMP, NGF or hypoxia in PC12 cells), is located far upstream of the start site, at over 1500bp. This relatively large distance is likely to affect the ability of CRE-bound CREB to activate transcription. In the context of the isolated Bcl-2 P1 promoter of LB334, the Bcl-2 CRE is brought closer to the start site by 1280bp, and is responsive to hypoxia. In pBLCAT2 the

Bcl-2 CRE is inserted within 150bp of the start site and is responsive to not only hypoxia, but also to cAMP and NGF.

6 Discussion

Most of the cells that make up a eukaryotic organism contain the entire complement of DNA that constitutes the genome of that organism, yet each cell will have its own specific and variable pattern of gene expression. In order for this to occur, gene expression is tightly regulated at a number of different levels, such as gene transcription, RNA processing and post-translational modification. The primary level of control is the regulation of gene transcription by *trans*-acting transcription factors, which bind to specific *cis*-acting elements within the promoter sequences of the regulated genes. The extracellular environment is able to influence the activity of specific transcription factors, and thus the regulation of specific genes, by transmitting signals from the cell surface to the nucleus by an array of intracellular signalling cascades.

The focus of this study has been the cyclic AMP response element (CRE), which is found in the promoter sequences of many different genes and is involved in the transcriptional regulation of those genes. As its name suggests, the CRE is sensitive to cAMP (Delmas *et al*, 1994), but is also sensitive to a number of other intracellular signalling pathways, such as calcium signalling, protein kinase C and various mitogen-activated protein (MAP) kinases (Sun *et al*, 1994; Hundle *et al*, 1995; Xing *et al*, 1998; Froedin and Gammeltoft, 1999). This is because these signalling pathways are able to induce the phosphorylation of CREB transcription factor, which binds constitutively to the CRE site, and is able to activate transcription when phosphorylated (Delmas *et al*, 1994).

The calcitonin/calcitonin gene-related peptide (CT/CGRP) gene has a CRE within its regulatory sequences (Watson and Latchman, 1995). In dorsal root ganglion neurons and PC12 cells this CRE site mediates transcription from the CT/CGRP promoter in response to cAMP and the neurotrophin nerve growth factor (NGF) (Watson and Latchman, 1995). The aims of this project were to identify the signalling mechanisms involved in NGF- and cAMP-mediated activation of the CT/CGRP promoter in PC12 cells.

The use of selective inhibitors against individual signalling enzymes has successfully enabled the identification of the signalling pathways responsible for the NGF- and cAMP-mediated induction of the CT/CGRP promoter. The second messenger cAMP generally mediates its effects through the enzyme PKA and CREB family transcription factors (Krebs and Beavo,

1979; McKnight *et al*, 1988; Mellon *et al*, 1989; Karin and Smeal, 1992). In the case of the CT/CGRP promoter, inhibition of the enzyme PKA completely abolished the effects of cAMP. The quenching of cellular CREB with dominant negative mutants also dramatically reduced CT/CGRP promoter activation in response to cAMP. This suggests that, as expected, cAMP-mediated activation of the CT/CGRP promoter in PC12 cells requires PKA and the transcription factor CREB.

NGF generally mediates its effects via a complex network of signalling cascades involving the MAP kinases and protein kinase C (Jing *et al*, 1992; Kaplan and Stephens, 1994; Heldin and Purton, 1996), which in turn activate a number of different transcription factors, including those of the CREB/ATF family (Riccio *et al*, 1997). Inhibition of PKC, c-Jun N-terminal kinase and p38 MAP kinase signalling pathways had no effect on the NGF-mediated activation of the CT/CGRP promoter. However, inhibition of various enzymes of the p42/p44 MAP kinase signalling cascade either abolished or dramatically reduced NGF-stimulated promoter induction, confirming that NGF exerts its influence upon the CT/CGRP promoter by activating the p42/p44 MAP kinase signalling cascade. The enzymes tested include the p42/p44 MAP kinases themselves, their upstream effectors Ras and MEK-1 and their downstream effector RSK-2. Inhibition of RSK-2 did not reduce the effects of NGF on the promoter, possibly because other RSK enzymes rather than RSK-2 mediate the effects of NGF, or because its loss was compensated for by other RSKs. This could be tested by the inhibition of various combinations of RSK proteins in similar experiments. Interestingly, inhibition of the enzyme PKA considerably reduced the effects of NGF. This is probably because PKA is required for MAP kinase signalling by aiding in the translocation of MAP kinases to the nucleus, although this was not tested.

As with cAMP-mediated CT/CGRP promoter activation, the quenching of cellular CREB with dominant negative mutants dramatically reduced CT/CGRP promoter activation in response to NGF, suggesting that CREB is involved in promoter activation in PC12 cells. However, this project has revealed that the CT/CGRP CRE site does not bind to CREB in *in vitro* electrophoretic mobility shift assays (EMSA) using PC12 cell protein extract. The dominant negative mutants used to quench CREB in these experiments are also known to quench certain proteins that can form dimers with CREB, such as other CRE-binding proteins and c-Jun, as well as CREB itself (Walton *et al*, 1992; Yang *et al*, 1996; Ahn *et al*, 1998). It is therefore possible that a CREB-related protein rather than CREB may be responsible for the CRE-mediated activation of the promoter. Alternatively, the CRE-binding protein in question may simply be an isoform of CREB not recognised by the antibody used in the EMSAs. This could be confirmed using a broader range of antibodies in EMSAs, and by testing the ability

of NGF and cAMP to activate transcription from the CT/CGRP promoter in CREB-deficient PC12 cells.

Whatever the identity of the CRE-bound protein, it is necessary for NGF-mediated CT/CGRP promoter activation. In PC12 cells at least, the CRE is also sufficient to induce transcription from a heterologous promoter in response to NGF. However, in dorsal root ganglion (DRG) neurons the CRE-binding factor is not sufficient for this; at least one otherDNA-binding factor co-operates with the CRE-binding factor to activate transcription in response to NGF (Watson and Latchman, 1995), although the nature and function of this second factor is not known. Since the p42/p44 signalling cascade is essential to NGF-mediated CT/CGRP promoter activation in PC12 cells, it is possible that, as well as CREB, this factor could be phosphorylated by the downstream effectors of these MAP kinases in DRG neurons. Transfections need to be carried out in DRG neurons to determine whether p42/p44 MAP kinases are pivotal in the NGF-mediated transcription from the CT/CGRP promoter, and the nature of the second factor also warrants investigation. The latter could be facilitated by identifying its DNA-binding site by site-directed mutagenesis of the 68bp region to which it is known to bind (Watson *et al*, 1995). As yet there is no direct evidence to suggest that NGF regulates the other factor(s) involved in any way; it may be a constitutively active factor even in the absence of NGF, which would make CREB phosphorylation the key event in NGF-stimulated CT/CGRP promoter activation.

The second aim of this study was to investigate the differential regulation of three different CRE sites by NGF, cAMP and hypoxia. As described above, the CT/CGRP CRE is sensitive to NGF and cAMP in PC12 cells. The tyrosine hydroxylase gene also has a CRE site which is sensitive to cAMP (Piech-Dumas and Tank, 1999) and to low oxygen levels (Beitner-Johnson and Millhorn, 1998) in PC12 cells. Thus in these cells the CRE is capable of responding to three different stimuli in these promoters. The Bcl-2 promoter has a CRE site, but this site was thought not to be responsive to cAMP in PC12 cells, and its role in NGF-mediated promoter activation was questionable (Liu *et al*, 1999; Riccio *et al*, 1999). Bcl-2 is up-regulated in certain neuronal cells in response to hypoxia (Bossenmeyer-Pourie and Daval, 1998), and it was thought that the CRE site may mediate this signal. Since PC12 cells can be manipulated more easily than primary neuronal cultures, the effects of hypoxia on the Bcl-2 promoter were tested in these cells. Unfortunately, promoter studies have shown that transcription from the full-length Bcl-2 promoter cannot be induced by hypoxia in PC12 cells, therefore they cannot be used as a model for studying the effects of hypoxia on neuronal Bcl-2 expression. Interestingly though, an isolated fragment of the Bcl-2 P1 promoter is sensitive to hypoxia in PC12 cells, and this effect is mediated by the CRE site. In addition,

when the isolated CRE site is linked to a heterologous promoter it can confer hypoxia-responsiveness to that promoter. This shows that the Bcl-2 CRE is capable of responding to hypoxia but its effects are repressed in the full-length Bcl-2 promoter. The Bcl-2 CRE is also capable of conferring cAMP-sensitivity to a heterologous promoter, yet cannot exert its effects on the full-length promoter since the Bcl-2 promoter is not activated by cAMP in PC12 cells. The full-length Bcl-2 promoter is sensitive to NGF in PC12 cells, but it is still under debate as to whether this effect is mediated, at least in part, by the CRE site. This study has shown that the Bcl-2 CRE is certainly capable of conferring NGF-sensitivity to a heterologous promoter, even if it does not do so in the context of the promoter.

In summary, the Bcl-2 CRE site is sufficient to confer NGF-, cAMP and hypoxia-responsiveness to a heterologous promoter in PC12 cells, even though cAMP and hypoxia have no overall effect on Bcl-2 promoter stimulation.

This study has also shown that the CT/CGRP promoter is sensitive to NGF and cAMP, but not to hypoxia. This is mirrored by the activity of the CT/CGRP CRE site; when linked to a heterologous promoter, the CT/CGRP CRE is able to confer both NGF and cAMP-sensitivity to that promoter in PC12 cells, but is unable to confer a response to hypoxia. This shows that the inability to respond to hypoxia lies within the sequence of the CRE site itself. A reason for this could be the proteins that bind to the element; EMSA experiments have shown that the protein-binding pattern of the CT/CGRP CRE site is very different to that of the other two CRE sites, which are responsive to hypoxia. It is possible that whilst hypoxia-responsive protein(s) can bind to the Bcl-2 and TH CRE sites, the same protein(s) cannot bind to the CT/CGRP CRE site.

Finally, the TH consensus CRE is capable of conferring cAMP- and hypoxia-responsiveness to a heterologous promoter, and is also capable of conferring an increase in basal transcription. The TH CRE cannot, however, confer NGF-responsiveness to a heterologous promoter. This correlates with the fact that the TH CRE contributes to basal TH expression as well as cAMP- and hypoxia-stimulated TH transcription in PC12 cells. Since the CRE site does not respond to NGF, it is unlikely that the TH promoter is regulated by NGF in a CRE-dependent manner in these cells, although it is possible that the CRE may be partially involved but not sufficient for any response to this stimulus.

In conclusion, this project has shown that the stimuli to which these CRE sites respond are determined by a combination of many different factors; the sequence of the CRE site itself, the proteins to which it is bound, the signalling pathways to which those factors respond, the promoter within which it is located and not least, the cell type studied.

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