

**MECHANISMS REGULATING VASOACTIVE INTESTINAL
POLYPEPTIDE EXPRESSION IN CULTURED DORSAL
ROOT GANGLION NEURONS**

by

Stephen P Dobson

Thesis submitted for the Degree of Doctor of Philosophy

University of Edinburgh

January 1996



I declare that this thesis has been composed by me and that the studies presented are the result of my own independent investigation.

This work has not been and is not currently being submitted for candidature in any other degree or professional qualification.

Stephen P. Dobson (Candidate)

Dr. Peter K. Mulderry (Supervisor)

Dr. Anthony Harmar (Supervisor)

Prof. George Fink (Supervisor)

ACKNOWLEDGEMENTS

I would like to thank the Medical Research Council for the award of a research studentship and Professor George Fink for the opportunity to study in the MRC Brain Metabolism Unit.

I would also like to thank Dr Peter Mulderry for all his help and advice during the last three years and for teaching me so much. Thanks are also due to Dr. Tony Harmar for critical reading of this thesis.

I would like to thank Drs. Janice Patterson and John Quinn for their enormous help during my three years in Edinburgh and Elma Clark, Christine Morrison and everyone else at the BMU who made life at the Royal Ed so much fun.

Finally I would like to thank Angela Ison and all of my friends at George square without whom this thesis would never have been written.

Some of the results presented in this thesis have been published as follows:

- Dobson, S.P., Mulderry, P.K., Quinn, J.P., Morrow, J.A., Gozes, I. and Harmar, A.J. (1993) Rat VIP cyclic AMP responsive element mediates expression in neonatal rat dorsal root ganglion neurons. *Society for Neuroscience Abstracts* **19**: 289.5.
- Dobson, S.P., Quinn, J.P., Morrow, J.A. & Mulderry, P.K. (1994) The rat vasoactive intestinal polypeptide cyclic AMP response element regulates gene transcriptional responses differently in neonatal and adult rat sensory neurons. *Neuroscience Letters* **167**: 19-23.
- Mendelson, S.C., Morrison, C.F., McAllister, J., Paterson, J.M., Dobson, S.P., Mulderry, P.K. & Quinn, P.J. (1994) Repression of preprotachykinin-A promoter activity is mediated by a proximal promoter element. *Neuroscience* **65**: 837-847.
- Morrison, C.F., McAllister, J., Dobson, S.D., Mulderry, P.K. & Quinn, J.P. (1994) An activator element within the preprotachykinin-A promoter. *Molecular and Cellular Neurosciences* **5**: 165-175
- Mulderry, P.K., Dobson, S.P., Chapman, K.E., Quinn, J.P., Lyons, V., Morrow, J.A., Gozes, I. & Harmar, A.J. (1994) Investigation of neuropeptide gene transcription in sensory neurons transfected by microinjection of reporter plasmids. *Gene Therapy* **1 (Suppl 1)**: S22.
- Mulderry, P.K. and Dobson, S.P. (1995) Regulation of VIP and other Neuropeptides by c-Jun in Sensory Neurons: Implications for the Neuropeptide Response to Axotomy. *European Journal of Neuroscience*. Manuscript submitted.

ABSTRACT

The neuropeptide vasoactive intestinal polypeptide (VIP) is expressed in dorsal root ganglion (DRG) neurons of the rat in response to peripheral axotomy (Nielsch and Keen, 1989, Shehab et al., 1986), where it may influence neuronal survival (Brenneman and Eiden, 1986), or modulate nociceptive input into the spinal cord (Wiesenfeld-Hallin et al., 1990). As this increase is paralleled by an increase in mRNA (Noguchi et al., 1989), it is likely that regulation occurs at the level of transcription. The aim of the present study was to identify regions of DNA within the VIP gene that may be responsible for the spontaneous expression of VIP. The expression of a number of neuropeptides, including VIP, in cultured DRG neurons from adult rats parallels that seen in axotomised DRG neurons *in vivo* (Mulderry and Lindsay, 1990). Cultured DRG neurons were therefore used as a model system with which to study mechanisms involved in the spontaneous expression of VIP. Previous experiments have shown that expression of the oncogene *c-jun* is required for spontaneous expression of VIP in cultured adult rat DRG neurons (Mulderry and Dobson, 1995, submitted). Since *c-Jun* is capable of forming a complex that can bind DNA to activate transcription (Curran and Franzen, 1988, Nakabeppu et al., 1988) it is likely that *c-Jun* containing complexes bind to sequences in the promoter region of the rat VIP gene to direct spontaneous expression. One such sequence is a putative cAMP responsive element (CRE) found between -94bp and -67bp relative to the start of transcription (Giladi et al., 1990), that shows homology to a sequence from the human VIP gene known to bind *c-Jun* containing complexes (Fink et al., 1991). Using an electrophoretic mobility shift assay the present study has shown that the rat VIP CRE is capable of binding *c-Jun* in a heterodimer with *c-Fos*. To determine the importance of these

proteins in binding to the VIP CRE, an attempt was made to compete them off the endogenous rat VIP CRE. DRG neurons were transfected with constructs containing copies of the CRE ligated into the plasmid pUC18. Quantitative analysis of the effects of transfection on endogenous VIP immunoreactivity, showed that the CRE containing construct caused a selective reduction in VIP expression. Proteins binding to the CRE are therefore important for spontaneous VIP expression.

To determine whether the rat VIP CRE is all that is necessary for spontaneous VIP expression it was analysed, using reporter constructs, for its ability to mediate patterns of gene expression analogous to those seen for endogenous VIP. The present study showed that the CRE is capable of increasing gene transcription from a heterologous c-fos promoter in neonatal but not adult rat DRG neurons in response to stimuli that raise cAMP and intracellular calcium. Therefore the VIP CRE may be responsible for the synergistic increase in VIP expression that occurs in neonatal rat DRG neurons in response to these same stimuli (Mulderry, 1993). Since the CRE was shown to be incapable of increasing expression from the c-fos promoter in the absence of stimuli other sequences together with the CRE must be involved in spontaneous VIP expression. The present study has shown that reporter constructs containing up to 1697bp of the endogenous rat VIP gene, 5' from the transcription start site (including the CRE), together with the first exon (151bp) and approximately 950bp of the first intron, do not contain enough sequence to direct spontaneous expression of VIP.

The present study suggests that the spontaneous expression of rat VIP is dependent on protein complexes binding to the CRE, and that these complexes probably contain c-Jun. Although the CRE alone is capable of mediating the response of VIP to cAMP and calcium and may mediate developmental differences in VIP expression, sequences are required in

combination with the CRE for spontaneous VIP expression. At least some of these sequences exist outside the 2800bp fragment tested in this study.

ABBREVIATIONS

A	adenine
α	alpha
Ac	acetate
APS	ammonium persulphate
ATF	activating transcription factor
ATP	adenosine-5'-triphosphate
BAYK8644	methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate
β	beta
BDNF	brain-derived neurotrophic factor
bp	base pair(s)
BSA	bovine serum albumin
bzip	basic leucine zipper domain
C	cytosine
C/EBP	CAAT box/enhancer binding proteins
Ca^{2+}	calcium ion
cAMP	adenosine 3',5'-cyclic monophosphate
CBP	Creb binding protein
cDNA	copy DNA
$\text{CG}\alpha$	choriogonadotropin α gene
CGRP	calcitonin gene related peptide
Ci	curies
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRE	cAMP-response element
CREB	cAMP-response element binding protein
dA	2'-deoxyadenosine
dATP	2'-deoxyadenosine-5'-triphosphate
dC	2'-deoxycytidine
dCTP	2'-deoxycytidine-5'-triphosphate
dG	2'-deoxyguanosine
dGTP	2'-deoxyguanosine-5'-triphosphate
dH ₂ O	distilled water
DNA	deoxyribonucleic acid

DRG	dorsal root ganglion neuron
dT	2'-deoxythymidine
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
E.coli	Escherichia coli
EDTA	sodium ethylenediamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
EtBr	ethidium bromide
fmol	femto moles
G	guanine
γ	gamma
g	gram
GALV	gibbon ape leukaemia virus
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesuphonic acid
K ⁺	Potassium ion
kb	kilobase pair(s)
KCl	potassium chloride
l	litre(s)
LIF	leukaemia inhibitory factor
M	molar
m	metre(s)
mA	milliamp(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
μ	micro
N	unspecified nucleotide
n	nano
NGF	nerve growth factor
NPY	neuropeptide Y
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PHI	peptide histidine isoleucine
PHM	peptide hidtidine methionine
PKA	protein kinase A
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonylfouride
pol II	RNA polymerase II
PPT	preprotachykinin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s	second(s)
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulphate
SOM	scanning optical microscope
SP	substance P
SST	somatostatin
T	thymidine
TAS	TBP associated proteins
TBE	tris-borate/EDTA
TBP	TATA-box binding protein
TEMED	N,N,N',N'-tetramethylethylene diamine
TPA	12-O-tetradecanoyl-phorbol-13 acetate
TRE	phorbol ester-response element
Tris	tris (hydroxymethyl) aminoethane
TTX	tetrodotoxin
V	volt
v/v	volume/volume
VIP	vasoactive intestinal polypeptide
vol	volume
w/v	weight/volume
³² P	phosphorous 32 radioisotope
³⁵ S	sulphur 35 radioisotope

TABLE OF CONTENTS

Declaration	
Acknowledgements	
Publications arising from this thesis	
Abstract	
Abbreviations	

CHAPTER 1: INTRODUCTION

1.1	Sensory Neurons	1
1.2	VIP and its functions	2
1.3	The response of peripheral neurons to axotomy	5
1.4	Mechanisms of action of transcription factors	9
1.4.1	Cyclic AMP response elements (CREs)	12
1.4.2	Transcription factors that bind the CRE	15
1.5	Mechanisms of calcium and cAMP signalling	17
1.5.1	cAMP	17
1.5.2	Calcium	18
1.6	Regulation of VIP expression in DRG	21
1.6.1	Neurotrophic regulation of VIP expression	21
1.6.2	Calcium and cAMP dependent regulation of VIP expression	24
1.6.3	Transcription factor expression in response to axotomy	26
1.6.4	Transcriptional versus non-transcriptional regulation of VIP	27
1.7	A Model system for the study of VIP gene expression in DRG	27
1.8	Expression of the VIP gene following axotomy: a hypothesis	30
1.9	Aims of this thesis	31

CHAPTER 2: THE ROLE OF CRE BINDING PROTEINS IN THE EXPRESSION OF VIP BY ADULT RAT DRG NEURONS IN CULTURE

2.1	INTRODUCTION	32
2.2	METHODS	34

2.2.1	Microinjection of DRG neurons with plasmids containing multiple copies of the rat VIP CRE and quantitation by immunofluorescence	34
2.2.2	Electrophoretic mobility shift assay (EMSA)	35
2.3	RESULTS	36
2.3.1	Immunofluorescence	36
2.3.2	EMSA	38
2.4	DISCUSSION	39

CHAPTER 3: EXPRESSION OF REPORTER CONSTRUCTS CONTAINING THE RAT VIP CRE IN PC12 CELLS AND DRG NEURONS

3.1	INTRODUCTION	44
3.2	METHODS	45
3.2.1	Reporter Constructs	45
3.2.2	Transfection of PC12 cells	47
3.2.3	Microinjection	48
3.3	RESULTS	49
3.3.1	PC12 Cells	49
3.3.2	Neonatal Rat DRG Neurons	49
3.3.3	Adult Rat DRG Neurons	50
3.4	DISCUSSION	52
3.4.1	PC12 Cells	52
3.4.2	DRG Neurons	53

CHAPTER 4: THE ROLE OF 5' FLANKING SEQUENCES FROM THE RAT VIP GENE IN MEDIATING VIP GENE EXPRESSION

4.1	INTRODUCTION	57
-----	--------------	----

4.2	METHODS	58
4.2.1	Construct Design	58
4.2.2	DRG neurons; transfection and treatment	60
4.3	RESULTS	61
4.3.1	Response to forskolin and depolarisation	61
4.3.2	Response to NGF and LIF	62
4.4	DISCUSSION	63
4.4.1	Reporter construct expression	63
4.4.2	Response to LIF	63
4.4.3	Response to NGF	64
4.4.4	Response to 10 μ M forskolin and 40mM K ⁺	65

CHAPTER 5: SEQUENCE ANALYSIS OF THE 5' FLANKING REGION OF THE RAT VIP GENE

5.1	INTRODUCTION	67
5.2	METHODS	68
5.2.1	Construct sequencing	68
5.2.2	Search for consensus transcription factor binding sites	68
5.3	RESULTS	68
5.3.1	Sequencing	68
5.4	DISCUSSION	69
5.4.1	LIF response element	70
5.4.2	AP1 element	70
5.4.3	AP2 element	71
5.4.4	E Box	72
5.4.5	C/EBP site	74
5.4.6	Conclusions	74

CHAPTER 6: OVERALL DISCUSSION AND FUTURE DIRECTIONS	76
APPENDIX: MATERIALS AND METHODS	82
A.1 Solutions	82
A.2 Lambda DNA preparations from plate lysates	84
A.3 Minipreparation of plasmid DNA	85
A.4 Large scale preparation of plasmid DNA	86
A.5 Restriction enzyme digests of DNA	87
A.6 Recovery of DNA from agarose gels	87
A.7 Generating blunt ends following restriction enzyme digest	88
A.8 DNA ligation	88
A.9 Preparation and transformation of competent <i>E. coli</i>	89
A.10 Horizontal agarose gel electrophoresis	89
A.11 Vertical gel electrophoresis	90
A.12 Autoradiography	91
A.13 Multimerisation of DNA oligonucleotides	92
A.14 <i>In vitro</i> -transcription/translation	92
A.15 Electrophoretic mobility shift assay	93
A.16 Labelling of double stranded DNA oligonucleotide probes	94
A.17 Dideoxy sequencing	94
A.18 PC12 cells maintenance and electroporation	95
A.19 Preparation of Dorsal Root Ganglion Neurons	96
A.20 Microinjection	97
A.21 Depolarisation and forskolin treatment of DRG neurons	98
A.22 Chloramphenicol acetyl transferase assay	98
A.23 β Galactosidase assay	99
A.24 Immunocytochemistry	100
A.25 Confocal microscopy	100
A.26 Statistics	101
BIBLIOGRAPHY	102
PUBLICATIONS	

CHAPTER 1

INTRODUCTION

1.1 Sensory Neurons

Primary afferent neurons with perikarya in the dorsal root ganglia (DRG) mediate the transmission of sensory information from the periphery and viscera to the spinal cord. Depending on the type of stimulus to which they respond, sensory nerve fibres have been categorised into three groups: large myelinated (A-beta), small myelinated (A-delta) and unmyelinated (C-fibres). A-beta fibres respond to light pressure whereas A-delta and C-fibres can respond to pressure, thermal and chemical stimuli. Each fibre in these groups may respond to only one or to several of these stimuli. Sensory neurons that respond with increasing frequency to stimuli that have the potential to cause tissue damage (painful stimuli) are termed primary afferent nociceptors and belong to the A-delta or C-fibre groups.

Sensory neurons synthesise a number of neuropeptides, of which four or more may coexist in the same neuron (Cameron, et al., 1988). These neuropeptides can be released by action potentials, from both the peripheral and central terminals and exert effects on post synaptic and presynaptic-receptors (Levine, et al., 1993). The released peptides also affect neutrophils and macrophages and play a role in the inflammatory response (Hartung, et al., 1986, Payan, et al., 1984). Some, for example neurokinin A, a tachykinin related to substance P, are relatively stable and are thought to be able to act on cells some distance away from their point of release (Levine, et al., 1993). The same peptides are also neurotransmitters. Substance P, for example released at central terminals has a predominantly excitatory effect on dorsal horn neurons (Henry, 1978, Sastry, 1979, Fleetwood-Walker, et al., 1990) although it is also capable of indirectly inhibiting a subpopulation of neurons (Fleetwood-Walker, et al., 1990). The DRG peptides also have neuromodulatory actions. For example, calcitonin gene related peptide (CGRP) exerts only limited effects

on its own, yet potentiates the effects of substance P on dorsal horn neurons (Wiesenfeld-Hallin, et al., 1984, Woolf and Wiesenfeld-Hallin, 1986).

Plasticity of peptide expression may be an important aspect of primary afferent function since peptide concentrations change in response to nerve damage or tissue injury. Injection of formalin into the hind limb results in a short-lived pain syndrome associated with increased expression of substance P in DRG neurons (Noguchi, et al., 1988). Adjuvant induced arthritis increases the concentrations of substance P and CGRP, but not somatostatin, in DRG neurons that innervate the affected joints (Smith, et al., 1992). In contrast, sciatic nerve section (axotomy) is associated with decreases in the levels of substance P, CGRP and somatostatin (Inaishi, et al., 1992, Nielsch and Keen, 1989, Noguchi, et al., 1990, Villar, et al., 1989). At the same time sciatic nerve section results in increases in the peptides galanin, vasoactive intestinal polypeptide (VIP) and neuropeptide tyrosine (NPY) which are not normally found in DRG neurons (Nielsch and Keen, 1989, Villar, et al., 1989, Wakisaka, et al., 1991, Doughty, et al., 1991, Frisé, et al., 1992, Hökfelt, et al., 1987, Kashiba, et al., 1992, Noguchi, et al., 1989, Shehab and Atkinson, 1986, Shehab, et al., 1986). Peripheral fibre transection has been proposed as a model for chronic neuropathic pain as it can lead to animals biting and removing their toes (autotomy), a process not merely due to numbing of the limb (Devor, 1991).

1.2 VIP and its functions

VIP is a 28 amino acid peptide, originally isolated from the porcine gastrointestinal tract by Said and Mutt (1970) due to its vasodilatory properties. It shows structural similarities with a number of other

gastrointestinal hormones such as secretin, glucagon and gastric inhibitory peptide (Mutt, 1988).

cDNA cloning studies demonstrated that VIP is derived from a precursor that generates, in addition to VIP, 27 amino acid peptides designated PHM (peptide histidine methionine) in man and PHI (peptide histidine isoleucine) in the rat (Itoh, et al., 1983, Bloom, et al., 1983). The VIP gene is composed of seven exons each coding for a distinct functional domain of the VIP precursor (Fig. 4.2). In the human VIP gene, exon 1 (165bp) codes for the 5' untranslated region of the mRNA, exon 2 (117bp) codes for the signal peptide, exon 3 (123bp) codes for an N-terminal peptide, exon 4 (105bp) codes for PHM, exon 5 (132bp) codes for VIP, exon 6 (89bp) codes for the C-terminal peptide and exon 7 (723bp) codes for the 3' untranslated region of the mRNA. The rat VIP gene (Fig. 4.2) has a very similar structure to the human gene. The human VIP gene promoter region contains three TATA boxes (the consensus sequence capable of binding RNA polymerase II), one at -28bp from exon 1 (the start of transcription) and one each at position -145bp and -155bp (Itoh, et al., 1983). TATA boxes have been located at corresponding positions in the rat VIP gene suggesting they play an important role in gene regulation and have thus been conserved in evolution. Similarly a putative cAMP response element (CRE) at position -80 is found in an identical position in both the human and rat genes (Giladi, et al., 1990).

VIP is widely distributed in the peripheral and central nervous system and in endocrine cells where it exerts a wide variety of biological actions including vasodilation, increased cardiac output, bronchodilation, hyperglycemia (Said and Mutt, 1970, Said and Mutt, 1988) smooth muscle relaxation (Piper, et al., 1970), secretory processes in the gastrointestinal tract (Reid, et al., 1988) and glycogenolysis in the liver (Karins and Said,

1973) and cerebral cortex (Magistretti, et al., 1981). The VIP content in various brain areas changes during development (Nobou, et al., 1985, Said, 1984) reaching a peak concomitantly with synapse formation in the rat brain (Gozes, et al., 1987) and decreasing as the brain ages (Gozes, et al., 1988). Administration of VIP antagonists *in vivo* has been reported to result in neurological deficits and to impairment of neuronal development, acquisition of reflexes and learning and memory mechanisms (Gozes, et al., 1990, Hill, et al., 1991, Hill, et al., 1994, Panlilio, et al., 1990, Glowa, et al., 1992). In the suprachiasmatic nucleus (SCN), rhythmical alteration in the levels of VIP mRNA occur in response to day-night changes (Okamoto, et al., 1991) suggesting a role for VIP in changes associated with the circadian clock. VIP appears to have proliferative, growth factor and neurotrophic actions, some of which appear to be mediated through indirect cellular interactions. Electrical blockade of spinal cord neurons in culture has been shown to accelerate neuronal death, a process that can be prevented by the addition of VIP (Brenneman and Eiden, 1986). This action of VIP is dependent on the presence in the culture of non-neuronal cells and it is thought that VIP interacts with high affinity receptors on glial cells to induce the secretion of neuronal survival factors (Brenneman, et al., 1987). One of the factors released is an interleukin-1-like substance that has been shown to increase glial proliferation and may play an important developmental role (Brenneman, et al., 1992). VIP produces a small but significant increase in the number of glial fibrillary acidic protein positive cells in spinal cord cultures (Brenneman, et al., 1990); it also exerts mitogenic effects on embryonic neurons in the superior cervical ganglion (Pincus, et al., 1990), on keratinocytes and on lung cancer cells (Haegerstrand, et al., 1989, Moody, et al., 1992), where it may act as an autocrine regulator.

This thesis is focused on the investigation of mechanisms regulating VIP expression in cultured DRG neurons. These mechanisms may be relevant to the expression of VIP as part of the axotomy response and may play a role in the regulation of other genes induced following axotomy. It is also possible that similar mechanisms regulate VIP expression in a number of the systems described above and may, therefore, have far reaching implications.

1.3 The response of peripheral neurons to axotomy

Following damage to a peripheral nerve a number of physical changes occur both at the site of damage and within the cell bodies and axons of the relevant neurons (for a review see Pleasure, 1994). The cut ends of both parts of the axon immediately lose axoplasm until becoming sealed off by membrane fusion. They then retract from one another and begin to swell, largely due to materials carried to the site by axonal transport and axoplasmic flow. Degeneration proceeds for a short distance in the proximal segment, usually only as far as the point of origin of the first axon collateral. However if the cell body dies then degeneration spreads down the remainder of the proximal segment. Conversely degeneration is widespread within the distal segment following a process termed Wallerian degeneration (after the 19th century physician Augustus Waller). The axon swells, becomes beaded and fragments are absorbed by local phagocytes derived from Schwann cells that have undergone a transient phase of intense proliferation. In many cases however the connective tissue sheath surrounding the nerve may remain intact. The proximal segment is then capable of regenerating and reconnecting to its previous sites as long as its cell body remains alive. Regenerating axons can run along the connective tissue sheath, which acts as a conduit leading the growing axons back to

the peripheral target. A growth promoting environment composed of proliferating Schwann cells and Schwann cell derived matrix (including collagen, fibronectin and laminin) aids the regeneration process.

Axotomy involves dramatic changes in the expression of a number of genes (Lasek and Hoffman, 1976, Wong and Oblinger, 1990, Koo, et al., 1988, Hökfelt, et al., 1994, Troy, et al., 1990, Wong and Oblinger, 1990, Woolf, et al., 1990). Some of these changes are assumed to act to minimise damage to the organism as a whole and to promote survival and regeneration of the injured neuron. Thus axons regenerating from DRG neurons in culture do so faster if the DRG neuron is axotomised *in vivo* up to two weeks previously (a conditioning lesion). It would appear that the neuron responds to the conditioning lesion with a program of events that aid its regeneration (Forman, et al., 1981, Oblinger and Lasek, 1984). It has also been proposed that neuronal injury results in decreased expression of substances required for chemical transmission and an increase in those required for cell survival (Kreutzberg, 1982, Lieberman, 1971). However, the functional importance of many of the changes in gene expression are poorly understood.

Consistent with an increased requirement of structural proteins for axonal replacement, axotomised peripheral neurons show increased expression of cytoskeletal proteins. For example, increased tubulin expression occurs in rat motor neurons, sympathetic neurons and dorsal root ganglion (DRG) neurons following injury (Lasek and Hoffman, 1976, Wong and Oblinger, 1990, Koo, et al., 1988). Similarly, expression of the intermediate-filament protein peripherin is upregulated in rat motor neurons and dorsal root ganglion (DRG) neurons following injury (Troy, et al., 1990, Wong and Oblinger, 1990). The cytoskeletal polymers formed provide

structure, motility and stability to the axon. They may also promote axon elongation and facilitate regeneration (Oblinger, et al., 1989).

The expression of a number of genes involved in chemical transmission across synapses is reduced following nerve injury. For example, expression of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines, is reduced in sympathetic ganglia following axotomy resulting in a decrease in the amount of available neurotransmitter (Cheah and Geffen, 1973). Similarly, axotomy of DRG neurons results in decreased expression of substance P and calcitonin gene related peptide (CGRP) (Inaishi, et al., 1992, Nielsch and Keen, 1989, Noguchi, et al., 1990, Villar, et al., 1989).

In contrast, axotomy of DRG neurons results in an increase in the neuropeptides galanin, vasoactive intestinal polypeptide (VIP) and neuropeptide tyrosine (NPY) (Nielsch and Keen, 1989, Villar, et al., 1989, Wakisaka, et al., 1991, Doughty, et al., 1991, Frisén, et al., 1992, Hökfelt, et al., 1987, Kashiba, et al., 1992, Noguchi, et al., 1989, Shehab and Atkinson, 1986, Shehab, et al., 1986). These neuropeptides newly synthesised in response to injury can be transported both to the central projection of the DRG and to peripheral sites of nerve injury (Villar, et al., 1989, Shehab and Atkinson, 1986, Anand, et al., 1990, McGregor, 1984) where they may mediate both central and peripheral responses to axotomy. The function of the injury induced peptides are currently under active investigation (Hökfelt, et al., 1994). Galanin expression, for example, may be important in preventing self mutilation following axotomy and thus may play a role in preventing chronic neuropathic pain (Ji, et al., 1994, Verge, et al., 1993). Similarly NPY has antinociceptive effects when applied intrathecally due to its ability to block sensory input or transmission at the spinal level. This effect has been shown to be enhanced following

axotomy of the sciatic nerve (Xu, et al., 1994). Conditioning stimuli applied to the sciatic nerve increase the excitability of the hamstring flexor reflex. This effect is mediated by substance P released from DRG neurons at their central terminals. VIP has been reported to take over the role of substance P in mediating afferent induced reflex facilitation following axotomy (Wiesenfeld-Hallin, et al., 1990) and may play a role in neuropathic pain associated with nerve injury. Biochemical studies have shown that VIP can stimulate glycogenolysis and increases glucose utilisation in injured neurons (Magistretti, et al., 1981, McCulloch and Kelly, 1983, Magistretti and Schorderet, 1984). VIP is also capable of stimulating glial cells to increase their production of neuronal survival factors (Brenneman, et al., 1987, Brenneman, et al., 1990). Thus VIP may enhance neural regeneration through its neurotrophic actions and by stimulating glucose metabolism.

Other proteins showing increased expression following axotomy of DRG neurons include nitric oxide synthase (NOS), the enzyme responsible for the synthesis of nitric oxide (NO), the growth associated protein GAP-43 and the transcription factors c-Jun, Jun D and CREB (Woolf, et al., 1990, Verge, et al., 1992, Chong, et al., 1992, Herdegen, et al., 1992). The functional role of NO is unknown. It is possible that it promotes regeneration by increasing blood flow to the injured neuron due to its potent vasodilatory properties (Moncada, et al., 1991). NO also stimulates glial cell guanosine 3',5'-cyclic monophosphate (cGMP) second messenger systems and may cause release of neurotrophic factors that aid neuronal survival (Morris, et al., 1992). However, NOS expression is also thought to be associated with neuronal death (Wu and Li, 1993) and may play a role in the death that occurs in 25% of axotomised DRG neurons (Aldskogius, et al., 1985). GAP-43 is a phosphoprotein transported to

regenerating axons in the periphery (Bisby, 1988) where it is thought to influence neurite elongation and synaptic formation (Benowitz and Routtenberg, 1987).

Transcription factors expressed following axotomy may play a role in regulating and co-ordinating the expression of a number of genes. Their role in the axotomy response is discussed further in section 1.6.3 and chapter 2. The mechanisms by which they may act are described in the following sections.

It would appear that peripheral neurons mount an integrated response to injury involving numerous changes in gene expression. How these changes are initiated and co-ordinated are not clear. However, it is likely that a study of mechanisms responsible for the expression of individual genes will lead to an understanding of common pathways responsible for the co-ordinated expression of a number of genes in axotomised sensory neurons.

1.4 Mechanisms of action of transcription factors

The transcriptional selectivity of eukaryotic genes is mediated by a variety of complex control sequences usually located within the 5' flanking region (for a review see Johnson and McKnight, 1989). Combinations of these control sequences, for example enhancers or transcriptional activators, in tandem with promoter elements allow multiple distinct regulatory proteins to bind and co-ordinately regulate RNA synthesis via their interactions with the RNA polymerase II (pol II) transcriptional machinery. Fractionation of HeLa cell transcriptional machinery has shown it to be composed of seven general factors: TFIIA, TFIIB, TFIID, TFIIE, TFIIH and TFIIJ (Conaway and Conaway, 1993, Zawel and Reinberg, 1993). In the absence of an activator such as the transcription factors CREB, Jun or

Fos, these factors assemble into a pre-initiation complex in a defined order initiated by the binding of TFIID to the TATA motif (Zawel and Reinberg, 1993). TFIID is a multi-subunit protein containing a DNA binding subunit, termed TATA binding protein (TBP) and multiple TBP associated proteins (TAS) (Dynlacht, et al., 1991). Although TBP can mediate basal transcription by pol II, the TAFs are necessary for a response to activators (Dynlacht, et al., 1991). Two models exist to explain the mechanism of activator induced gene transcription. One suggests that the activator stimulates the step-wise formation of the pol II complex from individual components. Alternatively the complex may be pre-assembled in solution and the activator recruits and stabilises its binding to the DNA. Most experiments have suggested that activators increase the number of pol II transcription complexes instead of increasing the rate of their formation (Johnson and Krashnow, 1992). A transcriptional coactivator which provides a link between the activator CREB and the pol II transcription complex has recently been discovered (Arias, et al., 1994, Kwok, et al., 1994, Chrivia, et al., 1993). Experiments have shown that following binding of CREB to its DNA recognition sequence (the CRE), phosphorylation of CREB increases its affinity for a CREB binding protein CBP. An activation domain of CBP is then thought to be directly able to interact with TFIIB which then interacts with the TATA box binding protein TBP. By interacting with TFIIB, the CBP protein is thought to be able to recruit the pol II transcription factor complex to the promoter region of the DNA resulting in enhanced gene transcription. CBP has also been implicated in mediating the actions of the transcriptional activator c-Jun, as signal dependent phosphorylation of c-Jun at serine 63 and serine 74, increases its affinity for CBP (Arias, et al., 1994).

It is possible that proteins binding to different regulatory sequences may synergise in their ability to activate pol II. For instance proteins binding to the CRE can synergise with the LIM family transcription factor Isl-1 to promote expression of the somatostatin gene in pancreatic islet cells (Comb, et al., 1988, Vallejo, et al., 1992) and an AP2 element can act synergistically with the CRE sequence of the human proenkephalin gene (Hyman, et al., 1989).

Transcription factors recognise DNA sequences by forming specific structures (motifs) that are conserved between binding proteins. Several different motifs exist, for example, the helix turn helix motif, the zinc finger motif, the leucine zipper motif and the helix loop helix motif (Johnson and McKnight, 1989). The helix loop helix motif consists of two α helices bridged by a sharp β turn. One helix is oriented in a manner allowing close positioning next to the major groove of DNA with specificity due to small differences in the shape or projection of the helix-turn-helix motif. This motif has been shown to be responsible for the DNA recognition characteristics of homeobox proteins concerned with developmental regulation of gene expression (Johnson and Herskowitz, 1985). The zinc finger motif is characterised by an ordered arrangement of histidines and cysteines forming a tetrahedral coordination complex (zinc finger) with a zinc ion (Brown, et al., 1985). This type of motif is found in the nuclear receptor family of proteins that recognise steroid and thyroid hormones. Following ligand binding the complex binds to a specific DNA sequence to regulate transcription. DNA specificity is determined by spacing and orientation of the zinc fingers. The helix loop helix is composed of two α helices separated by a loop of variable length (Murre, et al., 1989). They comprise a family of transcription factors able to form homodimers or heterodimers via interactions between their helix loop helix motifs. Many members of this

family have a domain of basic amino acids immediately upstream of the helix loop helix domain that determines DNA binding specificity. Members of the E box family of binding proteins are known to form helix loop helix motifs (Murre, et al., 1989, Begley, et al., 1992). The motif termed the leucine zipper is formed by the hydrophobic interactions between α helices of two polypeptide chains. The helices, each containing a heptad repeat of leucines, are flanked by highly basic regions thought to directly interact with DNA (Landschulz, et al., 1989). Protein dimerisation brought about by formation of the leucine zipper is necessary for DNA interactions (Landschulz, et al., 1989, Gentz, et al., 1989). The leucine zipper family of transcription factors includes CREB, Fos and Jun.

1.4.1 Cyclic AMP response elements (CREs)

A number of genes are known to respond to cAMP, for example VIP, somatostatin, tyrosine hydroxylase, leu-enkephalin, choriogonadotropin α ($CG\alpha$), c-Fos and phosphoenolpyruvate carboxykinase (Montminy, et al., 1986, Hayakawa, et al., 1984, Silver, et al., 1987, Sheng, et al., 1990). By analysing the response of reporter constructs to cAMP, together with mutational analysis of sequences within these constructs, short DNA sequences have been identified that confer a response to cAMP. These genes often contain an exact or nearly perfect copy of an 8-bp palindrome 5'-TGACGTCA-3' (a cyclic AMP response element: CRE) in their 5' flanking regions (Montminy, et al., 1986, Comb, et al., 1986, Tsukada, et al., 1987, Fink, et al., 1988). However, the minimal sequence required for cAMP responsiveness is not known. For instance, while the 8bp core sequence, 5'-TGACGTCA-3', alone is sufficient to confer cAMP responsiveness on the $CG\alpha$ gene promoter the activity of this sequence is highly dependent on neighbouring nucleotides. Nucleotides flanking the CREs of either rat

glucagon or bovine parathyroid hormone considerably reduce the activity of the CG α CRE while flanking sequences from the somatostatin CRE have no effect on the CG α CRE activity (Deutsch, et al., 1988). Similarly, the 10-bp sequence 5'-CTGACGTCAG-3' from the rat somatostatin gene does not confer cAMP responsiveness on the SV40 promoter although increasing its size to include 21bp of flanking sequence does result in a response to cAMP. An 8-bp sequence 5'-TGACGTCT-3' from the human VIP gene is considerably less active than an extended 17-bp sequence from the same gene which contains the sequence 5'-CGTCA-3', 5 nucleotides upstream from the TGACGTCT sequence. This CGTCA sequence has been shown to be required for full cAMP responsiveness of the human VIP gene (Tsukada, et al., 1987). The two sequences from the human VIP gene 5'-CGTCA-3' and 5'-TGACGTCT-3' have also been shown to act synergistically with each other in their response to cAMP.

One possible explanation for the large effect of neighbouring nucleotides on CRE activity is that the consensus sequence is actually larger than the 8bp suggested. Mutational analysis suggests that nucleotides several bases removed from the 8bp consensus CRE are involved. DNase 1 footprinting studies (which identify protein binding sites) have suggested that proteins are capable of binding to sequences adjacent to the 8bp core and as such may regulate cAMP responsiveness (Montminy and Bilezikjian, 1987). Further complications arise due to the fact that some genes contain the identical 8bp palindrome in their 5' flanking sequence, but are not known to be responsive to cAMP, for example the bovine parathyroid hormone gene and the rat glucagon gene (Weaver, et al., 1984). It is possible that active CREs possess a different secondary structure to inactive CREs, a property that may be conferred by flanking sequences. The presence of a short homopurine sequence, GAGAGAGA,

just 3' of the CRE in both the human and rat somatostatin genes suggests that in these genes at least secondary structure of flanking sequences is important.

The human enkephalin CRE has been shown to be capable of exhibiting a spontaneous conformational change from a duplex to a hairpin formation (McMurray, et al., 1991). Mutations that are expected to enhance hairpin (cruciform) formation have also been shown to increase the enhancer like activity of the enkephalin gene (McMurray, et al., 1991). Thus hairpin formation may allow alternative transcriptional regulation through altered protein binding. One of the requirements for hairpin formation is the presence of a palindrome. As many CRE sequences contain perfect or near perfect palindromes, it is conceivable that they also possess the ability to form cruciform structures resulting in alternative protein DNA complex formation. Cruciform structure is probably greatly affected by flanking sequences that can destabilize the palindrome dependent structure. Alternatively sequences flanking inactive CREs may contain repressor activity by binding to proteins that interfere with the activation of transcriptional machinery.

When a mutant CRE like palindrome is placed in tandem with a perfect CRE it is often able to increase cAMP responsiveness, while having no effect when present on its own (Deutsch, et al., 1988). It is possible that palindrome recognising proteins, for example CREB, could positively co-operate with each other when bound to adjacent sequences. As a full turn of β DNA helices occurs every 10bp it is likely that binding sites must also occur at 10bp intervals for full co-operativity to be observed. Experiments have shown that this is the case for repressor binding to λ DNA which shows co-operativity when the binding sites are 10bp apart (Whitson, et al., 1987). However for co-operativity between domains of the SV40 enhancer

and multiple regulatory elements of the mouse metallothionein-I gene, requirements for spacing intervals are far less rigid (Searle, et al., 1984).

It is known that the sequence between -94 and -67 of the human VIP gene (Fig. 1.1) is capable of mediating a response to cAMP when in the context of heterologous promoters and is capable of enhancing basal stimulation in certain cell types (Tsukada, et al., 1987, Deutsch, et al., 1988). It is possible that a similar sequence from the rat VIP gene (Fig. 1.1) will mediate the increased transcription of VIP axotomised DRG neurons or a response to elevated cAMP.

1.4.2 Transcription factors that bind the CRE

CREB, Fos and Jun belong to a class of transcription factors that contain a conserved basic leucine zipper domain (bzip) required for DNA recognition, DNA binding and protein dimerisation (Landschulz, et al., 1989, Gentz, et al., 1989). The bzip containing transcription factors can be divided into three families depending on the similarities of their structures, their ability to dimerise with other bzip containing proteins and their DNA binding specificities. The first family is that of the cAMP-response element binding proteins (CREB) and activating transcription factor (ATF) proteins, which bind to CRE sequences and regulate transcriptional responses to cAMP and also to certain viral proteins (Hai, et al., 1989). The second family is that of the Fos and Jun related oncoproteins which can heterodimerise to form the AP1 transcriptional activating complex that can respond to 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induced stimuli and bind to AP1 related sequences comprising of the 7 nucleotides TGAC/GTCA (Curran and Franzen, 1988). Members of this family have also been shown to be capable of binding to similar CRE sequences (Nakabeppu, et al., 1988, Halazonetis, et al., 1988). The third family is

composed of CAAT box/enhancer binding proteins (C/EBPs), which can bind to CAAT box and related enhancer sequences to regulate gene expression during cellular differentiation or in response to inflammatory cytokines (Landschulz, et al., 1989). A great deal of heterodimerisation can occur between proteins within each family via leucine zipper interactions. In some cases proteins from different families are able to heterodimerise, however, cross family dimerisation is more limited. Thus CREB and ATF1 form heterodimers (Hoeffler, et al., 1991) while ATF2, 3 and 4 can heterodimerise with Fos, Jun or Fra-1 *in vitro* (Hai and Curran, 1991). Similarly, C/EBP β has been shown to heterodimerise with C/ATF (a member of the ATF family) (Vallejo, et al., 1993). Experiments have shown that heterodimers can have different DNA binding affinities than either of their parent homodimers (Halazonetis, et al., 1988, Hai and Curran, 1991). It is also conceivable that inter and intra family heterodimerisation results in complexes with different transcriptional regulatory activities or complexes that respond differently to various signal transduction pathways. Activation of different signalling pathways may result in the formation of different complexes leading to alternative regulation. This is the case for the AP1 complex that has been shown to vary in composition in PC12 cells in response to NGF and TPA (Quinn, 1991). As well as activators of gene expression, proteins inhibiting expression are capable of binding to CRE elements. For example CREB 2 has been shown to significantly repress expression of CRE dependent transcription (Karpinski, et al., 1992), as has ATF1 (Lemaigre, et al., 1993) and CREM (CRE modulating protein) (Foulkes and Sassone-Corsi, 1992). Similarly a complex composed of c-Fos and Jun B has been shown to inhibit expression of the proenkephalin gene by binding to its CRE like sequence (Kobierski, et al., 1991).

Combinations of all these effects greatly increase the number of possibilities for control at the gene transcriptional level mediated by CRE like sequences.

1.5 Mechanisms of calcium and cAMP signalling

1.5.1 cAMP

In eukaryotes the most common mechanism whereby the second messenger cAMP acts is by stimulating the activity of the cAMP dependent protein kinase A (PKA). Following cAMP binding to the regulatory subunit of PKA the enzyme dissociates to yield a regulatory subunit dimer and two identical catalytic subunits which catalyse the transfer of a phosphate group from ATP to serine or threonine residues on specific protein substrates (McKnight, 1991, Taylor, 1989). The catalytic subunit is necessary for activation of cAMP induced gene expression (Montminy, et al., 1986, Grove, et al., 1987). Once released from the regulatory subunit, the free catalytic subunit can translocate from the cytoplasm to the nucleus where it phosphorylates nuclear factors involved in transcriptional control (Adams, et al., 1991, Meinkoth, et al., 1990). In mammals, the best characterised transcriptional response to cAMP is mediated by CREB and its family members (Montminy and Bilezikjian, 1987). Members of the CREB family of transcription factors possess a domain that contains consensus phosphorylation sites for a variety of protein kinases including PKA, protein kinase C, casein kinase I and II and others (Brindle and Montminy, 1992). PKA activates CREB by phosphorylation at a single serine residue at position 133 (Gonzalez and Montminy, 1989) through a mechanism that apparently does not change the affinity of CREB for its DNA binding site in the gene promoter (the CRE) but can increase transcription. A number of other proteins have also been implicated in cAMP dependent regulation of

gene expression. For instance, the transcription factors Jun D and Jun B have been shown to be involved in cAMP dependent proenkephalin transcription (Kobierski, et al., 1991).

1.5.2 Calcium

Alterations in the free cytoplasmic calcium concentrations bring about changes in numerous cellular processes, including changes in gene transcription. Initial studies on transcriptional activation by calcium employed the PC12 cell line. In these cells, KCl-evoked depolarisation induces expression of the fos proto-oncogene (Sheng, et al., 1990, Morgan and Curran, 1986). This effect is dependent on the influx of calcium through dihydropyridine-sensitive calcium channels as well as on the activity of the intracellular calcium binding protein calmodulin (Morgan and Curran, 1986, Greenberg, et al., 1986). A CRE like element in the promoter region of the c-fos gene functions as a calcium response element (Sheng, et al., 1990). This element binds CREB and has the ability to mediate activation of the c-fos gene in response to either cAMP or calcium (Sheng, et al., 1990). In addition phosphorylation of CREB at serine 133 is required for both cAMP and calcium induced stimulation of c-fos expression (Sheng, et al., 1991). However, as membrane depolarisation has little effect on cAMP metabolism in PC12 cells it is unlikely that PKA activation leads to CREB phosphorylation in response to calcium influx (Sheng, et al., 1990, Van Nguyen, et al., 1990). Instead as CREB has been shown to be an excellent substrate for calcium/calmodulin dependent protein kinase it may be phosphorylated by this kinase in response to increased calcium concentrations (Sheng, et al., 1991, Dash, et al., 1991). Calcium may also stimulate gene transcription by activating transcription factors capable of binding to sites other than the CRE. For example, c-fos gene constructs in

which the CRE element has been mutated without altering the rest of the construct are still responsive to depolarisation (Sheng, et al., 1990). The most characterised alternative element is the phorbol ester-response element (TRE), that shares homology with the CRE element and can bind proteins of the AP1 complex, (c-Fos and c-Jun heterodimers) (Lee, et al., 1987). This element is also able to bind distinct complexes containing other members of the Fos and Jun families, such as Jun B (Ryder, et al., 1988), Jun D (Hirai, et al., 1989, Ryder, et al., 1989) and a Fos related antigen Fra1 (Cohen and Curran, 1988). Alternative experiments using macrophage and fibroblast cell lines have shown that calcium stimulates c-Fos transcription by relieving a block in mRNA elongation within the first intron. This blockage is removed in a calcium dependent manner, using activators of PKC and PKA or a calcium ionophore (Mechti, et al., 1991, Collart, et al., 1991).

It appears that pathways used by calcium and cAMP to regulate gene expression may be dependent, at least in part, on common regulatory mechanisms. However, when cells are treated with stimuli that increase intracellular levels of both cAMP and calcium a greater than additive (synergistic) increase in the levels of gene expression can occur. For instance, the VIP gene, c-Fos gene, human chorionic gonadotrophin gene and the human proenkephalin gene have all been reported to respond with a synergistic increase in expression to combined activation of cAMP and calcium dependent signalling pathways (Sheng, et al., 1990, Van Nguyen, et al., 1990, Mulderry, 1993, Andersen, et al., 1988). This suggests that phosphorylation of CREB at serine 133 is not the only mechanism whereby both these signalling pathways are regulating gene expression.

Calcium and cAMP signalling pathways have been shown to interact at several other levels in neurons. For instance, activation of dopamine D1

receptors results in a cAMP and PKA-mediated increase in the activity of a dihydropyridine-sensitive calcium current in bovine chromaffin cells (Artalejo, et al., 1990). In certain neurons, including pyramidal and granule cells of the hippocampus, a calcium activated adenylate cyclase is expressed at high levels. Stimulation of these cells by glutamate results in an NMDA receptor induced calcium influx followed by accumulation of cAMP (Chetkovich, et al., 1991, Xia, et al., 1991). Cross talk between the two pathways may also occur at the level of the protein kinase, as it has been postulated that a calcium dependent activation of the protease calpain results in the degradation of the regulatory subunit of PKA and subsequent activation of the catalytic subunit (Aszodi, et al., 1991). The two pathways may also result in alternative phosphorylation of transcription factors involved in gene expression at sites other than serine 133 of CREB. Multiple phosphorylation by the two different pathways may result in antagonistic or synergistic activation of gene transcription.

It would appear that integration of multiple signals can regulate gene expression as shown by the synergistic effect of cAMP and calcium pathways. One situation in which synergy may play an important role is the seizure model. During a seizure synchronous firing of many neurons occur, resulting in the release of numerous transmitters such as glutamate, capable of depolarising target neurons and other neuropeptides capable of activating the cAMP or protein kinase C pathways. The high levels of proenkephalin mRNA induction reported in the hippocampus in response to seizures are consistent with a synergistic activation of expression by cAMP and calcium (White and Gall, 1987).

1.6 Regulation of VIP expression in DRG

1.6.1 Neurotrophic regulation of VIP expression

Many of the biochemical changes seen following axonal damage can be reproduced in the absence of physical nerve damage by applying vinblastine, an inhibitor of axoplasmic transport, to the peripheral nerve (Kashiba, et al., 1992, Keen, et al., 1989). This results in decreased expression of substance P and CGRP with a concomitant increase in the expression of VIP and galanin. One interpretation of these data is that neuropeptide expression is dependent on regulatory factors originating in the periphery and carried to the cell bodies of DRG neurons by retrograde axoplasmic transport. One possible candidate for such a factor is NGF, a well-characterised trophic factor known to be important for the development and maintenance of sensory neurons (Johnson, et al., 1986). NGF is produced by target tissues, internalised at the axonal endings and transported retrogradely to the cell body in the DRG (Yip, et al., 1984, Richardson and Riopelle, 1984). Following axotomy, the peripheral supply of NGF is effectively cut off, although Schwann cells in the distal stump of the nerve begin to synthesise NGF (Heumann, et al., 1987) and the peripheral axon contains NGF receptors along its length (Richardson and Riopelle, 1984). Thus it is likely that the cell body continues to receive some NGF although in very much reduced quantities.

The application of NGF to a cut peripheral nerve of adult rats prevents the cell loss usually seen in DRGs (Otto, et al., 1987, Arvidsson, et al., 1986) and more than reverses the down regulation of substance P and CGRP neuropeptides caused by axotomy (Inaishi, et al., 1992, Fitzgerald, et al., 1985, Wong and Oblinger, 1991). Similarly experimental deprivation of NGF *in vivo* by immunising animals against NGF (Keen, et al., 1989) results in reduced substance P expression. However, this appears to have

no effect on VIP expression in DRG neurons (Keen, et al., 1989). Whereas adult rat DRG neurons in culture require the presence of NGF for continued expression of both substance P and CGRP, VIP expression appears to be independent of NGF (Mulderry and Lindsay, 1990). Thus the effects of peripheral axotomy on substance P and CGRP expression appear to be due to a reduction in available NGF while VIP would appear to be dependent on other factors. There is, however, some evidence that NGF may suppress the expression of VIP in sensory neurons. Application of anti-NGF antibodies to the sciatic nerve has been reported to increase VIP expression in the dorsal horn of the spinal cord in adult rats in the absence of nerve injury. This VIP probably originates from DRG neurons (Knyihár-Csillik, et al., 1991). Also VIP expression in cultured neonatal rat DRG neurons is suppressed by NGF (Mulderry, 1994). It would seem likely that while NGF may play a role in VIP expression NGF deprivation alone is insufficient for spontaneous VIP expression in adult rat DRG neurons following axotomy.

DRG neurons are responsive to a number of neurotrophic factor proteins other than NGF and these may also play a role in VIP expression following axotomy. For instance, embryonic chick DRG neurons can be kept alive in culture by other members of the NGF-related neurotrophin family, including brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (Kalcheim, et al., 1987, Lindsay, et al., 1985, Maisonpierre, et al., 1990, Rosenthal, et al., 1990). In addition, two cytokine-related proteins ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF, also known as embryonic stem cell differentiation inhibiting activity or sympathetic neuron cholinergic differentiation factor), promote the survival of embryonic mammalian DRG neurons in culture (Barbin, et al., 1984, Murphy, et al., 1993, Murphy, et al., 1991) and LIF will promote the survival

of axotomised rat DRG neurons *in vivo* (Cheema, et al., 1994). It is possible that one or more of these alternative neurotrophic factors are responsible for VIP expression following axotomy. Receptors for all these factors are expressed in postnatal rat DRG neurons (Hendry, et al., 1992, Ip, et al., 1993, Mu, et al., 1993) and binding results in internalisation and retrograde axoplasmic transport of the factors to the cell body (Hendry, et al., 1992, Curtis, et al., 1993, DiStefano, et al., 1992). It has also been shown that nerve injury results in increased retrograde transport of CNTF and LIF in sensory neurons and that expression of LIF is increased in the distal section of the damaged nerve (Curtis, et al., 1993, Curtis, et al., 1994).

A spontaneous increase in VIP expression occurs in sympathetic neurons grown in dissociated cell or explant cultures, or when these neurons are axotomised. Expression is reduced when non-neuronal cells are removed and increased following the addition of medium conditioned by ganglionic non-neuronal cells. This increase is due to LIF produced at elevated levels in non-neuronal cells following axotomy or in culture (Sun, et al., 1994). In this context it is interesting to note that many genes respond to infection, inflammation or other pathological conditions, with a change in gene transcription termed an acute phase response. Several cytokines have been shown to regulate these responses, for instance LIF, interleukin 1 and 6 and glucocorticoids (Brasier, et al., 1990, Baumann and Wong, 1989, Gauldie, et al., 1987). Relevant here is the fact that human VIP 5' flanking region has been shown to contain an acute phase response element, a consensus sequence that is thought to bind proteins that can regulate gene transcription in response to inflammation (Symes, et al., 1994). Furthermore CNTF and LIF have been shown to increase the expression of VIP in a human neuroblastoma cell line (NBFL) by acting through a short DNA sequence containing this same element (Symes, et

al., 1994, Symes, et al., 1993). It is possible that increased LIF expression, following axotomy of DRG neurons, results in changes in transcription factor binding to an acute phase response element of the rat VIP gene. This in turn could lead to increased VIP expression. However, adult rat DRG neurons in culture show no change in VIP expression following addition of exogenous BDNF, NT-3, CNTF or LIF (Mulderry, 1994). LIF does increase VIP expression in neonatal rat DRG neurons cultured in the presence of NGF (Mulderry, 1994) suggesting that LIF may be important for VIP expression in neonatal rat DRG neurons.

1.6.2 Calcium and cAMP dependent regulation of VIP expression

Due to the large electrochemical gradient across the plasma membrane it has been calculated that intracellular calcium will reach millimolar levels in the terminal 0.5mm of a cut axon (Meri, et al., 1983). Calcium is an important regulatory ion acting on numerous proteins in neuronal cells. Therefore, many of the neuronal changes that occur following axotomy may be the result of alterations in intracellular Ca^{2+} levels. For instance, chelating extracellular calcium can prevent peripheral nerve degeneration. Conversely, elevating intracellular Ca^{2+} with the ionophore A23187 results in axoplasmic disintegration similar to degeneration (Schlaepfer, 1974, Schlaepfer, 1977). In contrast calcium is required for neuronal survival and a sustained increase in intercellular Ca^{2+} will increase the survival of neurons in culture (Collins, et al., 1991). Changes in intracellular calcium concentrations can lead to changes in gene expression (see section 1.5.2) suggesting the possibility that calcium may regulate VIP expression following axotomy. VIP expression can be stimulated in DRG neurons by applying capsaicin (a neurotoxin) to an intact peripheral nerve (Anand, et

al., 1990). Capsaicin induces calcium influx into DRG neurons in culture (Wood, et al., 1988, Winter, 1987) and VIP expression may be regulated by capsaicin in these neurons through activation of calcium dependent protein kinase C. An increase in protein kinase C activity induced by the phorbol ester TPA, results in a specific increase in expression of VIP in both chromaffin cells and neuroblastoma cells (Agoston, et al., 1992, Pruss, et al., 1985). Similarly, depolarisation with 40mM K⁺ has been shown to increase VIP expression in rat superior cervical ganglion (sympathetic) neurons (Sun, et al., 1992). Since this treatment is known to open voltage sensitive calcium channels (Van Nguyen, et al., 1990) and can lead to a sustained increase in intracellular calcium (Collins, et al., 1991) it seems likely that VIP expression is being increased by calcium.

VIP expression can also be increased in a wide variety of cells by treatment with drugs that elevate intracellular cAMP concentrations. Hayakawa et al (1984) have shown that in human neuroblastoma cells VIP gene transcription can be induced substantially by treatment with cAMP analogues. Thus for example 1mM dibutyryl cAMP stimulates the synthesis of pre-pro VIP-PHM-mRNA by up to 11 fold (Hayakawa, et al., 1984). Similarly, treatment of bovine chromaffin cells with the diterpene forskolin, an activator of adenylate cyclase (Seamon, et al., 1981), results in a 20 fold increase in VIP expression. Elevated cAMP levels have also been shown to increase expression of human VIP reporter constructs transfected into either HeLa or PC12 cells (Fink, et al., 1991), suggesting that expression of endogenous VIP could be increased by stimuli that raise cAMP levels. Axotomy has been shown to increase protein kinase A (PKA) like activity in certain neurons (Hall and Kosik, 1993). As PKA is directly regulated by cAMP (see section 1.5.1) it is possible that cAMP dependent signalling

pathways are responsible for changes in gene expression following axotomy of DRG neurons.

1.6.3 Transcription factor expression in response to axotomy

Axotomy of peripheral neurons leads to changes in the expression of a number of transcription factors some of which could be involved in regulating expression of VIP. For instance, transection of the rat sciatic nerve results in an increase in c-Jun immunoreactivity, in the ipsilateral DRG, that is visible (by immunocytochemistry) after 10h and remains elevated for over 300 days (Herdegen, et al., 1992). This is probably due at least in part to an increase in transcription as c-Jun mRNA has been shown to increase in these neurons following axonal damage (Jenkins and Hunt, 1991). Similarly increased expression of both Jun D and CREB protein occurs in DRG neurons *in vivo* following axotomy (Herdegen, et al., 1992). Increased expression of Jun D protein becomes visible at 15h and declines to control levels by 100 days. In contrast the detectable increase in CREB expression does not occur until 10 days after transection and reaches its maximum by 100 days. It has been suggested that the Jun proteins may play a role in the immediate response to axotomy while all three transcription factors may be involved in long term cell repair and survival (Herdegen, et al., 1992). This effect is specific for c-Jun, Jun D and CREB as no increase in expression of Jun B, Fos or the transcription factor Krox 24 could be detected in DRG neurons following axotomy (Herdegen, et al., 1992). It is likely that increased expression of transcription factors results in variations in the composition of transcription complexes binding to control DNA sequences.

Transcription factors induced by axotomy are likely to play an important role in the regulation of genes associated with regeneration (Leah, et al.,

1991, de Felipe, et al., 1993, Herdegen, et al., 1993, Herdegen, et al., 1993). As such they may be involved in the increased expression of proteins such as neuropeptides and cytoskeletal elements that occurs following axonal damage of DRG neurons. Jun D, c-Jun and CREB can all form complexes capable of binding to a CRE consensus sequence *in vitro* (Nakabeppu, et al., 1988, Fink, et al., 1991). Therefore, it is possible that by binding to the rat VIP CRE these transcription factors are involved in VIP gene expression following axotomy.

1.6.4 Transcriptional verses non-transcriptional regulation of VIP

Spontaneous VIP expression in adult rat DRG neurons following axotomy is paralleled by a similar increase in VIP mRNA levels making it seem likely that regulation occurs at the level of transcription (Noguchi, et al., 1989). As VIP can be regulated by cAMP and calcium at the level of transcription and spontaneous expression of VIP may be due to transcription factors expressed in response to axotomy (sections 1.6.3 and chapter 2) it would seem likely that spontaneous VIP expression is due to increased transcription. It is possible however that expression is due to differential stability of mRNA, or alternative splicing as discussed further in chapter 6.

1.7 A Model system for the study of VIP gene expression in DRG

In order to determine mechanisms responsible for VIP induction in DRG neurons following axotomy, DRG neurons in culture were used as a model system. Growth of DRG neurons in primary culture causes axotomy of all neurons which then display many of the properties of DRG neurons axotomised *in vivo*. Thus, for example, within 4 days in culture, adult rat

DRG neurons grown in the absence of nerve growth factor (NGF), show a decrease in expression of the neuropeptides substance P and CGRP while at the same time VIP which is initially undetectable increases substantially (Mulderry, 1993, Mulderry and Lindsay, 1990). VIP is expressed in DRG neurons of both adult and neonatal rats following nerve injury (Anand, et al., 1990). However, mechanisms leading to VIP expression are different between adult and neonatal rat DRG neurons in culture (Mulderry, 1993, Mulderry, 1994). Although adult rat DRG neurons spontaneously express VIP, in culture, the level of expression can be increased by exposure to drugs that raise intracellular calcium or cAMP individually during the first 4 days in culture (40mM K⁺ or 10 μ M forskolin). When cells are grown in the presence of both 40mM K⁺ and 10 μ M forskolin during the first 4 days in culture the resulting VIP concentrations are only slightly higher than with forskolin alone and much less than would be expected if the effects of depolarisation and forskolin were additive (Mulderry, 1993).

In contrast, VIP is not normally expressed in neonatal rat DRG neurons grown in culture. However, expression can be stimulated by the addition of drugs that increase intracellular cAMP or calcium concentrations. Cultured newborn rat DRG neurons express detectable levels of VIP when grown in the presence of either 40mM K⁺ or 10 μ M forskolin alone (Mulderry, 1993). However, when grown in the presence of 40mM K⁺ and 10 μ M forskolin together, concentrations of VIP obtained are significantly higher than the sum of those resulting from forskolin or depolarisation alone. This indicates a synergistic interaction between the two stimuli that does not occur in cultured adult rat DRG neurons (Mulderry, 1993).

The effects of forskolin on VIP expression in adult and neonatal rat DRG neurons could be reproduced using 8-bromoadenosine 3',5'cyclic monophosphate, a membrane permeable cAMP analogue, or the cAMP

phosphodiesterase inhibitor, IBMX. This suggests that the effect of forskolin is due to an increase in intracellular cAMP. The synergistic response to forskolin and depolarisation in neonatal rat DRG neurons could be blocked by nifedipine, a calcium channel antagonist, which inhibits calcium channel opening under depolarising conditions. The synergistic response can also be mimicked by using forskolin together with Bay K 8644, a dihydropyridine-sensitive (L-type) voltage gated calcium channel agonist, which stimulates calcium channel opening at negative membrane potentials. These results suggest that the effect of depolarisation on VIP expression is due to an influx of calcium through voltage sensitive calcium channels (Mulderry, 1993).

It is not known whether the spontaneous expression of VIP in adult rat neurons in culture is dependent on increased cAMP or an influx of calcium. However, the loss of a synergistic response to combined cAMP and calcium dependent pathways in cultured adult rat DRG neurons parallels the appearance of spontaneous VIP expression making it seem likely that the two are related. Both cAMP and calcium can activate signalling pathways leading to increased gene expression (see section 1.5). Therefore, even if VIP expression in cultured adult rat DRG neurons is not dependent on elevated intracellular cAMP and calcium it could be dependent on a constitutive activation of later stages in these pathways. Constitutive activation of these later stages could prevent further expression due to combined cAMP and calcium. A constitutive activation occurring in cultured adult but not neonatal rat DRG neurons could explain why VIP is spontaneously expressed in cultured adult but not neonatal rat DRG neurons. It could also explain why the synergistic response to combined cAMP and calcium dependent pathways only occurs in cultured neonatal rat DRG neurons.

Calcium and cAMP dependent pathways are known to be able to increase gene transcription by acting on transcription factors such as those that bind to cyclic AMP responsive elements (CREs) (see section 1.4.2). The last stage at which pathways leading to spontaneous or regulated expression could converge is therefore at the point of transcription factor binding to a CRE. The CRE located in the rat VIP gene 5' from its transcription start site (Giladi, et al., 1990), may therefore be involved in both the spontaneous expression of VIP in cultured adult rat neurons and the response to cAMP and calcium that occurs in cultured neonatal rat DRG neurons.

There is a strong precedence for the role of the CRE in both the spontaneous and synergistically regulated VIP expression described above. A CRE element can confer a synergistic increase in expression of the proenkephalin gene in response to combined forskolin and depolarising concentrations of K⁺ in PC12 cells (Van Nguyen, et al., 1990). Also CREs including the human VIP CRE can increase the basal levels of reporter gene expression (Deutsch, et al., 1988).

1.8 Expression of the VIP gene following axotomy: a hypothesis

Following the above discussion it is possible to propose a tentative hypothesis showing mechanisms mediating the spontaneous expression of VIP in cultured DRG neurons. This is shown in a schematic form in Fig. 1.2. Stimulation of neurons with agents that activate cAMP and calcium signalling pathways may lead to activation of transcription factors that bind to the VIP CRE. Through interactions with the transcriptional machinery this then leads to an increase in VIP transcription. The actions of cAMP and calcium on CRE regulated expression may be synergistic in neonatal rat DRG neurons. Plating adult rat DRG neurons in culture is thought to have

an action similar to that of axotomy and leads to spontaneous VIP expression. This may be may be due to constitutive activation of elements within the cAMP and calcium dependent pathways (see section 1.7).

It is also possible that transcription factors such as c-Jun, which show increased expression following axotomy, lead to increased expression of VIP. These transcription factors may act by binding to sequences in the VIP gene, perhaps even the CRE, to regulate transcription. The rat VIP CRE is therefore likely to play a major role in the regulation of VIP expression in cultured DRG neurons and following axotomy of DRG neurons *in vivo*.

1.9 Aims of this thesis

The aim of this thesis is to determine mechanisms responsible for the spontaneous expression of VIP that occurs in cultured adult rat DRG neurons with particular reference to the CRE. It is hoped that this will shed light on the mechanisms responsible for VIP induction following axotomy *in vivo*. These mechanisms may also play a role in the regulation of other genes as part of the axotomy response.

Figure 1.1. Human and rat VIP CRE sequences

A. The rat VIP CRE containing sequence from position -94 to -67 relative to the transcription start site. Linkers allowing cloning into the unique *Sa*I restriction site of the *c-fos* minimal promoter are indicated in bold type.

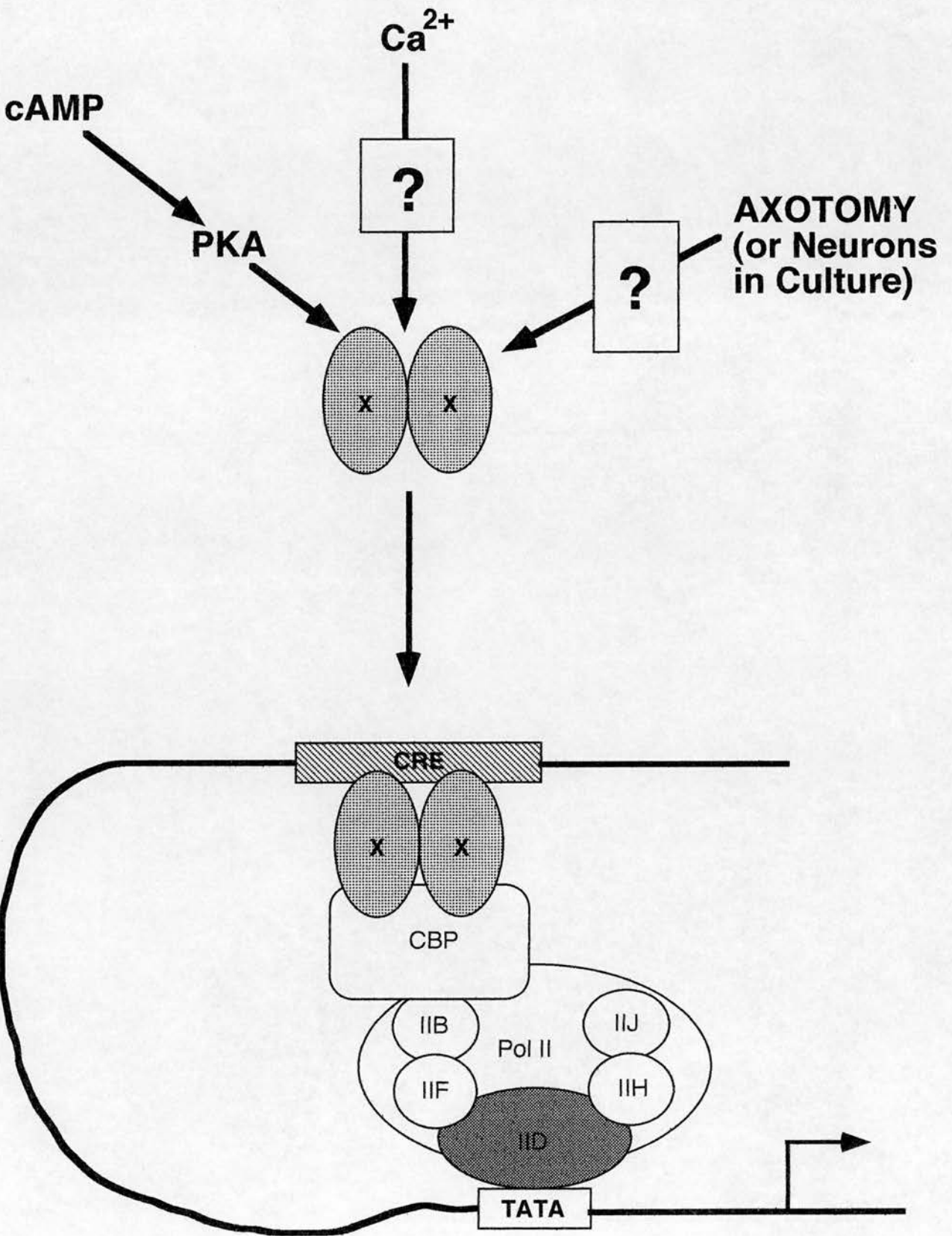
B. The human VIP CRE sequence from position -94 to -67 relative to the transcription start site.

Differences between the two sequences are underlined.

- A 5' -TCGACCATGGGATCACCCTGTGACGTCTTTCA-3'
GGTACCCTAGTGGTGACACTGCAGAAAGTAGCT-5'
- B 5' -CCATGGCCGTCATACTGTGACGTCTTTC-3'

Figure 1.2. Possible mechanisms leading to spontaneous expression of VIP in cultured DRG neurons

It is possible that three main pathways exist for the regulation of VIP at the level of transcription in cultured DRG neurons. The first two are the cAMP and calcium signalling pathways which converge to act on transcription factors that bind the VIP CRE leading to increased gene expression. The actions of these two pathways are synergistic in neonatal but not adult rat DRG neurons. The third pathway results from placing the neurons in culture (thought to have an action similar to that of axotomy) which causes spontaneous VIP expression in adult rat DRG neurons. Axotomy may result in increased expression of transcription factors that increase VIP transcription, perhaps by binding to the CRE, or it may act on components of the cAMP/calcium pathway to activate transcription. The latter case may explain why adult rat neurons spontaneously express VIP but don't respond synergistically to cAMP and calcium. PKA refers to protein kinase A. X=unknown transcription factor capable of binding to the CRE. IIB, F, H and J refer to transcription factor complexes involved in the transcriptional machinery. CBP= CREB binding protein. See text for further details.



CHAPTER 2

THE ROLE OF CRE BINDING PROTEINS IN THE EXPRESSION OF VIP BY ADULT RAT DRG NEURONS IN CULTURE

2.1 INTRODUCTION

As described in chapter 1, the regulation of VIP production in sensory neurons differs strikingly between neonatal and adult rats. When placed in culture or axotomised *in vivo*, DRG neurons from adult animals spontaneously express VIP peptide and mRNA (Nielsch and Keen, 1989, Kashiba, et al., 1992, Noguchi, et al., 1989, Mulderry and Lindsay, 1990), probably as the result of an increase in VIP gene transcription resulting from changes in transcription factor binding to the VIP gene promoter (see section 1.6.4). In contrast, DRG neurons from neonatal animals do not spontaneously express VIP in culture, but a synergistic increase in VIP expression occurs in response to combined activation of cAMP and calcium dependent signalling pathways (Mulderry, 1993). The response of DRG neurons from neonatal animals is likely to involve transcription factors binding to the VIP CRE (see section 1.5). I propose that the same or similar transcription factors may be constitutively activated in cultured adult rat neurons, leading to spontaneous VIP expression through interactions with the CRE (see section 1.7 and Dobson, et al., 1994). Axotomy results in increased expression, in DRG neurons, of a number of transcription factors that are capable of binding to CRE like sequences, including c-Jun Jun D and CREB (Herdegen, et al., 1992, Nakabeppu, et al., 1988, Fink, et al., 1991) (see section 1.7). Heterodimers between c-Jun and Fos are capable of forming an AP1 transcriptional activation complex (Nakabeppu, et al., 1988, Halazonetis, et al., 1988) and can also bind to the human VIP CRE *in vitro* (Fink, et al., 1991). A cut or crush injury to the sciatic nerve leads to a rapid increase in mRNA and immunoreactivity for c-Jun in axotomised DRG neurons (Herdegen, et al., 1992, Jenkins and Hunt, 1991, de Felipe, et al., 1993, Herdegen, et al., 1993, Herdegen, et al., 1993, Robinson, 1994).

Levels of c-Jun remain elevated until regeneration has been completed (Leah, et al., 1991, de Felipe, et al., 1993). An increase in c-Jun expression also occurs in adult rat DRG neurons when placed in culture (de Felipe and Hunt, 1994). Micro-injection of an antisense c-Jun oligonucleotide into adult rat DRG neurons in culture resulted in significant and selective decreases in c-Jun and VIP immunoreactivities (Mulderry and Dobson, 1995, submitted: Fig. 2.1). c-Jun is thus a strong candidate for one of the transcription factors that may interact with the CRE to regulate VIP expression.

The aim of the work described in this chapter was to ascertain the role of the CRE in the regulation of VIP expression in adult rat DRG neurons in culture. To determine the effects of CRE binding proteins on VIP expression, a plasmid containing multiple copies of an oligonucleotide corresponding to the rat VIP CRE was microinjected into DRG neurons. I reasoned that CRE sequences in the microinjected plasmid should compete with the endogenous VIP CRE, reducing transcription factor binding to the endogenous CRE and decreasing expression of VIP. A number of studies have reported the use of decoy oligonucleotides to reduce gene expression by competition with endogenous genes or exogenous reporter constructs for transcription factors (Dash, et al., 1990, Lamb, et al., 1990, Bielinska, et al., 1990, Tanaka, et al., 1994). In parallel experiments, electrophoretic mobility shift assays (EMSAs) were performed to determine the ability of the AP-1 complex formed by c-Fos/c-Jun heterodimers to bind to the rat VIP CRE *in vitro* (see Appendix A.15).

2.2 METHODS

2.2.1 Microinjection of DRG neurons with plasmids containing multiple copies of the rat VIP CRE and quantitation by immunofluorescence

Synthetic double-stranded oligonucleotides corresponding to the rat VIP CRE (nucleotides -94 to -67) were synthesised (Oswel DNA Services, University of Edinburgh) with a short 5' extension (TCGA) to allow insertion into the unique Sal 1 site in the plasmid vector pUC18 (Fig. 1.1). This vector contains no promoter regions that would bind proteins involved in transcription. Following ligation the construct obtained was sequenced and found to contain three tandem repeat copies of the CRE oligonucleotide ligated into the Sal 1 site. DRG neurons were plated out on coverslips (as described in appendix A.19) that had previously been engraved with a small square. After leaving overnight to attach, in the presence of 50ng/ml NGF, all cells inside the engraved square were microinjected with construct with no bias towards cell size or position. On the third day after injection, neurons were fixed and stained for VIP immunofluorescence (as described in appendix A.24). After first visualising stained cells under phase contrast, cells that appeared healthy, i.e. possessing an apparently intact plasma membrane, a clearly differentiated nucleus and lacking a granular cytoplasm, were digitally photographed for immunofluorescence using an MRC 600, Biorad laser scanning optical microscope (SOM) as described in appendix A.25. Only healthy cells were photographed with no bias for size, distribution or levels of immunofluorescence. For each coverslip approximately 100 neurons from the engraved region (injected neurons) and a similar number from outside the engraved region (uninjected

neurons) were photographed. The fluorescence image obtained consisted of 384 X 512 pixels (field area 105 X 140 μ m) with 256 levels of grey. Higher values indicating stronger immunofluorescence. A phase-contrast image of the same field was collected at the same time to help in later identifying the boundaries of cells that contained only low levels of fluorescence. The image analysis software (NIH Image) was used to calculate the total value of all the pixels in each cell. This was used as an index of the VIP content of the cell (appendix A.25). Total fluorescence levels (total pixel values) for individual cells were then ranked and a non-parametric Mann-Whitney U test was used to determine significance of difference between injected and uninjected neurons. A non-parametric test was chosen as the majority of DRG neurons do not stain for VIP immunoreactivity and VIP immunofluorescence is not distributed normally. The critical value for significance was taken as $P < 0.05$.

Quantitative immunofluorescence was chosen as a preferable technique over alternative methods such as in-situ hybridisation. Due to the wide range of values attributable to each pixel value and the large number of pixels per cell it is likely that immunofluorescence is a more sensitive technique than in-situ hybridisation. It is also quicker to perform than the in-situ hybridisation technique. As Immunofluorescence had been used to demonstrate changes in c-Jun protein levels following injection of antisense c-Jun oligonucleotide (Mulderry and Dobson, 1995), this technique was already optimised in our laboratory.

2.2.2 Electrophoretic mobility shift assay (EMSA)

To determine the ability of AP-1 complexes containing c-Jun to bind to the rat VIP CRE *in vitro*, EMSA experiments were performed as described in appendix A.15. Expression constructs encoding mouse c-Jun

(Nakabeppu, et al., 1988) and mouse c-Fos (Halazonetis, et al., 1988) were obtained from Dr. Daniel Nathans; John Hopkins University School of Medicine, Baltimore, USA and from Dr. Philip Leder; Howard Hughes Medical Institute, Harvard Medical School, Boston, USA respectively. c-Jun and c-Fos constructs were transcribed and translated using a Promega rabbit reticulocyte lysate *in vitro* translation kit as described in Appendix A.14. Translated material was then used in EMSA experiments to determine the ability of c-Fos/c-Jun heterodimers to bind to a double stranded oligonucleotide containing the rat VIP CRE (5'-CCATGGGATCACCCTGTGACGTCTTTCA-3'). An oligonucleotide from Gibbon Ape Leukaemia Virus (GALV: 5'-GCAGAAATAGATGAGTCAACAGC-3') containing an AP-1 motif was used as a positive control for AP-1 activity and an oligonucleotide containing an E-box motif from the preprotachykinin-A (PPT) promoter (5'-GGAGAGTGTCACGTGGCTCTCCCTCGA-3') was used as a negative control (see chapter 5.4.4). In some experiments a rabbit polyclonal anti-c-Jun antiserum (Santa Cruz Biotechnology, Inc.) was used to demonstrate "supershifts" (see appendix A.15) providing confirmation of c-Jun binding to oligonucleotides in the EMSA.

2.3 RESULTS

2.3.1 Immunofluorescence

In initial experiments, a range of concentrations of CRE containing construct were microinjected into DRG neurons (2.2 μ g/ml, 22 μ g/ml, 44 μ g/ml, 88 μ g/ml and 116 μ g/ml) and VIP immunofluorescence was determined by eye with each cell being scored as either positively or

negatively stained. Although this method was subjective and large numbers of neurons have to be counted to provide statistically significant results, it provided a rapid indication of the lowest concentration of construct that produced a significant effect. It was considered desirable to inject the lowest possible concentration of plasmid DNA to prevent effects due to non-specific binding of proteins that may be involved in transcription. When counting cells stained for VIP by eye only those that contained clear strong staining in the cytoplasm (Fig. 2.2), together with staining in the processes (usually concentrated in varicosities) and an absence of staining from the nucleus were scored as positively stained. This staining characteristic proved consistent with previous studies (Mulderry, 1993, Mulderry, 1994). The lowest concentration of CRE containing construct to give an observable decrease in VIP immunofluorescence when microinjected into DRG neurons was $22\mu\text{g/ml}$. This concentration was used in all further experiments. Preabsorption of the VIP antiserum with synthetic VIP peptide completely abolished VIP staining in DRG neurons confirming the specificity of staining.

Using the SOM, a wide variety of VIP immunofluorescence levels were obtained in uninjected neurons indicating a wide variation of VIP concentrations between cells. It was not possible to determine from this data whether VIP expression occurred predominantly in neurons of a certain size (Fig. 2.3). There was no significant difference between the size of neurons in the injected and uninjected populations indicating that cell size could not account for differences in VIP immunofluorescence between injected and uninjected neurons.

Microinjection of the pUC18 plasmid construct ($22\mu\text{g/ml}$) alone into the neurons had no significant effect on VIP immunofluorescence compared to uninjected neurons (Table 2 and Fig. 2.4). However microinjection of the

pUC18 plasmid construct containing 3 copies of the rat VIP CRE (22 μ g/ml) resulted in a significant decrease in VIP immunofluorescence compared to uninjected neurons, $P=0.039$ or $P=0.0128$ two tailed test, for two separate experiments: Table 2.1 and Fig. 2.4. This corresponds to a 15-18% decrease in mean total VIP immunofluorescence in injected cells when compared to uninjected controls.

To test the selectivity of the construct containing multiple copies of the rat VIP CRE element its effect on substance P and CGRP immunofluorescence was also determined. No significant difference occurred between injected and uninjected populations of neurons stained for either substance P or CGRP. Injection of pUC18 into these neurons also did not cause any significant effect on either substance P or CGRP immunofluorescence.

2.3.2 EMSA

When the rabbit reticulocyte transcription/translation mix alone was incubated with radiolabelled oligonucleotides corresponding to the GALV AP1, the VIP CRE or the PPT E-box, reproducible retardation of a number of bands, corresponding to nonspecific binding by proteins in the *in vitro* translation and transcription mix was observed (Fig. 2.5 lanes 13, 14 and 15). When mouse c-Jun was translated *in vitro* it was able to produce an extra retarded band of higher molecular weight when in the presence of the GALV AP1 oligonucleotide, but not in the presence of the VIP CRE oligonucleotide (lanes 1 and 7 respectively), indicating that c-Jun could bind as a homodimer specifically to the AP1 element but not to the VIP CRE. This c-Jun band could be removed by co-incubation with an antibody against c-Jun, producing a faint supershifted band of higher molecular weight, lane 2. As expected mouse c-Fos on its own was not able to bind to

either the AP1 element or the CRE (lanes 3 and 9). However, when mouse c-Fos and mouse c-Jun were co-translated very strong binding to the AP1 element and moderately strong binding to the CRE element was observed (lanes 4 and 10 respectively). This corresponds to binding of c-Fos and c-Jun heterodimers which could be supershifted to a small extent using antibodies to either c-Fos or c-Jun (see appendix A.15 and lanes 5, 6, 11 and 12). Binding was shown to be specific for the CRE and the AP1 element as no binding to the PPT E-box motif could be detected (lane 16).

2.4 DISCUSSION

These results showed that microinjection of multiple copies of the rat VIP CRE into adult rat DRG neurons in culture selectively reduced the expression of VIP but not of PPT or CGRP. This suggests that the microinjected CRE is competing for proteins binding to the endogenous VIP CRE and that these proteins are required for spontaneous VIP expression in culture. The lack of effect of the pUC18 plasmid alone on VIP expression, shows that proteins important for VIP expression are binding specifically to the CRE and not to the pUC18 plasmid.

The modest decrease in VIP immunofluorescence observed following injection (a 15-18% decrease in mean total fluorescence) may reflect the low transfection efficiency, since only a proportion of injected cells will have been successfully transfected with DNA (Mulderry, et al., 1993) and this assay is unable to distinguish between successfully or unsuccessfully transfected cells. There is also a variation in the volume of solution delivered to individual cells by the microinjection procedure so that the concentration of plasmid in individual cells after injection varies over a

broad range. Alternatively it is possible that the decrease in VIP immunofluorescence is modest because the CRE is only partially responsible for VIP induction. Alternative sites may exist elsewhere in the VIP gene that also play a role in spontaneous VIP expression. Co-injection of the decoy CRE with decoy oligonucleotides corresponding to alternative sites (for instance those suggested in chapter 5), into DRG neurons, may have a much larger effect on VIP expression than the decoy CRE alone.

A larger decrease in VIP immunofluorescence may be obtained if only successfully transfected cells are counted. This could be achieved by co-injection of the CRE containing pUC18 construct with fluorescently labelled marker DNA to permit the detection of successfully transfected cells. The marker DNA would have to have a different emission wavelength to the streptavidin-linked fluorescein isothiocyanate used for VIP immunofluorescence. This could be accomplished by using for example, Rhodamine conjugated marker DNA. Control cells would also have to be injected with the marker DNA alone.

EMSA experiments showed that, although c-Jun could bind to the GALV AP-1 motif as a homodimer, it could bind to the VIP CRE only in the form of a heterodimer with c-Fos. c-Fos expression cannot be detected in DRG neurons *in vivo* under normal conditions or following axotomy (Herdegen, et al., 1992, Leah, et al., 1991) although a c-fos transgene has been shown to be expressed in DRG of transgenic mice following axotomy. However, it is not known this c-fos transgene is present in neuronal or non-neuronal cells of the ganglia (Smeyne, et al., 1993). Transient c-fos expression has also been observed in cultured DRG neurons (Lindsay, et al., 1990). It is possible that a transient expression of c-Fos during the early phase of c-Jun induction could result in stable c-Fos/c-Jun heterodimers that persist beyond the period of active c-Fos expression and contribute to the

regulation of VIP and other genes. Alternatively, it is possible that c-Jun can bind to the CRE in the form of a heterodimer with other members of the Jun family for instance Jun B, Jun D or the ATF/CREB families. Complexes containing either Jun B or Jun D are capable of binding to CRE sequences and c-Jun can heterodimerise with either Jun B or Jun D (Nakabeppu, et al., 1988). c-Jun will also heterodimerise with CREB, ATF2, 3 and 4, forming complexes that can bind DNA (Benbrook and Jones, 1990, Karin and Smeal, 1992). Both Jun D and CREB are expressed in DRG neurons (Herdegen, et al., 1992). Although the CREB/c-Jun complex has been shown to be incapable of activating transcription (Benbrook and Jones, 1990), it remains to be seen whether this is the case for other c-Jun containing complexes that bind to CREs.

These results together with data showing that VIP expression is dependent on c-Jun (Mulderry and Dobson, 1995 and Fig. 2.1) suggest that c-Jun, is involved in spontaneous VIP expression. Since c-Jun can bind to the CRE in the form of a heterodimer and proteins binding to the CRE are involved in the spontaneous expression of VIP, it is likely that the CRE is one site of action of c-Jun.

It is possible that c-Jun may also act indirectly by altering expression of proteins, including c-Jun itself, that subsequently regulate VIP expression or that c-Jun may act on sites in the VIP gene other than the CRE. Expression of c-Jun is known to be regulated through proteins binding to an AP-1 like element located in its promoter region (Angel, et al., 1988). It is possible that the CRE containing construct microinjected into DRG neurons competes for proteins that would normally regulate c-Jun expression. If this occurs, a resulting decrease in c-Jun expression could lead to a concomitant decrease in VIP expression. This is unlikely since previous work in our laboratory showed that a reduction in c-Jun

expression resulted in a large increase in CGRP immunofluorescence (Mulderry and Dobson, 1995) which was not observed in this study. To eliminate this possibility, the effects of the CRE containing construct on c-Jun expression should be examined.

Quantitative immunofluorescence of VIP peptide can not distinguish between loss of peptide to the neurites and a decrease in VIP expression, as measurements are only made on the cell body, the neurites being too long and inter-twined to make accurate measurements of VIP content per cell. However, neurons with peptide staining in the neurites invariably display cell body staining as well. Also, although VIP peptide is transported to the peripheral site of injury and to central terminals its concentration remains high in dorsal root ganglia axotomised *in vivo* (Villar, et al., 1989, Shehab and Atkinson, 1986). Changes in cell body staining are therefore likely to be a reliable index of changes in total peptide content. Much of the published evidence for altered peptide expression after axotomy *in vivo* also comes from changes in cell body immunostaining (Shehab and Atkinson, 1986).

The decrease in VIP immunoreactivity that occurs following microinjection of the CRE containing construct is most likely due to a decrease in transcription. To test this experiments to measure transcription will be required, such as nuclear run on assays, though these will be limited by the difficulty in isolating the large numbers of DRG neurons that would be required.

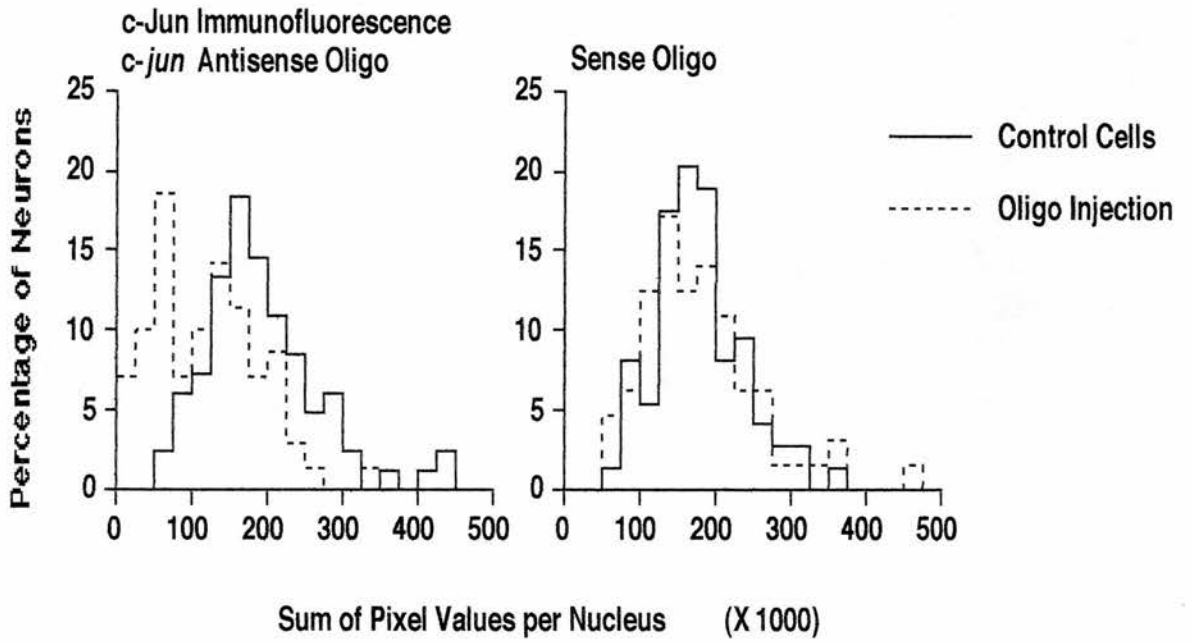
VIP and c-Jun show different patterns of expression in DRG neurons, c-Jun being expressed in approximately 80% of adult rat DRG neurons (de Felipe and Hunt, 1994) and VIP being expressed in approximately 30% of neurons (Mulderry, 1994) as arbitrarily determined by eye. The

heterogeneity of VIP immuno-staining compared to c-Jun immuno-staining suggests that factors other than c-Jun must regulate VIP expression.

Figure 2.1. *c-jun* antisense oligonucleotide injection blocks c-Jun and VIP expression in DRG neurons

Scanning optical microscope measurements of VIP immunofluorescence in DRG neurons after injection of a *c-jun* antisense oligonucleotide (left) or the complimentary sense oligonucleotide (right) compared with measurements from a similar number of control cells in each case. Independent experiments using similar methodology (P.K. Mulderry, unpublished) showed that the effects of the antisense oligonucleotide on VIP are selective in that they are not reproduced for substance P or CGRP. The graphs represent the frequency distribution of c-Jun or VIP immunofluorescence levels in individual neurons. Cells show a reduction of VIP immunoreactivity in response to injection of the antisense oligonucleotide ($p < 0.002$, Mann-Whitney U-test), but no change in response to the sense oligonucleotide. Overall reduction in VIP or c-Jun immunofluorescence is due to fewer cells exhibiting high levels of fluorescence and correspondingly more cells exhibiting low levels of fluorescence.

A



B

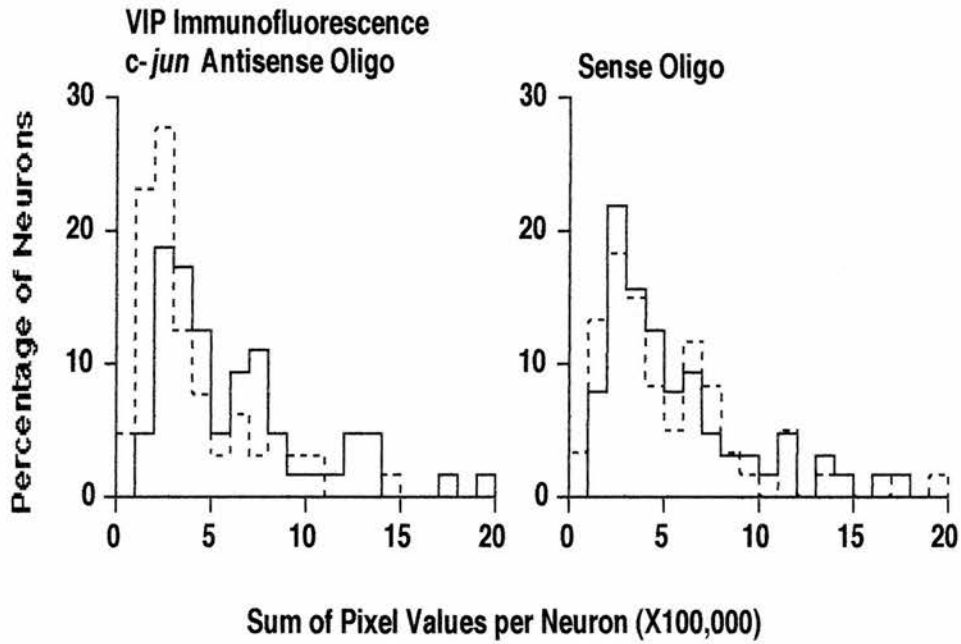
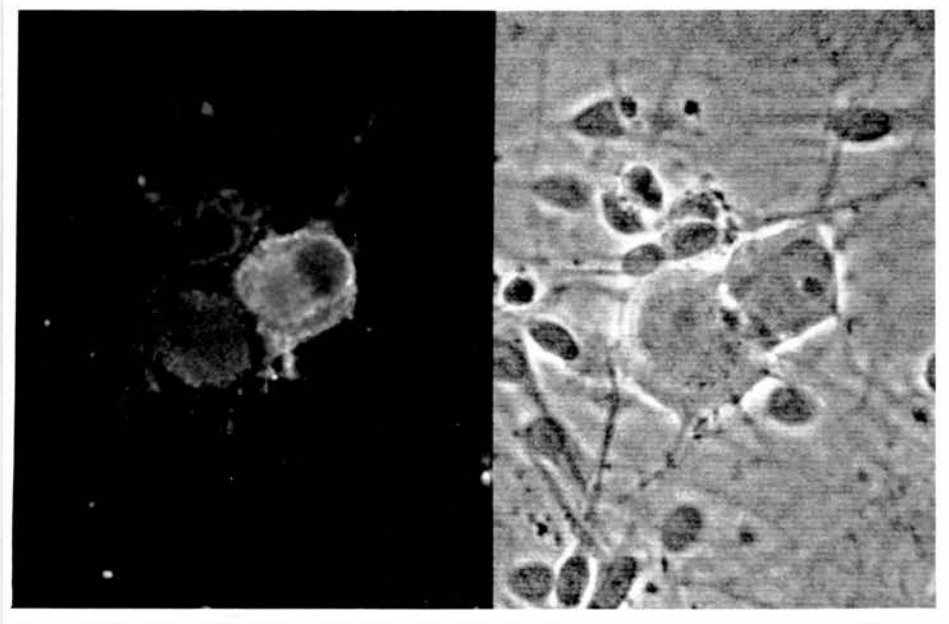


Figure 2.2. VIP immunofluorescence in adult rat DRG neurons

A. Digital photograph of 2 adult rat DRG neurons after 4 days in culture, stained with a rabbit antiserum against VIP. Photographs were taken using an MRC 600 biorad confocal laser scanning microscope. The neuron on the right shows high intensity staining, the one on the left shows lower intensity staining.

B. The same field seen under phase contrast.



A

B

Figure 2.3. Relationship between mean VIP immunofluorescence and cell size

Uninjected DRG neurons stained for VIP immunoreactivity were analysed for mean fluorescence and cell area. Values shown correspond to the number of pixels per cell (directly proportional to cell area) or the mean pixel intensity (VIP immunofluorescence). Results are for 196 uninjected neurons obtained from two coverslips. Cells on each coverslip were treated the same and were plated out, stained and analysed on identical days.

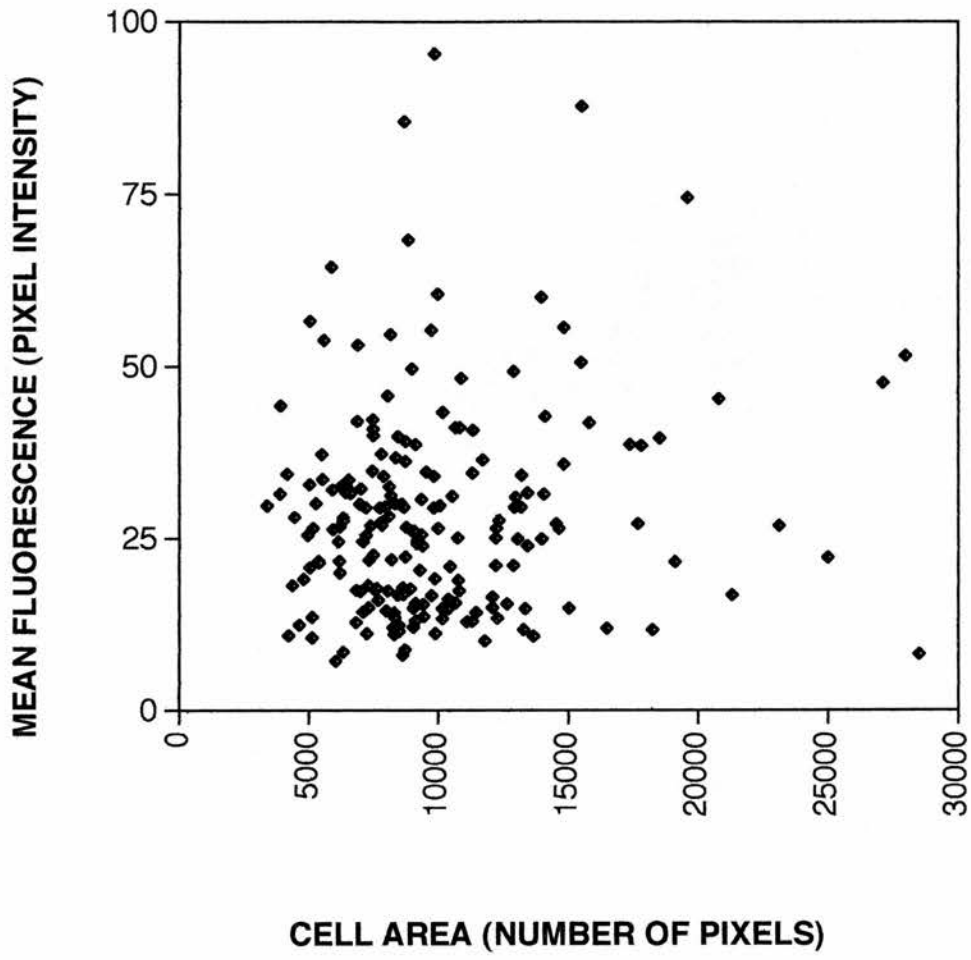


Figure 2.4. Injection of a CRE containing construct reduces VIP expression in DRG neurons

Scanning optical microscope measurements of VIP immunofluorescence in DRG neurons after injection of the CRE containing pUC18 construct (left) or pUC18 alone (right) compared with measurements from a similar number of control cells in each case. The graphs represent a frequency distribution of VIP immunofluorescence levels in individual neurons. Injection of the CRE containing pUC18 construct results in a significant shift to the left ($p=0.0128$ Mann-Whitney U-test), due to fewer cells exhibiting high levels of fluorescence and correspondingly more cells exhibiting low levels of fluorescence. There is no effect following injection of pUC18 alone.

CRE containing pUC 18

pUC18

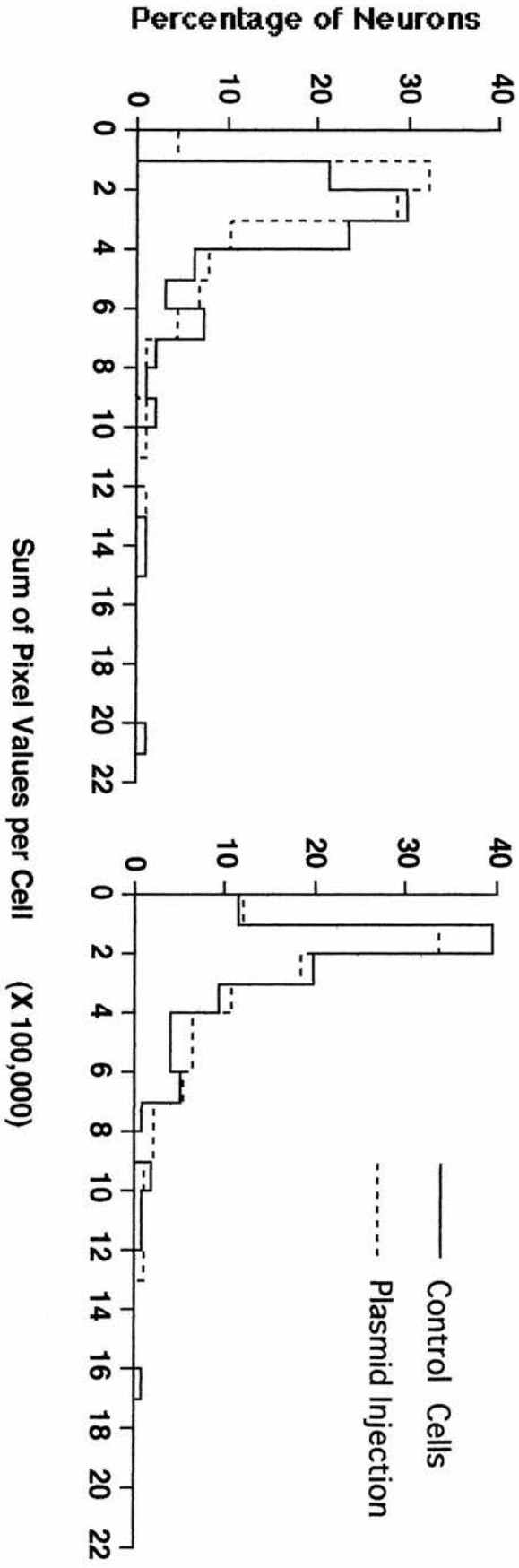


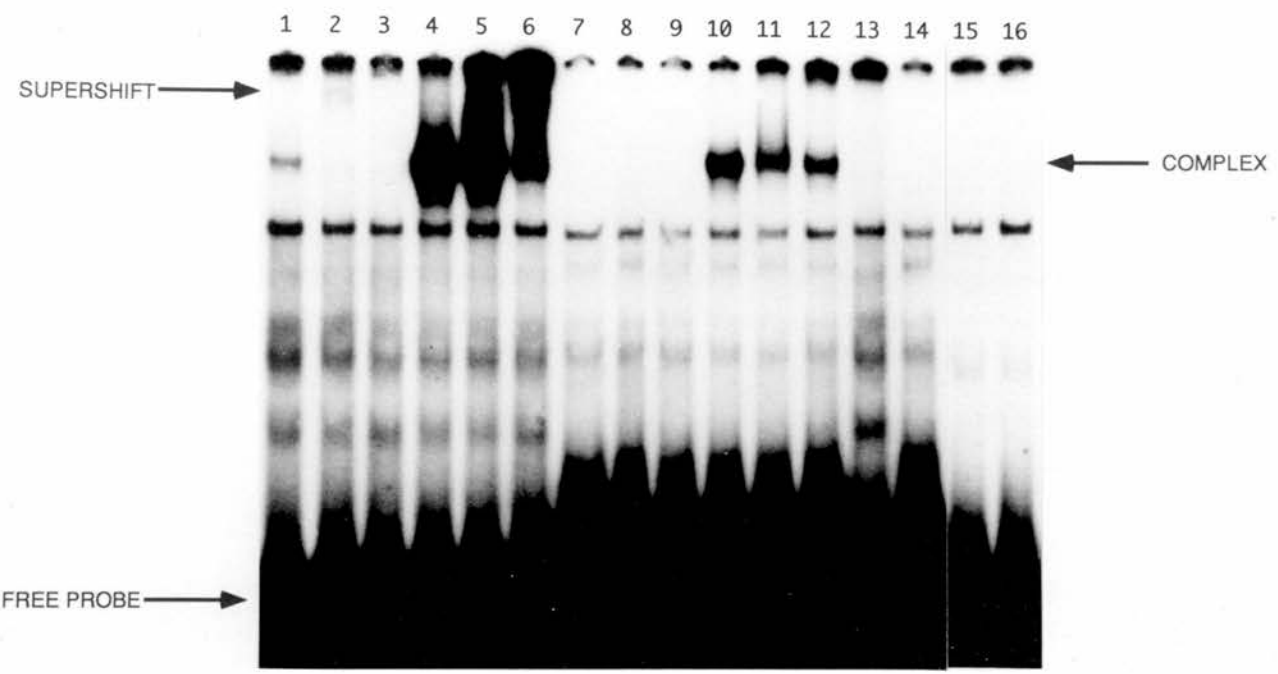
Table 2.1. Effects of a microinjected CRE containing construct on VIP, substance P and CGRP immunofluorescence

Scanning optical microscope measurements of VIP, substance P or CGRP immunofluorescence in DRG neurons after injection of the CRE containing pUC18 construct or pUC18 alone as indicated. In each case fluorescence levels of injected neurons were compared to those from a similar number of uninjected neurons. As absolute levels of fluorescence varied between each experiment values were normalised to those of uninjected cells (100%). The values shown for normalised average total fluorescence provide an indication of the size and direction of the change in immunofluorescence following injection. Significance of difference between injected and uninjected cells on the same coverslip were obtained using a Mann-Whitney U-test. pUC18 with or without the CRE had no effect on immunofluorescence levels of either substance P or CGRP. pUC18 alone also had no effect on VIP immunofluorescence. The CRE containing pUC18 construct did cause a significant decrease in VIP immunoreactivity. * = significant differences $p < 0.05$.

Antigen	Injection	N° of Cells	Mean total fluorescence (%Injected/ Control)	p
VIP	pUC18+CRE	87	82.6±6.4	0.0390*
	Uninjected	94		
VIP	pUC18+CRE	97	85±10.2	0.0128*
	Uninjected	104		
VIP	pUC18	92	103±7.8	0.410
	Uninjected	96		
VIP	pUC18	75	113±16.1	0.407
	Uninjected	97		
Substance P	pUC18+CRE	97	127±15.7	0.108
	Uninjected	101		
Substance P	pUC18	87	96±8.9	0.111
	Uninjected	91		
CGRP	pUC18+CRE	95	93.4±8.4	0.615
	Uninjected	86		
CGRP	pUC18	110	85.5±7.2	0.056
	Uninjected	104		

Figure 2.5. Electrophoretic mobility shift assay showing mouse c-Jun and c-Fos binding to the rat VIP CRE and GALV AP1 sequence

Double stranded oligonucleotides containing either the rat VIP CRE, 5'-TCGACCATGGGATCACCACTGTGACGTCTTTCA-3' (lanes 7-12 and lane 14), the GALV AP1, 5'-GCAGAAATAGATGAGTCAACAGC-3' (lanes 1-6 and lane 13), or the PPT E Box motif, 5'-GGAGAGTGTACGTGGCTCTCCCTCGA-3' (lanes 15 and 16) were 5' labelled with ³²P as described in appendix A.16. Labelled oligonucleotides (1ng) were incubated with mouse c-Jun or c-Fos (expressed using a rabbit reticulocyte transcription/ translation mix) as follows. Lanes 1 and 7, mouse c-Jun. Lanes 2 and 8, mouse c-Jun in the presence of an anti c-Jun antibody. Lanes 3 and 9, mouse c-Fos. Lanes 4, 10, 15 and 16, co-translated mouse c-Jun and c-Fos. Lanes 5 and 11, co-translated mouse c-Jun and c-Fos in the presence of an anti c-Jun antibody. Lanes 6 and 12, co-translated mouse c-Jun and c-Fos in the presence of an anti c-Fos antibody. Lanes 13 and 14, rabbit reticulocyte transcription/ translation mix, in the absence of expressed c-Fos or c-Jun.



CHAPTER 3

THE ROLE OF THE RAT VIP CRE IN REGULATING REPORTER GENE EXPRESSION

3.1 INTRODUCTION

The data presented in Chapter 2 suggest that factors binding to the CRE like sequence found at position -94 to position -67 of the rat VIP gene play a role in regulating expression of VIP in adult rat DRG neurons in culture. Previous studies have indicated that the CRE in the promoter of the human VIP gene can mediate the effects of cAMP and Ca²⁺ influx on gene expression in a variety of cell types (Tsukada, et al., 1987, Deutsch, et al., 1988, Fink, et al., 1991). Although not identical in sequence to the CRE found in the human VIP gene, it would seem likely that the rat VIP CRE is at least partly responsible for the synergistic response to forskolin and K⁺-evoked depolarisation seen in neonatal rat DRG neurons in culture. CRE like elements from a number of genes, including the human VIP gene, have also been shown to enhance the expression of heterologous reporter genes when transfected into appropriate cell lines (Vallejo, et al., 1992, Tsukada, et al., 1987, Deutsch, et al., 1988, Deutsch, et al., 1987, Delegeane, et al., 1987). The aim of the studies described in this chapter was to determine whether the isolated rat VIP CRE is capable of driving expression of a reporter gene in DRG neurons from adult and neonatal rats. The rat pheochromocytoma (PC12) cell line (Greene and Tischler, 1976) provides a clonal cell line model for the study of VIP expression in which bulk transfection methods can be used (Tsukada, et al., 1994). Therefore reporter constructs containing the rat VIP CRE were also transfected into PC12 cells.

Previous studies, described in Chapter 1, showed that VIP is expressed spontaneously in adult rat DRG neurons in culture, a phenomenon that may be directly relevant to the spontaneous expression that occurs in DRG neurons following axotomy *in vivo*. In cultured neonatal rat DRG neurons,

VIP expression can be induced synergistically by the combined effects of potassium evoked depolarisation (resulting in Ca^{2+} influx) and drugs that increase intracellular cAMP concentrations (Mulderry, 1993). These studies led me to the hypothesis that the pathways responsible for spontaneous VIP expression in adult rat neurons and for synergistically induced expression in neonates might converge on a common regulatory element which, being constitutively activated in adult neurons, is no longer capable of responding to combined activation of cAMP and Ca^{2+} -dependent signalling pathways (Dobson, et al., 1994). By comparing the role of the CRE in adult and neonatal rats, it was hoped that developmental alterations in the control of VIP expression, acting through the CRE, might become apparent.

3.2 METHODS

3.2.1 Reporter Constructs

Cells were transfected with plasmid constructs containing a synthetic oligonucleotide sequence corresponding to the rat VIP CRE inserted upstream of the c-Fos minimal promoter (Gilman, et al., 1986) driving expression of a chloramphenicol acetyl transferase (CAT) reporter gene. The reporter gene vector, FOS, containing the mouse c-Fos promoter (nucleotides -56 to +106 relative to the transcription start site, Fig. 3.1: Gilman, et al., 1986), linked to the CAT gene was used as the basis for reporter plasmid construction. The endogenous c-Fos promoter contains a CRE like element at position -69 relative to the transcription start site that is known to respond to membrane depolarisation and cAMP (Sheng, et al., 1990). This is removed in the minimal promoter and replaced with a SalI

site into which CRE containing oligonucleotides were cloned. Thus it is likely that sequences downstream from the CREs cloned into the FOS reporter construct are responsive to CRE regulated expression.

The CRE in the rat somatostatin gene (nucleotides -37 to -45), which is probably the best-characterised CRE and is known to be capable of mediating a response to forskolin when in the context of specific promoters (Montminy, et al., 1986, Deutsch, et al., 1988), was used as a positive control with which to compare the rat VIP CRE results (Fig. 3.2). A sequence from the rat PPT (preprotachykinin-A) gene (nucleotides -200 to -185) that shows homology with a CRE was also cloned into the same position in the FOS reporter construct (Fig. 3.2). The element from the PPT gene is known to direct high levels of expression of the FOS reporter construct when transfected into PC12 cells (Morrison, et al., 1994). The use of CRE-like sequences from the PPT and somatostatin genes, which are regulated quite differently to the VIP gene in DRG neurons (Mulderry and Lindsay, 1990, Noguchi, et al., 1993), provides an indication of the changes in transcriptional response that may occur as a result of nucleotide changes within the CRE sequence.

Synthetic double-stranded oligonucleotides corresponding to the rat VIP CRE (nucleotides -94 to -67), the rat somatostatin CRE (nucleotides -45 to -37) and the rat PPT CRE like sequence (nucleotides -200 to -185) were synthesised (Oswel DNA Services, University of Edinburgh) with 5' overhangs to allow insertion into the unique Sal I restriction site immediately upstream of the c-Fos promoter (Fig. 3.2). The sequences of all clones were determined by sequence analysis using a primer corresponding to a sequence within the c-Fos promoter.

In addition to a construct containing a single copy of the rat VIP CRE, a construct containing 3 tandem copies of the CRE, in the reverse orientation

to that found in the endogenous VIP CRE, was obtained. Since it is well documented that certain enhancer sequences, including CREs, can work in either orientation (Deutsch, et al., 1987) and that multiple regulatory sequences often synergise in their effects on transcription (Deutsch, et al., 1988), the activity of this construct was also examined.

3.2.2 Transfection of PC12 cells .

In initial studies, constructs containing the three CREs were transfected into the PC12 rat pheochromocytoma cell line (Greene and Tischler, 1976). FOS constructs containing other enhancer elements have previously been shown in our laboratory to respond to combined forskolin and K⁺-evoked depolarisation (Morrison, et al., 1994) when transfected into PC12 cells. Although PC12 cells do not normally express VIP, stimulation with forskolin or other agents that also raise intracellular cAMP results in an induction of VIP mRNA (Tsukada, et al., 1994). The human VIP gene promoter will also direct cAMP-dependent expression of a reporter gene when transfected into PC12 cells (Tsukada, et al., 1987, Tsukada, et al., 1994). PC12 cells may therefore contain transcription factors that are capable of binding to the rat VIP CRE to regulate gene expression in response to cAMP. Transfection of PC12 cells with constructs containing the CRE is likely to show whether the rat VIP CRE is an active DNA element capable of regulating transcription.

3x10⁶ cells were electroporated at 200V in the presence of 5µg of reporter construct DNA. 1.5x10⁶ undifferentiated cells were then plated out per petri-dish and left overnight in the presence of 50ng/ml NGF to attach. The following day the medium was removed and medium containing 40mM KCl, 10µM forskolin and 50ng/ml NGF was added. Although 10µM forskolin and 40mM K⁺ act to differentiate PC12 cells, the presence of NGF

in the medium results in the differentiation of all cells and should act to minimise differences between forskolin and 40mM K⁺ treated and untreated cells.

Leaving cells to recover from electroporation overnight, before addition of forskolin and 40mM K⁺, should also help to minimise any differences due to cell survival by allowing cells to die that may have been rescued by immediate addition of forskolin and K⁺.

Cells were left for 96h before lysates were assayed for CAT immunoreactivity using an enzyme-linked immunosorbent assay kit (Boehringer-Mannheim), see appendix A.22. Consistent transfection of PC12 cells was obtained, as measured by comparing CAT expression between parallel transfections.

3.2.3 Microinjection

DRG neurons were transfected with reporter constructs by microinjection of plasmid DNA. Using microinjection as a method for transfecting cells we were able to obtain high specificity for neurons combined with reasonable levels of expression in a CAT assay. A number of other transfection methods using a variety of calcium phosphate precipitation procedures and lipofection reagents have been attempted. These resulted either in low transfection efficiency or simultaneous transfection of non-neuronal cells (P. Mulderry and authors own unpublished observations). Microinjection was carried out as described in appendix A.20 and Mulderry *et al.* 1993. Cultures were plated on shallow 10mm diameter polystyrene wells at a density of 4000-10000 neurons per well. Three hundred and fifty neurons per well were injected with plasmid (appendix A.20). Following injections neurons were left overnight in normal medium before addition of medium containing 40mM K⁺ or 10 μ M

forskolin. For depolarisation experiments, medium containing 40mM K⁺ was prepared as described in appendix A.21 (Mulderry, 1993). Forskolin (Sigma), stock solution dissolved in ethanol at 10mM was added at 1:1000 to yield a final concentration of 10 μ M. All cells were supported in culture with NGF (50ng/ml) and NGF was present at all times before and after microinjection.

3.3 RESULTS

3.3.1 PC12 Cells

PC12 cells transfected with the control plasmid construct FOS, or the plasmid containing 1 copy of the rat VIP CRE element showed no significant increase in CAT immunoreactivity (under basal conditions or under conditions of combined forskolin and K⁺-evoked depolarisation) over the low levels detected in untransfected cells. Transfection of cells with the plasmid containing 3 tandem copies of the rat VIP CRE resulted in no significant increase in CAT immunoreactivity under basal conditions or when in the presence of 40mM K⁺. However in the presence of forskolin alone a significant level of expression could be detected that was approximately 14 fold greater than the expression levels under basal conditions. Combined forskolin and K⁺-evoked depolarisation resulted in an approximately 77 fold increase in expression above that obtained under basal conditions (Fig. 3.3).

3.3.2 Neonatal Rat DRG Neurons

Following injection of the control plasmid, FOS, into neonatal rat DRG neurons, there was no significant increase in CAT immunoreactivity (under basal conditions, or in the presence of forskolin or under depolarising

conditions alone) over the low levels detected in uninjected cultures. Under conditions of combined forskolin and depolarisation, however, significant concentrations of CAT immunoreactivity, approximately 4 fold greater than those obtained under basal conditions, were detected (Fig. 3.4). Injection of the plasmid containing one copy of the VIP CRE resulted in CAT expression significantly different from that seen with FOS only under conditions of combined forskolin and depolarisation, when CAT levels were approximately 4-fold higher than those in cultures injected with FOS (Fig. 3.4). Injection of the plasmid containing the somatostatin CRE resulted in significant levels of CAT expression under basal conditions. This was not significantly altered by forskolin or depolarisation alone, but was increased to levels similar to those seen with the VIP CRE construct under conditions of combined forskolin and depolarisation (Fig. 3.4).

3.3.3 Adult Rat DRG Neurons

Injection of FOS into adult rat neurons produced similar results to those seen in neonates. There was no significant CAT under basal conditions but combined depolarisation and forskolin treatment resulted in the appearance of detectable CAT immunoreactivity. Results obtained from adult rat cells injected with the VIP CRE construct differed from those obtained in neonates, however, in that CAT concentrations were not significantly different from those seen with the FOS plasmid under basal or stimulated conditions (Fig. 3.5). Adult rat neurons injected with the somatostatin CRE construct did not express significant levels of CAT under basal conditions but did express CAT at significantly higher level (approximately 1.6-fold) than FOS under conditions of combined depolarisation and forskolin treatment (Fig. 3.5). Adult rat neurons injected with the PPT construct expressed CAT at significant levels under basal

conditions. This level was approximately 1.3-fold greater than that obtained with FOS under conditions of combined depolarisation and forskolin treatment. Cells transfected with the PPT enhancer and incubated under conditions of combined depolarisation and forskolin treatment expressed CAT at approximately 2.2 fold greater levels than those obtained with FOS under the same conditions. Both neonatal and adult rat DRG neurons expressed CAT at high levels under conditions of combined depolarisation and forskolin treatment following injection of the construct containing 3 copies of the VIP CRE element. As absolute levels of CAT activity varied between experiments results from different experiments were normalised against values obtained with the construct containing 3 copies of the CRE under conditions of combined 10 μ M forskolin and 40mM K⁺ (normalised to 100 in Fig. 3.4 and 3.5).

If the absolute levels of expression of the FOS plasmid were much higher in DRG neurons from adult rats than in neonates, then this might obscure any effect of the VIP CRE and hence lead to the apparently different result. This could be due to the FOS plasmid expressing CAT at close to its maximum levels such that the CRE element could have little effect. Expression levels of the FOS plasmid were compared as directly as possible using adult and neonatal rat DRG cultures prepared, injected and assayed at the same time (Table 3.1). The results of this experiment showed that absolute levels of FOS expression differed by no more than 2-fold between neonatal and adult rat neurons, indicating that the different results obtained with the CRE-containing constructs are due to a differences in CRE function.



3.4 DISCUSSION

3.4.1 PC12 Cells

Constructs containing three tandem copies of the rat VIP CRE were capable of responding to forskolin and depolarisation, when transfected into PC12 cells, suggesting that the CRE can bind proteins required for this response and may play a role in the response of endogenous VIP to these stimuli in DRG neurons.

However, since the expression of CAT in cells transfected with the FOS construct was below the sensitivity of the assay under all experimental conditions, it is not possible to determine whether the CRE element can respond synergistically to combined forskolin and depolarisation or if the CRE acts simply to enhance the response of the FOS construct to forskolin and depolarisation. To distinguish between these possibilities it would be necessary to perform similar experiments with the CRE inserted into a different promoter that is capable of expressing detectable levels of CAT in PC12 cells under basal conditions.

It is not known why 3 copies of the CRE are required for a response to combined forskolin and depolarisation. It is possible that factors capable of binding to the CRE synergise (co-operate) in their stimulatory action on proteins that initiate transcription. Co-operativity between transcription factors has been observed for proteins binding to sites in the lactose repressor, such as the yeast transcriptional activators GAL 4 and GCN4 proteins (Giniger and Ptashne, 1988, Hope and Struhl, 1987). Co-operativity between non identical DNA binding proteins has also been reported in higher eukaryotic organisms (Reinberg and Roeder, 1987, Lu and Bensadoun, 1993). As a full turn of DNA helices occurs every 10bp it is likely that binding sites must also occur at 10bp intervals for full co-

operativity to be observed. Experiments have shown that this is the case for repressor binding to DNA which shows co-operativity when the binding sites are 10bp apart (Whitson, et al., 1987). It is possible that multimerisation of the CRE containing oligonucleotide can result in alternative positioning of different binding sites relative to one another perhaps allowing optimal co-operativity between two or more transcription factors. Alternatively 3 tandem copies may provide 3 or more binding sites for proteins. As each one of these binding sites will be in the presence of different flanking sequences and flanking sequences have shown to exert large effects on the activity of CRE sequences (Deutsch, et al., 1988) it is possible that one of these positions is more permissive for transcription than positions that occur in the construct containing 1 copy of the CRE. It is also possible that insertion of tandem copies of the CRE into a plasmid generates alternative and perhaps novel transcription factor binding sites, resulting in unpredictable regulation of the relevant reporter gene.

As the 3 tandem copies of the CRE are all in the reverse orientation, relative to the transcription start site and the orientation of CRE containing enhancers has been shown to result in differences in ability to activate transcription (Deutsch, et al., 1987), it is also possible that the orientation of the VIP CRE plays some role in its ability to stimulate transcription.

3.4.2 DRG Neurons

A single copy of the rat VIP CRE was capable of enhancing the response of the c-Fos promoter to forskolin and K⁺ in neonatal rat DRG neurons. This is the first demonstration that a single copy of this element can act as a functional enhancer. No such effect was seen in adult rat neurons, suggesting that the responsiveness of the CRE to cAMP and

depolarisation declines as the neurons mature, in parallel with the response of the endogenous VIP gene.

The construct (FOS) containing only a minimal c-fos promoter also exhibited a synergistic response to combined depolarisation and forskolin in neonatal and adult rat neurons. The cause of the FOS response in neonatal and adult rat neurons is unknown. This construct does not respond to combined depolarisation and forskolin treatment in transfected PC12 cells even though associated CREs do confer responsiveness to these stimuli (Morrison, et al., 1994).

It is not possible to say from these results whether the effect of the VIP CRE on CAT expression in neonatal rat neurons was due to a response on the part of the CRE itself to cAMP and depolarisation or only to an enhancement of the FOS response.

As the magnitude of the FOS response is virtually identical in neonatal and adult rat DRG neurons, the differences seen with the VIP CRE construct can be attributed to properties of the CRE. These results therefore support the view that the VIP CRE is likely to participate in regulating the endogenous VIP response to combined depolarisation and forskolin seen in neonatal rat neurons (Mulderry, 1993).

The different results obtained following transfection of either PC12 cells or neonatal rat DRG neurons with the construct containing a single copy of the CRE may be explained by different signal transduction pathways being available in these cell types allowing expression from the reporter construct only in neonatal rat DRG neurons. Alternatively the different results may reflect different roles for the CRE in these cell types or may merely reflect differences in the methods of transfection.

Results obtained with the somatostatin and PPT CREs in both neonatal and adult rat neurons were significantly different to those obtained with the

VIP CRE. These results suggest that different CREs can confer specific patterns of gene expression.

The rat VIP CRE (TGACGTCT) identified by Giladi et al. (Giladi, et al., 1990), bears a 7/8 homology with the somatostatin consensus CRE sequence (TGACGTCA) (Montminy, et al., 1986). However, it has been shown that mutating the final adenosine nucleotide of the somatostatin CRE into a thymidine, as in the VIP CRE, can affect its enhancer activity (Deutsch, et al., 1988). The different results obtained with the two CREs may therefore reflect this slight sequence difference. On the other hand, the human VIP CRE has been shown to contain two adjacent CRE-like sequences, one identical to that in the rat and another upstream of it, that are both required for full enhancer activity (Fink, et al., 1988). Our rat VIP CRE oligonucleotides were designed to include not only the consensus CRE identified by Giladi et al. (Giladi, et al., 1990) but also a second sequence (TGGGATCA) upstream of it that has 5/8 homology with the consensus CRE and whose position corresponds approximately to the second element in the human VIP gene (Fig. 1.1). It is possible that this sequence also contributes to the enhancer function of the CRE.

Although the PPT enhancer shows a 6/8 homology with the somatostatin CRE it also shows a 6/7 homology with a consensus AP1 binding site (TGAGTCA). Gel retardation assays have shown that the PPT element does not bind the same proteins as the somatostatin CRE in HeLa nuclear extract, but it does compete with a consensus AP1 element for binding proteins (Morrison, et al., 1994), suggesting a molecular basis for the different levels of CAT expression observed with the CRE-like sequence from the PPT gene.

Although the lack of effect of the VIP CRE on CAT expression in adult rat neurons is consistent with the absence of synergistic response to cAMP

and depolarisation by endogenous VIP in these neurons (Mulderry, 1993) the CRE did not direct spontaneous expression as is seen for endogenous VIP. The idea that spontaneous VIP expression in adult rat neurons and induced expression in neonatal rat neurons might both be mediated through the CRE is therefore not supported by the present results. It is likely that in adult neurons, the rat VIP CRE participates in the control of gene expression in association with other regulatory elements.

A difficulty with the interpretation of these results is that endogenous VIP expression is seen in only approximately 30 % of DRG neurons in culture (Mulderry, 1993). Since the microinjection technique is not selective for specific neuronal subpopulations (Mulderry, et al., 1993) and a difference in CRE function in DRG neurons at different stages of maturity has been shown, it is conceivable that CRE function also differs in different subpopulations of DRG neurons. Further studies involving localisation of reporter gene and endogenous VIP expression will be needed to determine whether this is so.

The mechanisms responsible for the synergistic response to cAMP and depolarisation and the reasons for its disappearance in mature neurons are unknown. One possibility lies in cAMP-sensitive Ca^{2+} channels whose abundance in rat DRG neurons declines as the neurons mature (Fedulova, et al., 1991). However, the uniform response of the FOS plasmid, to combined forskolin and depolarisation, in neonatal and adult rat neurons argues against this possibility and suggests that the different responses mediated by the VIP CRE may reflect differences in the cellular transcription factors that bind to the CRE. The nature of these factors remains to be determined.

Figure 3.1. c-Fos minimal promoter sequence

Nucleotides -56 to +109 relative to the start of transcription of the c-Fos promoter. Hatched box indicates the TATA box binding site for transcription factor II D. The transcription start site is indicated by an arrow and in the reporter plasmid FOS directs expression of the chloramphenicol acetyl transferase reporter gene.

-56

GTCATCCAT TCACAGCGCT TCTATAAAGG CGCCAGCTGA GCGCCTACT



ACTCCAACCG CGACTGCAGC GAGCAACTGA GAAGACTGGA TAGAGCCGGC

GGTTCCGCGA ACGAGCAGTG ACCGCGCTCC CACCCAGCTC TGCTCTGCAG

109

CTCCCACCAG TGICT

Figure 3.2. Reporter constructs containing the c-Fos promoter

Oligonucleotide sequences cloned into the unique *Sal* I restriction site 5' of the c-Fos minimal promoter (hatched box). The oligonucleotides containing the somatostatin CRE and the c-Fos minimal promoter cut with *Sal* I were blunt ended using Klenow before ligation (see appendix A.7). Numbers refer to nucleotides 5' from the transcription start site (arrow). Black box indicates site of the chloramphenicol acetyl transferase reporter gene. Arrows above the sequences indicate the core 8bp CRE motif.

TCG AAA TTG CGT CAT TTC
TT AAC GCA GTA AAG AGC T

PPT ENHANCER

GAT CGT GAC GTC AGG
CA CTG CAG TCC CTA G

SOMATOSTATIN CRE

TCG ACC ATG GGA TCA CCA CTG TGA CGT CTT TCA
GG TAC CCT AGT GGT GAC ACT GCA GAA AGT AGC T

VIP CRE

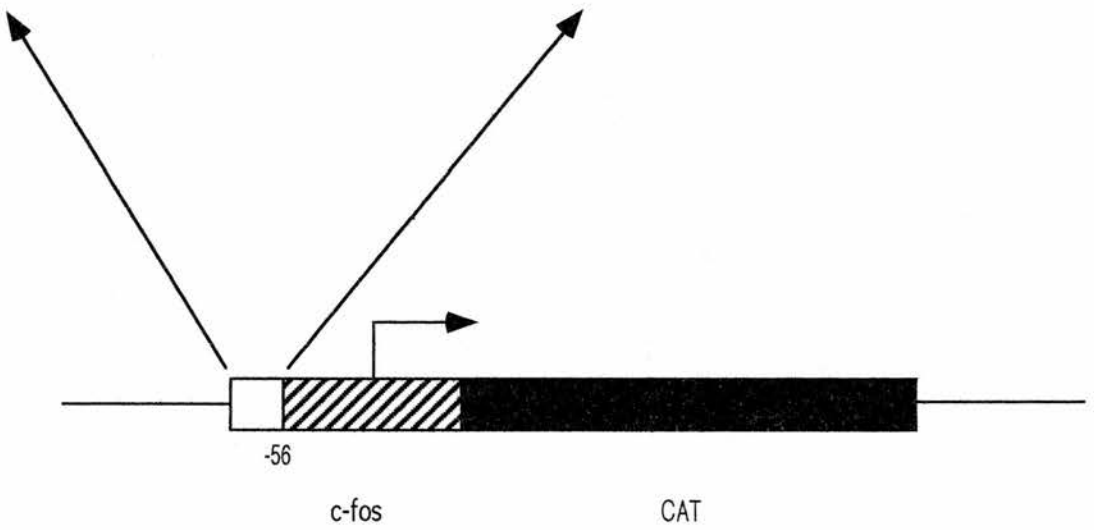


Figure 3.3. Effects of forskolin (10 μ M) and 40mM K⁺ on reporter gene expression in transfected PC12 cells

1.5x10⁶ PC12 cells were electroporated in the presence of 5 μ g of the respective reporter construct DNA. Cells were left overnight in normal medium before addition of medium containing forskolin or K⁺, as indicated. Cultures were then maintained in experimental media for a further three days before assaying for CAT immunoreactivity. NGF was present at all times after electroporation. ND. = not done. Each value is mean \pm standard error for 3 separate culture wells. *= Values significantly increased above those obtained for untransfected cells (P<0.05).

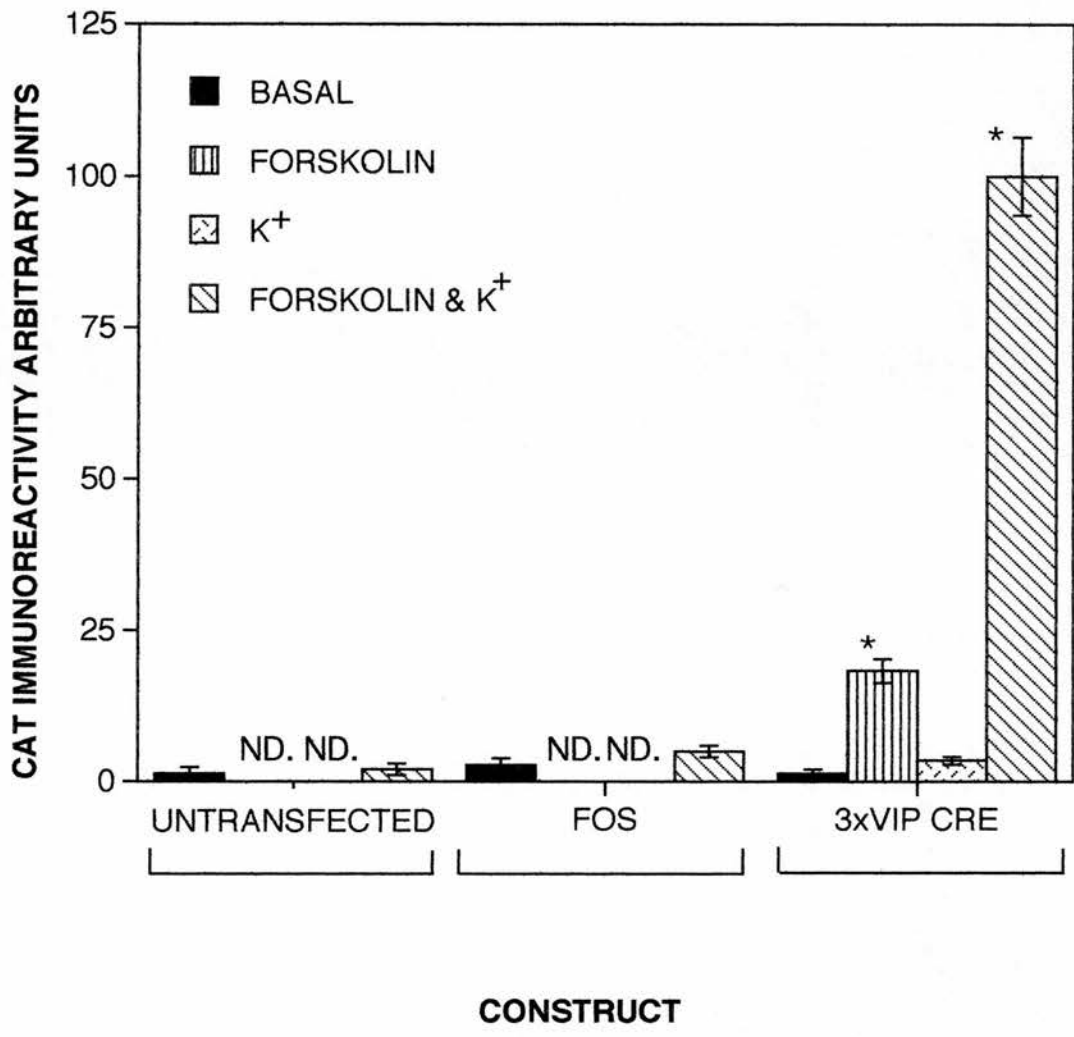


Figure 3.4. Effects of forskolin (10 μ M) and 40mM K⁺ on reporter gene expression in neonatal rat DRG neurons

Reporter constructs as described in the text were microinjected into neonatal rat DRG neurons. The following day cultures were treated as indicated and left for 72h before extracting and assaying for CAT immunoreactivity. ND. = not done. Each value is mean \pm standard error for at least 4 separate culture wells. As absolute levels of CAT immunoreactivity varied between experiments, results were normalised against values obtained with an internal control (cells microinjected with the construct containing 3 copies of the VIP CRE and stimulated with 10 μ M forskolin and 40mM K⁺). Background = cultures that had received no injections. *= Values significantly increased above those obtained for uninjected cells (P<0.05).

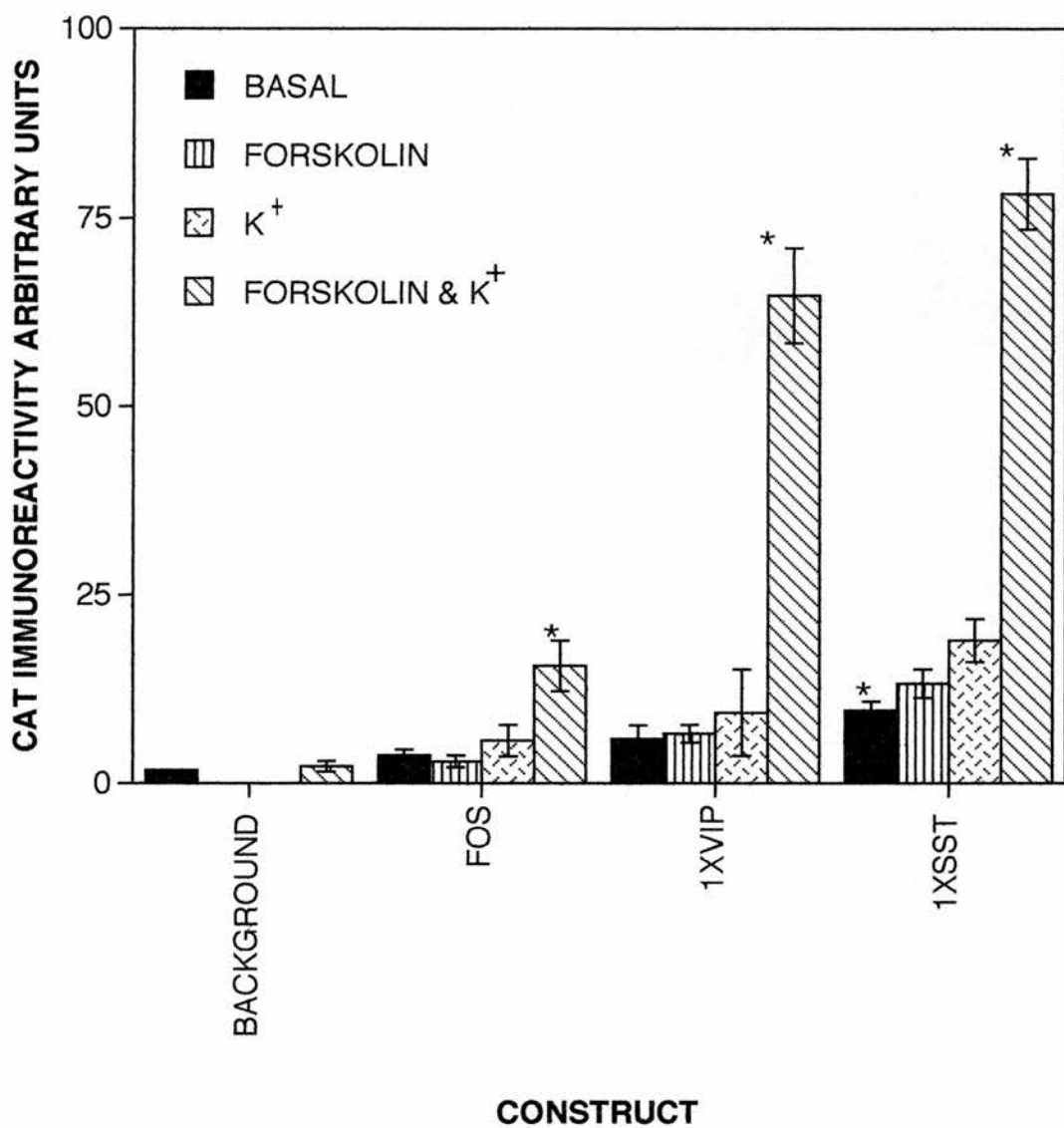


Figure 3.5. Effects of forskolin (10 μ M) and 40mM K⁺ on reporter gene expression in adult rat DRG neurons

Reporter constructs as described in the text were microinjected into adult rat DRG neurons. The following day cultures were treated as indicated and left for 72h before extracting and assaying for CAT immunoreactivity. Each value is mean \pm standard error for at least 4 separate culture wells. As absolute levels of CAT immunoreactivity varied between experiments, results were normalised against values obtained with an internal control (construct containing 3 copies of the VIP CRE stimulated with 10 μ M forskolin and 40mM K⁺). Background = cultures that had received no injections. * = Values significantly increased above those obtained for uninjected cells (P<0.05). ** = Values significantly increased above those obtained for the FOS construct under conditions of combined 10 μ M forskolin and 40mM K⁺ (P<0.05).

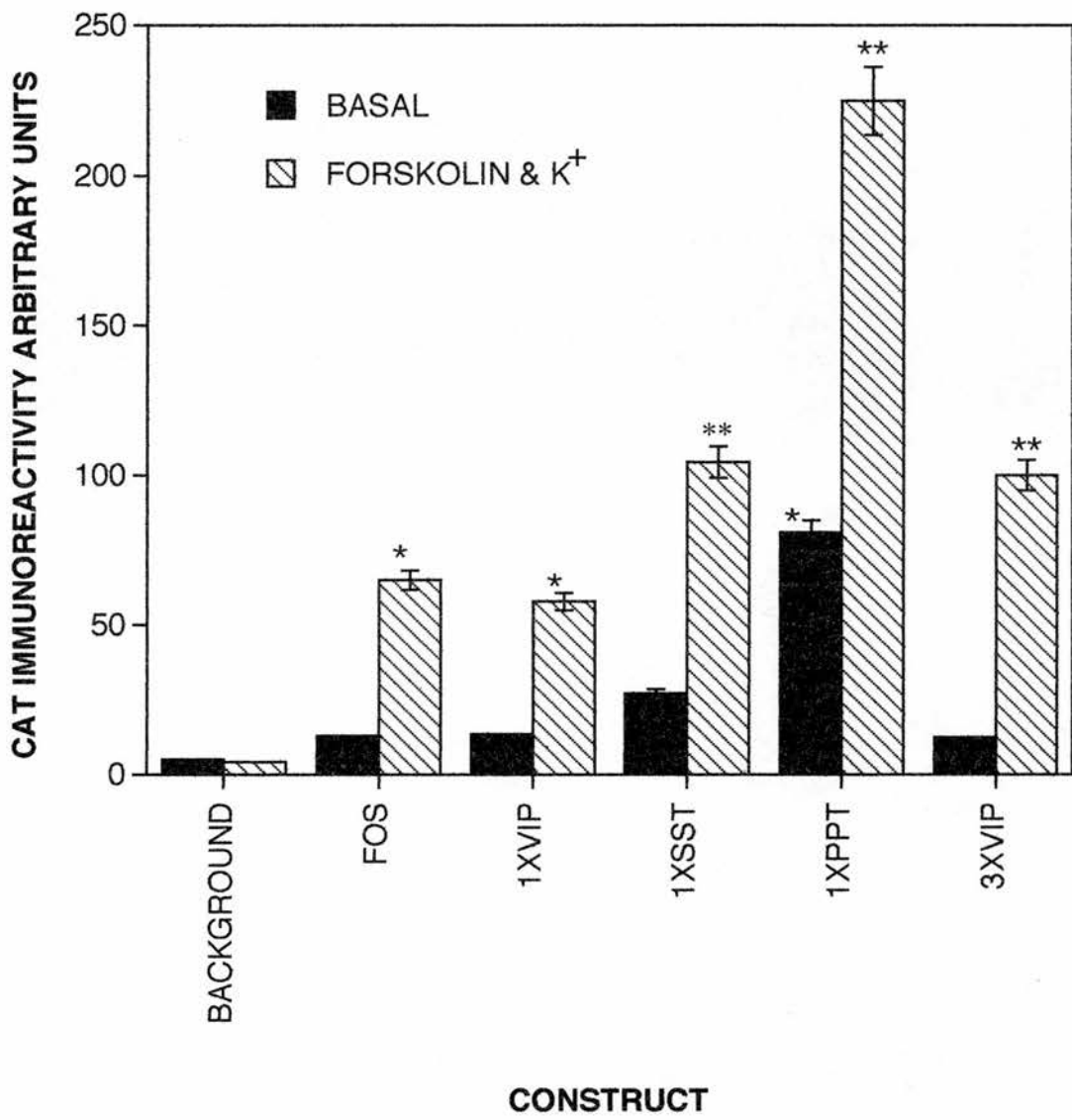


Table 3.1. Comparison of CAT immunoreactivity in neonatal and adult rat DRG neurons microinjected with FOS plasmid construct

Comparison of absolute levels of CAT immunoreactivity (pg/well), between adult and neonatal rat neurons, following microinjection with FOS plasmid. Adult and neonatal rat DRG neurons were plated out at similar densities on alternate days and 350 neurons/well were microinjected with FOS plasmid. Following stimulation with combined 10 μ M forskolin and 40mM K⁺ as indicated, extracts were assayed simultaneously for CAT immunoreactivity. Each value is the mean \pm standard error for four separate culture wells.

	NEONATAL RAT NEURONS	ADULT RAT NEURONS
Uninjected basal conditions	3.83 ± 1.82	8.40 ± 0.79
Neurons injected with FOS Basal conditions	13.06 ± 4.14	28.74 ± 5.26
Neurons injected with FOS 10µM forskolin + 40mM K ⁺	59.48 ± 18.20	109.86 ± 18.52

CHAPTER 4

THE ROLE OF 5' FLANKING SEQUENCES FROM THE RAT VIP GENE IN MEDIATING VIP GENE EXPRESSION

4.1 INTRODUCTION

Although the results described in chapter 3 suggest that the rat VIP CRE is capable of conferring a response to stimuli that raise cyclic AMP and intracellular calcium on the reporter construct FOS, the CRE alone is not capable of conferring spontaneous expression of the FOS promoter in adult rat DRG neurons. Thus, other regulatory sequences from the VIP gene are probably required, in combination with the CRE, for spontaneous VIP expression.

This chapter describes experiments in which a reporter construct containing 1697bp of upstream (5' flanking) sequence, including the rat VIP CRE and promoter (Giladi, et al., 1990, Lamperti, et al., 1991), the first exon of 151bp and approximately 950bp of the first intron, was transfected into DRG neurons.

Typically, sequences within a few hundred base pairs of the promoter regions of genes play a key role in regulating transcription (for a review see Johnson and McKnight, 1989). Previous experiments have demonstrated high levels of expression in DRG neurons of reporter constructs containing up to 3356bp of 5' and 527bp of 3' sequence from the rat PPT promoter (Mulderry, et al., 1993). Deletion studies using reporter constructs containing up to 5.2kb of 5'-flanking sequence from the human VIP gene show that multiple regulatory sequences exist in this region of the DNA (Waschek, et al., 1992).

As rat VIP expression has been shown to be inhibited by NGF in neonatal rat DRG neurons (Mulderry, 1994), NGF deprivation may play a role in VIP induction (see chapter 1.6.1). VIP has been reported to be induced by LIF in sympathetic superior cervical ganglion neurons (Sun, et al., 1994) and in neonatal rat DRG neurons grown in the presence of NGF

(Mulderry, 1994). As an induction of LIF expression occurs following axotomy of DRG neurons (Curtis, et al., 1993, Curtis, et al., 1994), LIF may be partly responsible for inducing VIP expression in axotomised DRG neurons (see chapter 1.6.1). The response of constructs to combinations of LIF and NGF was therefore determined.

4.2 METHODS

4.2.1 Construct Design

Three constructs containing either 1697bp , 960bp or 279bp of 5' flanking sequence from the rat VIP gene (termed construct 1697, construct 960 or construct 279 respectively) followed by exon 1 of 151bp, approximately 950bp of the first intron and a β galactosidase reporter gene (Fig. 4.1) were designed as described in Fig. 4.2 and Fig. 4.3. A full length clone of the rat VIP gene, approximately 15kbp, in bacteriophage Charon 4A was obtained from Professor Illana Gozes, Sackler School of Medicine, Tel Aviv, Israel (Giladi, et al., 1990). The host cells *Escherichia Coli* LE392 were infected with the phage stock and a large scale plate lysate preparation of the bacteriophage was grown up from a single clone (appendix A.2). This clone containing the endogenous promoter was digested with the restriction enzyme *EcoR1* to generate 7 fragments (Fig. 4.2). The largest two of 19,500bp and 10,500bp, correspond to the bacteriophage DNA (not shown in Fig. 4.2), while the remaining 5 fragments of 5200bp, 4300bp, 4000bp, 900bp and 700bp correspond to the genomic VIP DNA as described by Giladi et al (1990). The fragment of 5200bp, containing the transcription start site, the first two exons and approximately 3kb of 5' flanking sequence was isolated and cloned into the *EcoR1* site of the plasmid vector pUC18. The orientation of the fragment within this plasmid was determined by restriction mapping with

the enzyme *Pst1* which cuts once in the plasmid vector and at three positions in the 5200bp fragment. Orientation was also determined by sequence analysis as described in chapter 5. Both orientations were obtained. Digestion of the appropriate clone with *Pst1* yielded an approximately 2800bp fragment containing the VIP promoter that was cloned into the *Pst1* site of pUC18. Digestion with the enzymes *Nco1*, which cuts twice within the fragment and *Xba1* which cuts once within the polylinker of the pUC18 plasmid, were used to determine the orientation of the fragment. The 2800bp fragment was removed from the appropriate pUC18 clone using the restriction enzymes *HindIII* and *Xba1* that cut either side of the fragment within the pUC18 polylinker and ligated into plasmid prPPT- β GAL3 (Mulderry, et al., 1993) upstream of the *E. coli lacZ* gene (Fig. 4.3). Before ligation prPPT- β GAL3 was digested with *Hind III* and *Xba1* to remove a fragment containing a separate promoter. The 2800bp VIP fragment was ligated into the *Hind III* and *Xba1* sites of the 6726bp prPPT- β GAL3 plasmid vector in a known orientation. The final construct (construct 1697) contained 1697bp of 5' flanking sequence (including the CRE element and the endogenous VIP promoter) followed by the transcription start site, the first exon and approximately 950bp of the first intron (Fig. 4.3). The *lacZ* gene was located a further 500bp downstream. The first translation start site downstream of the VIP promoter in this construct is located in the β galactosidase gene, since the first exon of the VIP gene contains no translation start sites.

Construct 1697 was digested with the restriction enzymes *Apa1* and *Xba1* resulting in two fragments the smaller of which (1418bp) was discarded. The larger fragment was gel purified, the ends were filled in with klenow and ligated back together (appendix A.6-8) to generate construct 279 (Fig. 4.3).

Construct 1697 was digested fully with the enzyme Xba1 followed by a partial digest with the enzyme Nco1 which cuts in two positions within the VIP gene (Fig. 4.3). This resulted in 5 fragments (737bp, 867bp, 1604bp, 7922bp and 8789bp) in addition to the linearised plasmid (9526bp). The fragment of size 8789bp, generated by the removal of a 737bp Xba1-Nco1 fragment (Fig. 4.3), was isolated (see appendix A.5-6) the ends were filled in with klenow and ligated back together (appendix A.7-8) to generate construct 960 (Fig. 4.3).

4.2.2 DRG neurons; transfection and treatment

DRG neurons were prepared and transfected with constructs by microinjection as described in appendix A.19 and appendix A.20. Following microinjection of reporter constructs cells were left overnight in normal medium before addition of medium containing 40mM K⁺ and 10 μ M forskolin (as described in appendix A.21) or fresh normal medium. For comparisons between reporter constructs all cell were plated at a density of 4,000 cells per well and were supported in culture with NGF (50ng/ml). NGF was present at all times before and after microinjection.

In studies of the effects of NGF and LIF, cells were maintained in the absence of NGF or LIF (approximately 10,000 cells/well) for 2 days prior to microinjection. Following injection cells were then left 24h before addition of either NGF (50ng/ml), LIF (100U/ml) or a combination of the two. LIF concentrations are expressed in units of mouse embryonic stem cell differentiation inhibiting activity (Smith, 1991, Smith, et al., 1988). Cells were left in the presence of growth factor for 72 hours before harvesting and assaying for β galactosidase activity as described in appendix A.23.

Maintaining all cells under similar conditions prior to microinjection eliminates any differences in cell survival or morphology that may result in differences in microinjection efficiency between each well.

Statistical analysis of β galactosidase activity was performed using a 2-way analysis of variance for all combinations of injected construct and treatment of cells after injection. A post-hoc Student Newman-Keuls test was used to detect significance of individual comparisons. The critical value for significance of differences between group means was taken as $P < 0.05$.

4.3 RESULTS

4.3.1 Response to forskolin and depolarisation

When microinjected into neurons from adult rats and assayed under basal conditions none of the constructs expressed β galactosidase at levels significantly higher than the background level (presumably due to endogenous β galactosidase activity) obtained in uninjected cells (Fig. 4.4). However, significant levels of β galactosidase activity (approximately 8-fold higher than the non-specific levels obtained under basal conditions) were detected in cells microinjected with construct 1697 or construct 960 and stimulated with $10\mu\text{M}$ forskolin and 40mM K^+ (Fig. 4.4). In contrast, β galactosidase activity in neurons microinjected with construct 279 was not increased in the presence of forskolin and K^+ .

Similar results were obtained when constructs 1697, 960 and 279 were microinjected into neurons from neonatal rats: under basal conditions, none of the constructs expressed β galactosidase at levels significantly higher than the background level obtained in uninjected cells. Cells injected with constructs 1697 or 960 expressed significantly increased

levels of β galactosidase activity following stimulation with forskolin and K^+ , when levels were approximately 7.5-fold higher than those obtained under basal conditions. Cells injected with the construct 279 did not express significantly increased levels of β galactosidase in the presence of forskolin and K^+ , above those obtained when incubated under basal conditions alone (Fig. 4.5).

4.3.2 Response to NGF and LIF

In order to determine the effects of NGF and LIF on gene transcription, cells grown in the absence of NGF and LIF were transfected with construct 1697. DRG neurons from adult rats, transfected with this construct, did not express significantly increased levels of β galactosidase above the low levels obtained in uninjected cells, in response to NGF alone, LIF alone or a combination of NGF and LIF (Fig. 4.6). Cells maintained in the absence of either NGF or LIF did not express significantly altered levels of β galactosidase. Transfected cells could be made to express significantly increased levels of β galactosidase when cultured in the presence of $10\mu M$ forskolin and $40mM K^+$ when levels reached approximately 8-fold higher than those obtained under basal conditions, showing that the neurons had been transfected successfully.

Although up to 70% of DRG neurons from neonatal rats die when cultured in the absence of growth factors, a significant proportion do survive (Mulderry, 1994). However, this population of neurons did not express β galactosidase when transfected with the construct containing 1697bp of 5' flanking sequence and grown in the presence of either NGF alone, LIF alone or a combination of NGF and LIF. Cells maintained in the absence of NGF or LIF following microinjection, also did not show any significant difference in β galactosidase expression when compared to uninjected

cells. Again the construct could be induced in these cells to levels approximately 5.3 fold higher than those obtained under basal conditions, in response to combined stimulation with 10 μ M forskolin and 40mM K⁺ (Fig. 4.7).

4.4 DISCUSSION

4.4.1 Reporter construct expression

As no expression from any of these constructs could be detected in adult rat DRG neurons, under basal conditions, it appears that sequences in addition to those present in the 2800bp VIP gene fragment are required for the spontaneous increase in endogenous VIP expression that occurs in cultured DRG neurons. As expression could be detected from these constructs under conditions of forskolin and K⁺, the construct is known to be present in these cells. However, as no significant difference was obtained between transfected and untransfected cells the construct is not being transcribed at levels high enough to be detected in the absence of forskolin and K⁺.

4.4.2 Response to LIF

No response to LIF could be detected in adult or neonatal rat DRG neurons injected with construct 1697. Experiments performed by P. Mulderry (Mulderry, 1994) with the same preparation of LIF showed that endogenous VIP is regulated by LIF in neonatal rat DRG neurons and that the preparation of LIF was biologically active. It would be informative to examine the expression of construct 1697 in NBFL cells or sympathetic neurons where endogenous VIP gene expression has been shown to respond to LIF (Sun, et al., 1994). Although exogenous LIF has been

shown to have no effect on VIP expression in cultured adult rat neurons (Mulderry, 1994), it is possible that LIF may already be present endogenously at high enough levels to be partly responsible for the spontaneous expression seen in adult rat neurons. Experiments remain to be performed to see if removal of endogenous LIF from cultures of adult rat DRG neurons results in a reduction in VIP gene expression. If this is the case it is likely that construct 1697 does not contain all the sequences required for a response to LIF. This may also explain the lack of expression from this construct in neonatal rat DRG neurons where endogenous VIP is expressed when cells are cultured in the presence of LIF but not NGF.

4.4.3 Response to NGF

Although NGF has been shown to be capable of altering transcription levels of certain genes, by acting on response elements within 150bp 5' of the transcription start site (DeFranco, et al., 1993, Watson and Latchman, 1995) and NGF has been shown to inhibit VIP expression in DRG neurons from neonatal rats (Mulderry, 1994), no response to NGF could be detected on β galactosidase expression from construct 1697 in either adult (Fig. 4.6) or neonatal (Fig. 4.7) rat DRG neurons. Although no expression could be detected in the absence of NGF it is still possible that if longer constructs were made that did spontaneously express a reporter gene, expression could be inhibited by NGF. Results from this experiment provide no evidence that sequences found in construct 1697 may be involved in a transcriptional response to NGF.

It is possible that NGF may act postranscriptionally to regulate translation or post-translational processing as has been observed for other genes (Nishizawa, 1994), in which case NGF would be expected to have no effect on reporter gene expression.

4.4.4 Response to 10 μ M forskolin and 40mM K⁺

Although constructs 1697 and construct 960 expressed increased levels of β galactosidase in response to combined forskolin and K⁺, the shorter construct containing 279bp of flanking sequence did not respond, even though it contains the CRE sequence. This suggests that sequences between -279bp and -960bp are required, in addition to the CRE, for the response to forskolin and K⁺. These results contrast with those described in Chapter 3, where it was shown that the VIP CRE was capable of responding to combined forskolin and K⁺ when placed in the context of the c-fos promoter. There are two possible explanations of these apparent discrepancies:

(1) Alternative sequences may be present in the FOS construct that act together with the CRE to give a response to forskolin and K⁺. Alternatively, the structure of the c-Fos promoter, used in the FOS construct, may be such as to make these sequences unnecessary. The Fos promoter contains only a single TATA box between the CRE and the transcription start site (Fig. 3.1) (Gilman, et al., 1986), whereas the rat VIP promoter contains a TATA box on either side of the CRE and a CAAT box only 28bp 5' of the CRE (Giladi, et al., 1990). These different structures may affect the response of the CRE to forskolin and K⁺ (Deutsch, et al., 1988).

(2) Construct 279 may contain sequences capable of binding to proteins that repress gene transcription. This effect may be overcome by proteins that bind to sequences between -279bp and -960bp 5' from the transcription start site in response to forskolin and K⁺. A similar situation exists for the somatostatin gene, where a repressor is thought to bind to a sequence between -425 and -345, the effect of which is overcome by the presence of sequences between -750 and -425 (Vallejo, et al., 1992).

Such a repressor is probably not present in the Fos promoter, allowing CRE regulated gene transcription. A repressor may play an important role in preventing VIP gene transcription in DRG neurons *in vivo* before axotomy, when VIP expression can not be detected (Nielsch and Keen, 1989, Shehab and Atkinson, 1986).

It is not known whether these constructs respond synergistically to forskolin and K⁺ in a similar manner to endogenous VIP in neonatal rat DRG neurons, while at the same time showing a less than additive response to forskolin and K⁺ in adult rat DRG neurons. To determine if this is the case and therefore whether these sequences can direct similar developmental alterations on gene expression, seen for the CRE containing FOS construct in chapter 3, further experiments must be performed showing the response of these constructs to forskolin alone and K⁺ alone.

Differences in the developmental response of construct 1697 and endogenous VIP to forskolin alone and K⁺ alone would indicate that sequences outside the region studied are involved. However, the CRE is responsible for developmental alterations in reporter gene expression analogous to endogenous VIP expression (Dobson, et al., 1994 and chapter 3). Therefore the CRE is likely to play a large role in any developmental alteration of construct 1697 in response to forskolin and K⁺.

Figure 4.1. β Galactosidase expressing reporter constructs for VIP genomic sequences

Reporter constructs containing the rat genomic VIP promoter together with 950bp 3' from the transcription start site (arrow) and up to 1697bp 5' from the transcription start site as indicated. The β galactosidase reporter gene (filled box) is located a further 500bp downstream from the 3' end of the genomic VIP sequences. Hatched box indicates VIP genomic sequence. Single lines represent vector. Constructs are drawn to scale.

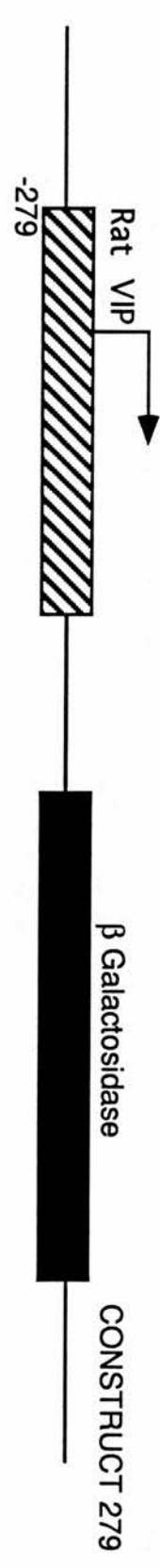
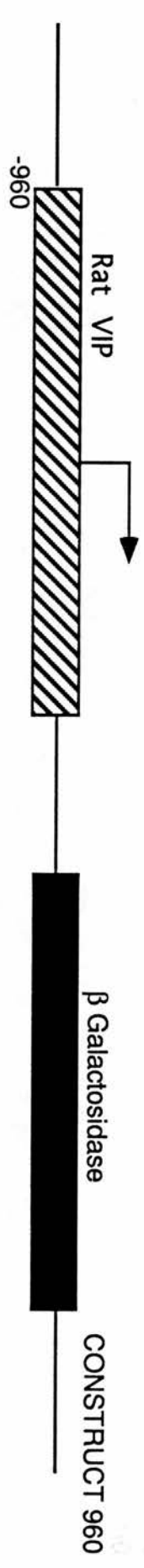
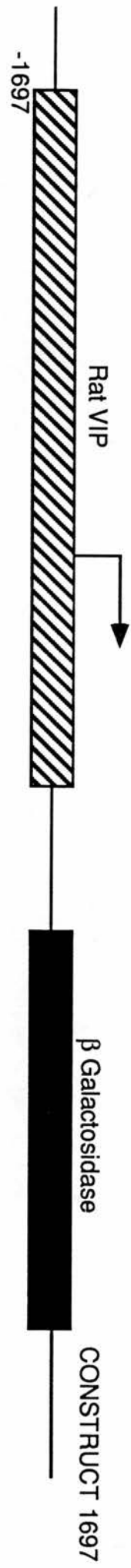


Figure 4.2. Structure of the rat VIP gene

A full length VIP genomic clone (15kb), showing the relative positions of the 7 exons (filled boxes). Digestion with the restriction enzyme *EcoR1* yielded 5 fragments, the largest of which (5200bp) was cloned into the *EcoR1* site of pUC18. This 5200bp *EcoR1* fragment was further digested with the enzyme *Pst1* to yield a fragment of 2800bp which was cloned into the *Pst1* site of pUC18 in either orientation. Orientation was determined as described in the text. The appropriate clone was digested with *Xba1* and *HindIII* which each cut once within the polylinker of pUC18. The resulting 2800bp fragment was then cloned into the plasmid prPPT- β GAL3 cut with *Xba1* and *HindIII* to yield plasmid construct 1697 (Fig 4.1). Restriction enzyme sites are indicated with text.

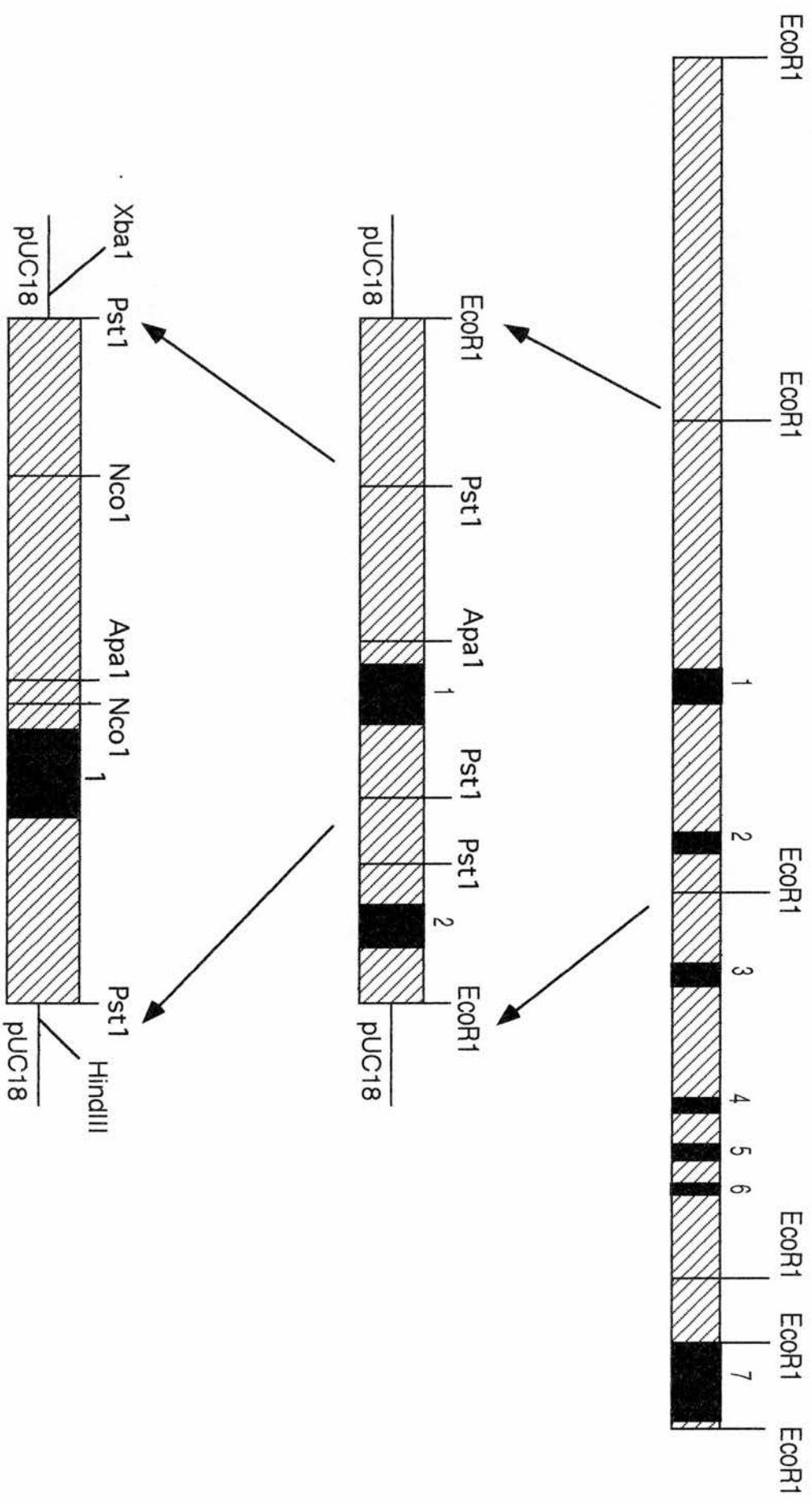
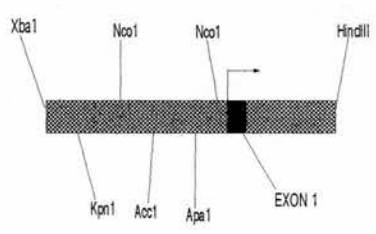
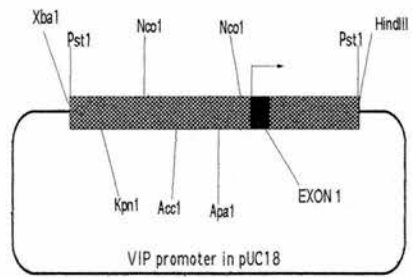


Figure 4.3. Construction of construct 960 and construct 279

The 2800bp *pst* 1-*pst* 1 genomic VIP fragment from the rat VIP gene was cloned into the vector prPPT-βGAL3 cut with *Xba* 1 and *Hind*III to generate construct 1697. Restriction sites are indicated by text. Filled box indicates the first exon of the VIP gene. Arrow indicates transcription start site. Removal of the 737bp *Xba* 1 to *Nco* 1 fragment (as described in the text) resulted in the formation of construct 960. Removal of the 1418bp *Xba* 1 to *Apa* 1 fragment resulted in the formation of construct 279.



prPPT-βGAL3 cut with HindIII/Xba1

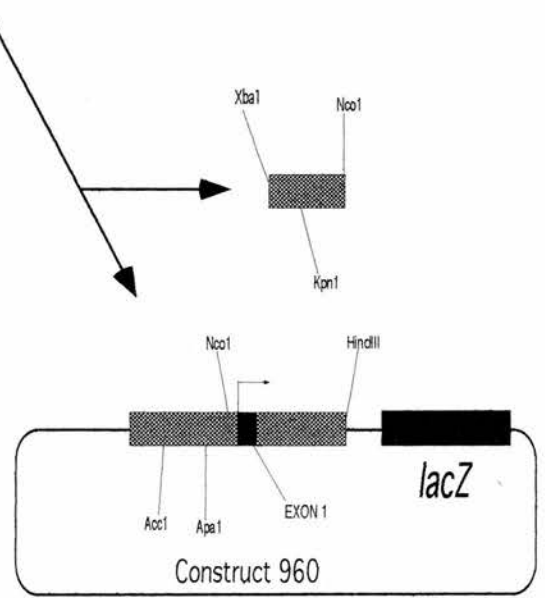
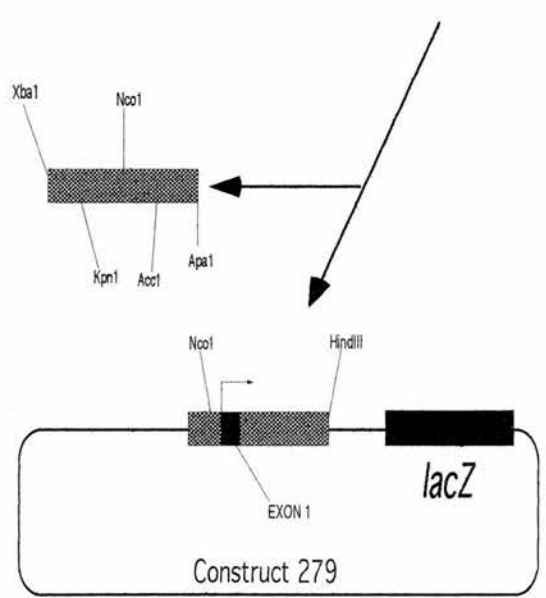
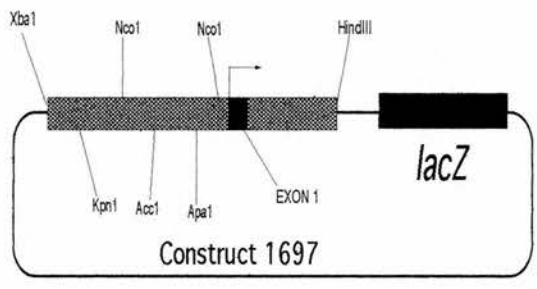


Figure 4.4. Effects of 10 μ M forskolin and 40mM K⁺ on expression of genomic VIP reporter constructs in adult rat DRG neurons

Following microinjection of neurons with reporter constructs 1697, 960 or 279, cells were left overnight before changing to experimental media as indicated. After 72 hours cultures were extracted and assayed for β galactosidase enzyme activity. Each value is mean \pm standard error for 3 separate culture wells. Uninjected = cells that have received no injections. *= Values significantly increased above those obtained for uninjected cells (P<0.05).

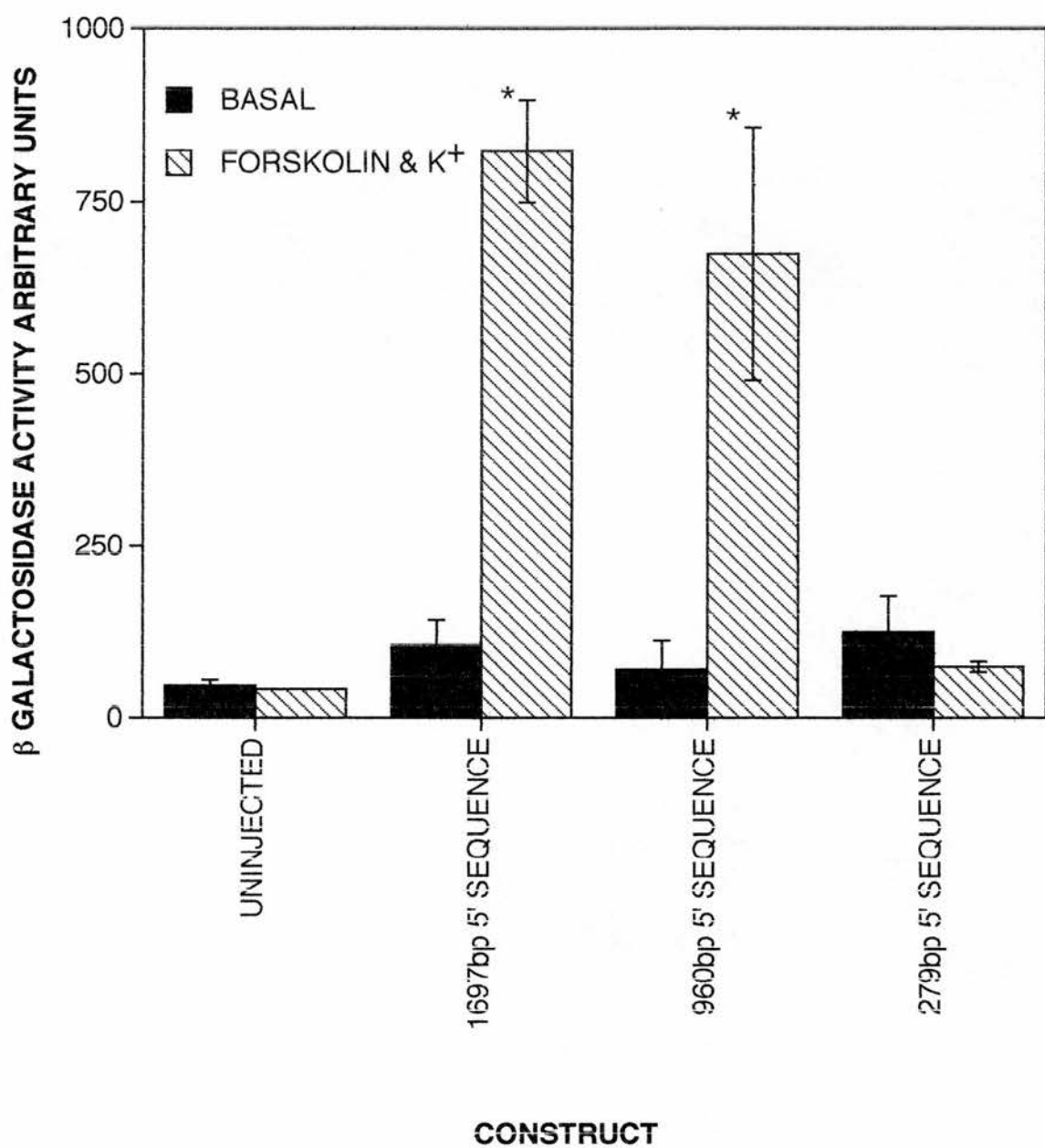


Figure 4.5. Effects of 10 μ M forskolin and 40mM K⁺ on expression of genomic VIP reporter constructs in neonatal rat DRG neurons

Following microinjection of neonatal rat DRG neurons with reporter constructs 1697, 960 or 279, cells were left overnight before changing to experimental media as indicated. After 72 hours, cultures were extracted and assayed for β galactosidase enzyme activity. Each value is mean \pm standard error for 3 separate culture wells. Uninjected = cells that have received no injections. *= Values significantly increased above those obtained for uninjected cells ($P < 0.05$).

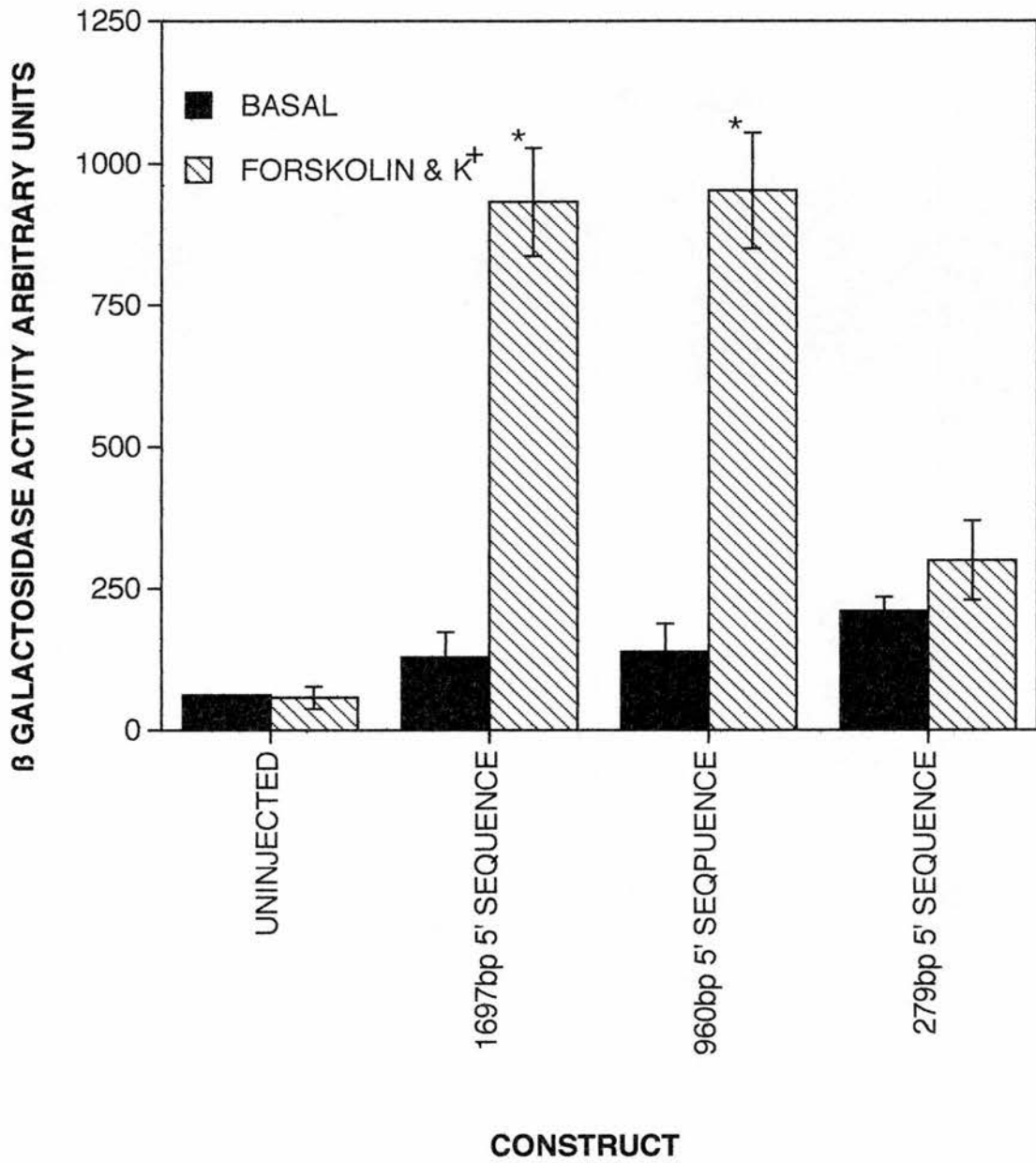


Figure 4.6. Effects of 50ng/ml NGF and 100U/ml LIF on expression of genomic VIP reporter constructs in adult rat DRG neurons

Cultured DRG neurons incubated for 2 days in the absence of added growth factors were microinjected with reporter construct 1697 and left overnight before changing to experimental media as indicated. After 72 hours cultures were extracted and assayed for β galactosidase enzyme activity. Each value is mean \pm standard error for 3 separate culture wells.

Uninjected = cells that have received no injections. * = Values significantly increased above those obtained for uninjected cells under basal conditions ($P < 0.05$).

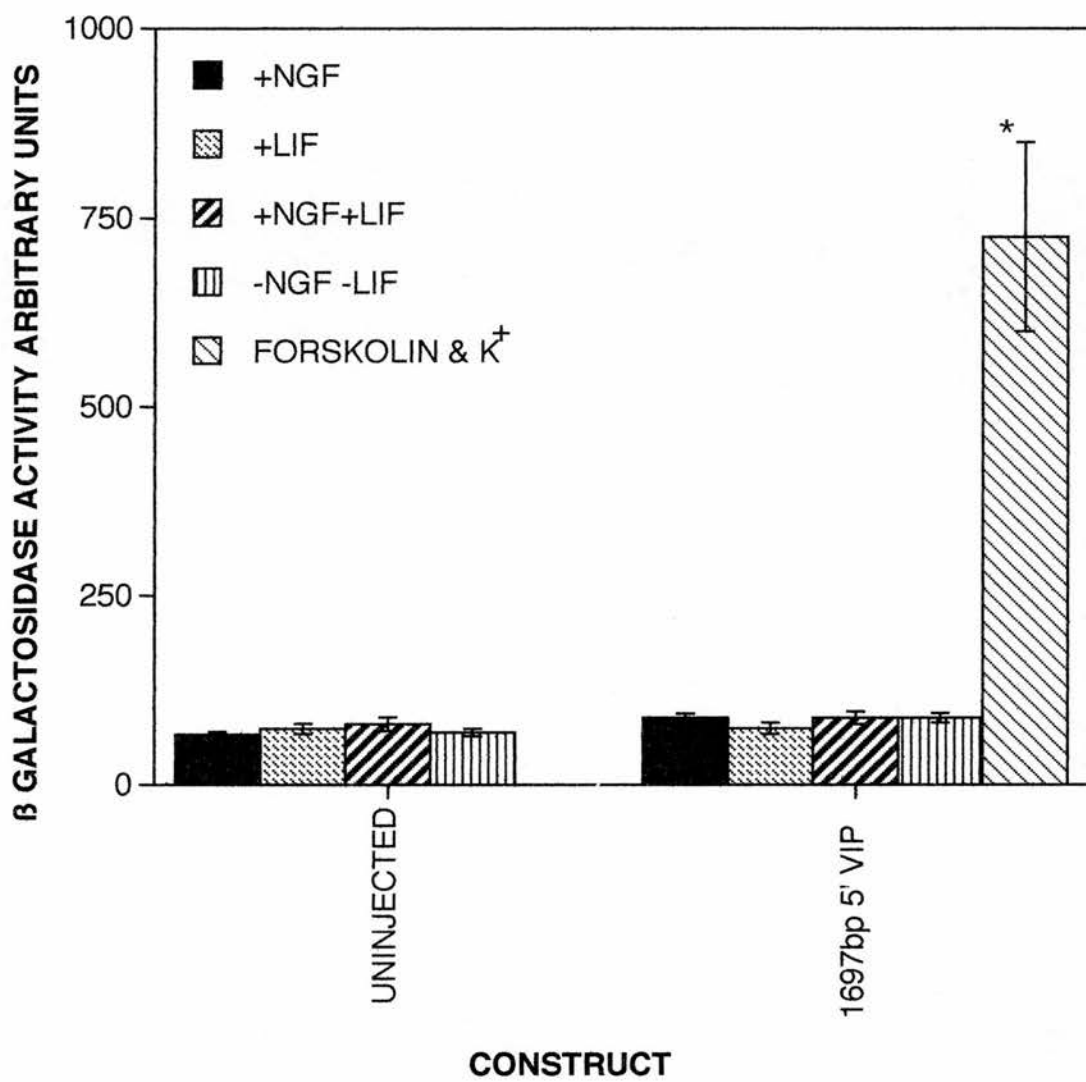
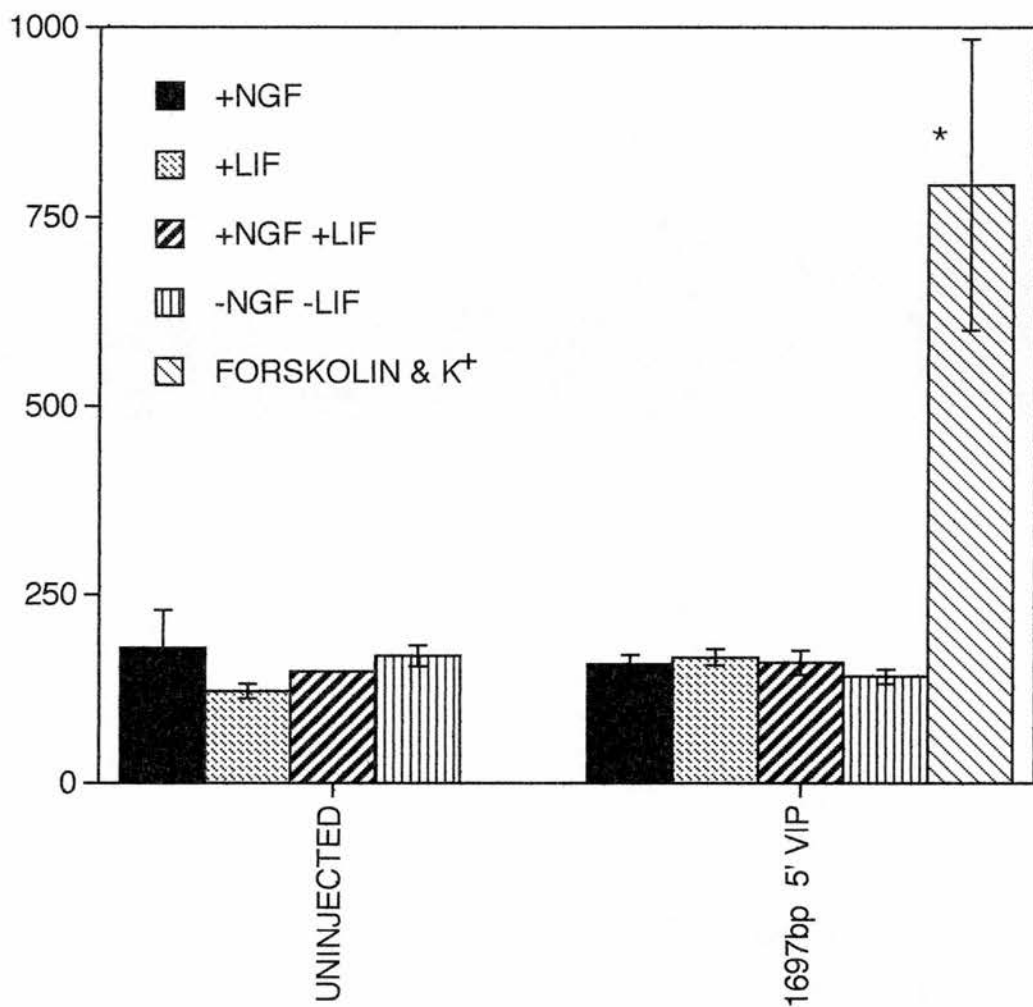


Figure 4.7. Effects of 50ng/ml NGF and 100U/ml LIF on expression of genomic VIP reporter constructs in neonatal rat DRG neurons

Cultured DRG neurons incubated for 2 days in the absence of added growth factors were microinjected with reporter construct 1697 and left overnight before changing to experimental media as indicated. After 72 hours cultures were extracted and assayed for β galactosidase enzyme activity. Each value is mean \pm standard error for 3 separate culture wells.

Uninjected = cells that have received no injections. * = Values significantly increased above those obtained for uninjected cells under basal conditions ($P < 0.05$).

β GALACTOSIDASE ACTIVITY ARBITRARY UNITS



CONSTRUCT

CHAPTER 5

SEQUENCE ANALYSIS OF THE 5' FLANKING REGION OF THE RAT VIP GENE

5.1 INTRODUCTION

The rat VIP CRE could not direct spontaneous expression of a reporter construct, in adult rat DRG neurons, when alone or in the context of up to 1697bp of VIP 5' flanking sequence (chapters 3 and 4). Therefore it would seem likely that sequences outside this region of the VIP gene are required for spontaneous expression. However, in view of the results obtained in chapter 2 it would seem likely that proteins capable of binding to the CRE are important for this expression. There are numerous examples of regulatory elements, including CREs, that synergise in their effects on transcriptional regulation (Vallejo, et al., 1992, Hyman, et al., 1989, Lu and Bensadoun, 1993). Thus it is possible that elements within the 1697bp region are required, together with the CRE and sequences outside the 1697bp region for spontaneous VIP expression.

This chapter describes the sequence analysis of 1697bp of 5' flanking sequence from the VIP gene. This information was obtained (1) in order to establish the structure and orientation of the reporter constructs used in chapter 4. (2) To extend our knowledge of the sequence of the 5' flanking region of the gene (only 242bp 5' of the transcription start site had been published), (3) to allow the identification of elements showing homology with consensus transcription factor binding sites that may be relevant for spontaneous VIP expression and (4) to identify regions of homology with the published sequence of the human VIP gene. Any sequences showing a high degree of homology between the rat and the human genes have been conserved in evolution and may play an important role in transcriptional regulation.

5.2 METHODS

5.2.1 Construct sequencing

The pUC18 vector containing 2800bp of the rat VIP gene (Fig. 4.2) was sequenced by the Sanger dideoxy method (appendix A.17) using the pUC/M13 -40 forward primer and the pUC/M13 reverse primers from Promega. The plasmid was digested with restriction enzymes that cut once within the polylinker of the pUC18 vector and at one or more sites within the VIP clone (*Kpn1*, *Acc1* and *Nco1* see Fig. 4.3). The resulting VIP clone fragment was removed and the remaining intact vector containing a shortened VIP fragment was religated and sequenced using the same primers. Fragments were aligned using the computer programme Genejockey II and gaps were filled in by designing primers to allow sequencing across gaps. Each fragment obtained was sequenced at least twice in a single direction. A total of 1910bp from the 5' end of the 2800bp fragment was sequenced.

5.2.2 Search for consensus transcription factor binding sites

In order to identify possible binding sites in construct 1697 that may be involved in interactions leading to spontaneous VIP expression a scan for putative transcription factor binding sites was carried out using the program SIGNAL SCAN provided by the UK. Human Genome Mapping Project Resource Centre (Prestridge, 1991).

5.3 RESULTS

5.3.1 Sequencing

Following sequencing of the 5' flanking region we were able to show that construct 1697 included sequences corresponding to the first exon

(151bp), 242bp of 5' flanking sequence and 61bp of the first intron of the published rat VIP sequence (Giladi, et al., 1990). Our sequence differed from the published sequence at 4 nucleotides, none of which were within the CRE sequence (Fig. 5.1). It is likely that the 4 differences represent errors in the published sequence. Our sequence showed greater homology to the published sequences of the mouse (Lamperti, et al., 1991) and human (Yamagami, et al., 1988) genes at two of the positions where there were discrepancies (between the two TATA boxes at positions -148 and -146: Fig. 5.1 and 5.2). To determine if these represent errors in the published sequence it will be necessary to isolate and sequence independent clones of the VIP gene.

A comparison of the 1910bp of rat VIP DNA sequenced in this study with the published 5' flanking sequence from the human VIP gene, using the UK. Human Genome Mapping Project Resource Centre, genetics computer group (Wisconsin) package, revealed a homology of 67.1% (Fig. 5.2). This is similar to the 67.8% homology observed between the 454 nucleotides of published rat 5' VIP sequence (Giladi, et al., 1990) and the published human VIP gene (Yamagami, et al., 1988).

5.4 DISCUSSION

Several consensus transcription factor binding sites were identified using the SIGNAL SCAN program. Identified sites are shown below. Positions refer to the number of nucleotides from the 5' end of the known sequence (nucleotide 1 corresponds to -1697 nucleotides 5' from the start of transcription). Numbers in brackets refer to the position relative to the transcription start site

5.4.1 LIF response element

A 180bp sequence from the human VIP gene has been shown to confer a response on a reporter construct to LIF when transfected into NBFL cells (Symes, et al., 1994). This sequence has been shown to contain at least 4 regions with homology to acute phase response elements termed G3, G4, G5 and G6.

G3; GATTCCTGGAATTAA

G4; CTGTCAACTGGGAAACAAA

G5; TTTCTGGTAACTGGATTAG,

G6; TTTACTGGATCAGTCTG

A sequence 100% homologous to G3 was found at position 424bp from the start of sequencing (-1273bp from the transcription start site). G3 has been shown to be absolutely required for the LIF response of reporter constructs transfected into NBFL cells (Symes, et al., 1994). A sequence showing a 16/19bp match with G4 was found at 468 (-1229bp) and an 18/19bp match for G5 at 521 (-1176bp). G6 shows the least homology with the rat VIP gene with a 10/17bp match at 573 (-1124bp) (Fig. 5.2). The position of all these elements relative to the transcription start site corresponds very closely with the positions of homologous elements within the human VIP gene. Although construct 1697 (Fig. 4.1) contains sequences necessary for a LIF response, no such response was obtained in DRG neurons transfected with construct 1697 (Fig. 4.6 and 4.7). Further experiments are necessary to determine whether this construct will respond to LIF in NBFL cells.

5.4.2 AP1 element

Three sequences showing homology to a consensus AP1 site (5'-TGAC/GTCA-3') (Curran and Franzen, 1988) were found at 546 (-1151bp

TGATTAA) , 953 (-744bp TGATTCA) and 1852 (+155bp TGAGTAA).

Although the last of these sites is downstream from the start of transcription, it is a property of enhancer proteins that they can act from long distances away (several hundred to thousand nucleotides) either upstream or downstream from the transcriptional start site (for review see Johnson and McKnight, 1989). As AP1 elements are known to bind the transcription factors Jun and Fos in the form of heterodimers and can also bind Jun/ATF heterodimers or Jun homodimers (Nakabeppu, et al., 1988, Halazonetis, et al., 1988, Hai and Curran, 1991) it is likely that the homologous sites described above will also bind these proteins. As Jun has been shown to be important for spontaneous VIP expression in DRG neurons (Peter Mulderry manuscript submitted), binding of Jun to any of these sites may be important for this expression. As AP1 elements are capable of binding many transcription factors that also bind to CRE sequences (see chapter 2 and Nakabeppu, et al., 1988) it is possible that these sites are also involved in the response of endogenous VIP to forskolin and K⁺.

The only putative AP1 element to be 100% conserved between the human and rat VIP sequence and thus most likely of these three sequences to be important in transcriptional regulation, is that at position 546 (-1151).

5.4.3 AP2 element

Three sequences were found showing homology to a consensus AP2 element (5'-CCCCAGGC-3') (Mitchell and Wang, 1987). CCCAAGGG at 1844 (+147bp), CCCCGCGC at 1807 (+110bp) and CCCATGGG at 1603 (-94bp). Of these sites, only the one at position 1603 (-94) is conserved between human and rat VIP. This position is adjacent to the TGACGTCTTT sequence of the CRE and the last 7 nucleotides (CCATGGG) of this putative AP2 site are included in the CRE containing oligonucleotide used

in the studies described in Chapter 3. The consensus AP2 sequence is thought to bind a 52kd protein known to bind to enhancer regions of the SV40 and human metallothionein IIA genes, stimulating RNA synthesis (Mitchell and Wang, 1987). An AP2 like element of the human proenkephalin gene has also been shown to act synergistically with an adjacent CRE to confer a maximal response to cyclic AMP and phorbol esters (Hyman, et al., 1989). DRG neurons are also known to express AP-2 binding proteins (Mitchell, et al., 1991). It is therefore conceivable that the sequence at position 1603 (-94bp) is capable of binding AP2 and may play a role in modulating responses of the CRE to forskolin and depolarisation. The AP-2 sequence may be required together with the CRE for the spontaneous VIP expression seen in adult rat DRG neurons. It is also possible that proteins binding to this AP-2 element could prevent the binding of proteins to the CRE element or vice versa. Further information on the possible role of the AP2 site in regulating the function of the adjacent CRE could be obtained by DNase 1 footprinting experiments to show which nucleotides are important for binding proteins in adult rat DRG neurons. Additional information could be obtained by performing electrophoretic mobility shift assays (EMSA) using consensus AP-2 or CRE oligonucleotides to compete with proteins in adult rat DRG neurons for binding to a radiolabelled oligonucleotide containing the CRE and AP2 sequences from the rat VIP gene. This would confirm whether AP-2 and/or CRE binding proteins are capable of binding to this sequence.

5.4.4 E-box

An element (CAGCTG) which corresponds to the consensus E-box motif CANNTG was identified at position 472 (-1225bp). The E-box motif is recognised by dimeric protein complexes composed of helix-loop-helix

and basic helix-loop-helix leucine zipper families of proteins (Murre, et al., 1989, Sawadogo and Roeder, 1985). Binding of these proteins to the E-box motif can result in a 10 to 20 fold increase in transcription (Sawadogo and Roeder, 1985). E-box binding proteins are known to be expressed in DRG neurons (Begley, et al., 1992). It is interesting that adult rat DRG neurons transfected by microinjection with a reporter construct containing the E-box motif CACGTG in front of the c-Fos promoter spontaneously express the corresponding reporter gene at high levels (Paterson, et al., 1995). If E-box binding proteins from adult rat DRG neurons are capable of binding to the sequence CAGCTG 5' from the rat VIP gene transcription start site they may have a strong effect on VIP expression.

Since no spontaneous expression was obtained from the constructs containing 1697bp of 5' flanking sequence (chapter 4) any stimulatory effects of the CAGCTG sequence may be antagonised by alternative binding sites or the sequence CAGCTG may be inactive in the context of the rat VIP gene. As the rat VIP CRE sequence has been shown to respond to forskolin and K⁺ when together with the c-Fos promoter, but not its endogenous VIP promoter, as discussed above, it is possible that the response with the CACGTG E-box sequence is only fortuitously obtained when using the c-Fos promoter and would not be obtained when in the context of the rat VIP promoter. Experiments to determine if this is true will require a fragment of the VIP gene that can be used in a reporter construct and that does direct spontaneous expression. Mutation of the E-box motif in this construct will determine its importance in the spontaneous expression of VIP. Similar experiments to those performed in chapter 2 could also be performed, but using this E-box motif instead of the CRE to compete binding proteins off the endogenous VIP gene.

5.4.5 C/EBP site

CAAT box/enhancer binding proteins (C/EBP) are a leucine zipper containing protein family originally identified due to interactions with the CCAAT motif of the herpes virus thymidine kinase promoter (Landschulz, et al., 1989, Graves, et al., 1986). It is possible that members of this family may bind to the sequence CAAT at position 1572 (-125bp) as identified by Giladi et al (Giladi, et al., 1990) or to a sequence identified at position 1861 (+164bp) as TCTTACTC which conforms to the consensus C/EBP site of TCN TACTC where N can be any of the four nucleotides (Costa, et al., 1988). It is known that certain members of the C/EBP family can dimerise with proteins capable of binding CRE elements resulting in a complex with altered DNA binding specificity (Vallejo, et al., 1992). Thus proteins binding to the CAAT box may compete with the CRE for CRE binding proteins. This may explain why the CRE containing FOS construct responds to forskolin and K⁺ while the CRE containing construct 279 does not. C/EBP α has been shown to be able to act as a repressor of transcription, when in the context of certain promoters (Lopez-Cabrera, et al., 1990) and may also be responsible for repressing the expression directed by construct 279 in response to forskolin and K⁺ as discussed previously in section 4.4.4.

5.4.6 Conclusions

To obtain a complete understanding of the regulatory sequences necessary for spontaneous VIP gene expression it will be necessary to create longer constructs that respond in a similar manner to endogenous VIP. Binding sites such as the CRE that are thought to play a role in this expression (see chapter 2) could then be mutated individually to determine

their effects. Although a number of sites that may be involved in spontaneous VIP expression have been identified in this study, the ability of any of these sites to regulate gene expression is unknown. This will require further analysis of their binding characteristics together with functional studies, for example of their ability to confer expression on a reporter construct or their ability to reduce VIP expression when microinjected into DRG neurons. Footprinting studies performed on the 2800bp fragment of the rat VIP gene will allow identification of sites within this sequence capable of binding transcription factors. However due to the heterogeneity of DRG neuronal cultures footprinting studies using extracts from these cells may be difficult to perform. Useful information may instead be obtained from footprinting studies performed with cultured HeLa cells, which are known to express a large array of transcription factors that are also expressed in DRG neurons, or with VIP producing cell lines such as the neuro-blastoma cell line SH-SY-5Y (Ross, et al., 1983). This approach has already been used successfully to characterise potential regulatory elements within the rat PPT gene (Mendelson and Quinn, 1995).

Figure 5.1 Comparison of Sequenced and Published Rat VIP 5' Flanking Region

Nucleotides are numbered from the start of the known sequence. The top line indicates previously published rat VIP sequence. The lower line indicates sequence obtained from clones containing fragments of the rat VIP gene as described in the text.

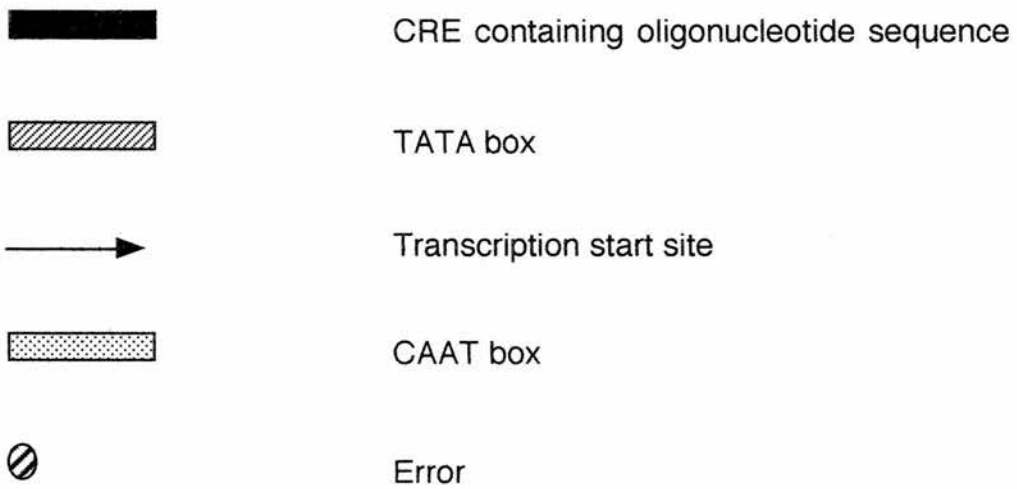
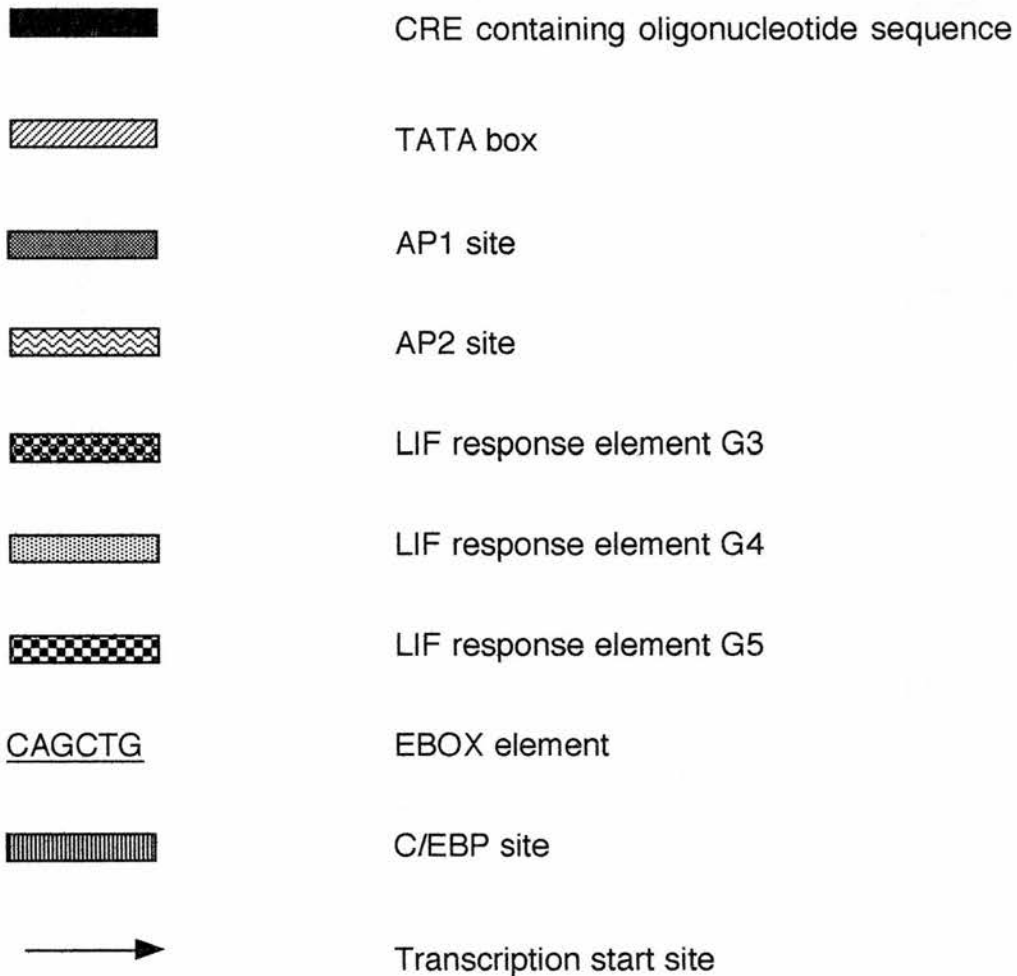


Figure 5.2. Comparison of rat VIP 5' flanking region sequenced in this study with previously published human VIP 5' flanking region

Previously published human VIP sequence is shown on the bottom line (Yamagami, et al. 1988). The top line indicates the 1910 nucleotides of sequence obtained from clones containing fragments of the rat VIP gene as described in the text. Nucleotides are numbered from the start of the known sequence. There is 67.1% homology between the two sequences.



1877 TTCCTCTTCGCCTTCGGGACTTTGAATAGGAGGG..... 1910

|| | || | | || || | | |

2125 TTTCCCTCCTGCCTCTGGAAC TTTCTCACAAAATAATGAATTTTCTCT 2174

CHAPTER 6

OVERALL DISCUSSION AND FUTURE DIRECTIONS

CHAPTER 6

This thesis has provided an insight into the role of 5' flanking sequences in the regulation of VIP gene expression in DRG neurons in culture (Mulderry and Lindsay, 1990). I have shown that proteins that bind to the CRE are required for VIP gene expression and have identified proteins that may fulfil this function. I have also demonstrated that sequences other than the CRE are required. The experiments described in Chapter 4 indicate that at least some of these sequences must be located outside the 2800bp sequence (1697bp 5' and approximately 1100bp 3' from the transcription start site) analysed in reporter constructs.

This study provides evidence for the importance of transcription factors which bind to the CRE in the regulation of VIP gene expression following axotomy. I have shown that the rat VIP CRE is a functional element that can act to increase gene expression in neonatal rat DRG neurons in response to cAMP and calcium dependent pathways. However, the VIP CRE does not respond to these stimuli in cultured adult rat DRG neurons, either in isolation or in the context of the endogenous VIP promoter. Therefore, if cAMP and calcium dependent pathways play a role in VIP expression by cultured adult rat DRG neurons it is unlikely that they regulate transcription factors binding to the CRE.

I have shown that c-Jun is a candidate transcription factor regulating VIP expression through binding to the CRE. However, it is not clear how a signal originating at the site of axotomy could regulate c-Jun or other transcription factors binding to the CRE. NGF and LIF, which are candidate mediators of such a response, are incapable of increasing gene expression by CRE-containing reporter constructs and appear to have no effect on VIP expression by cultured adult rat DRG neurons (Mulderry, 1994). However,

a CRE sequence from the CGRP gene has been shown to be necessary for NGF responsiveness of CGRP (Watson and Latchman, 1995). Therefore, it is possible that the CRE is required for NGF responsiveness of VIP by cultured neonatal rat DRG neurons (Mulderry, 1994).

Other possible mediators of the response to axotomy are GTP-binding proteins, which are known to mediate signal transduction in diverse cell types by a variety of receptors (Bourne, et al., 1990, Gilman, 1987). In their basal inactive state these proteins bind GDP. Following interaction with a receptor-ligand complex GDP is released. GTP then binds shifting the protein to its active conformation allowing it to activate various second messenger systems. Fast axonal transport is modulated by cycles of GTP hydrolysis and nucleotide exchange by one or more GTP-binding proteins (Bloom, et al., 1993). Changes in VIP and c-Jun expression very similar to those seen after peripheral axotomy can be produced without physical nerve damage by applying vinblastine, an inhibitor of axoplasmic transport (Kashiba, et al., 1992, Keen, et al., 1989, Leah, et al., 1991). As activation of GTP-binding proteins inhibits fast axonal transport it is possible that one of the initial stages in the axotomy response is activation of these proteins (Bloom, et al., 1993). Certain GTP binding proteins, for instance RAS, have also been implicated in the control of c-Jun expression by regulating the MAP kinase signalling pathways (Maruta and Burgess, 1994). Addition of inhibitors such as thiophosphorylated derivatives of GTP or the protein phosphatase CL100 that act on G proteins or MAP kinases respectively will help in the identification of pathways responsible for the axotomy response (Alessi, et al., 1993, Strittmatter, et al., 1992). It is possible that c-Jun may become phosphorylated, following axotomy or once cells are placed in culture, and may regulate its own expression by binding to an AP-1 like element located in its own promoter (Angel, et al., 1988). Candidate

proteins that may phosphorylate Jun include the stress-activated protein kinases known as JNKs (Kyriakis, et al., 1994).

The identity of additional sequences that must be involved, together with the CRE, in the expression of VIP following axotomy remain unknown. Experiments using reporter constructs containing sequences from the human VIP gene have shown that sequences more than 2.5kb 5' of the transcription start site are required for a basal level of VIP expression (Waschek, et al., 1988). A full understanding of the regulation of VIP gene expression will require the identification of reporter constructs which are expressed with the same tissue distribution and under the same physiological regulation as the endogenous VIP gene. Such constructs may require extensive regions of flanking DNA. Constructs may require sequences 3' from the transcription start site including introns before reporter gene expression parallels VIP expression. Introns can contain cis-acting regulatory elements, are capable of increasing transcriptional efficiency in transgenic mice and may be required for cell type specific gene expression (Aronow, et al., 1989, Brinster, et al., 1988). Experiments using transgenic mice show that sequences within the introns of the cytoskeletal gene peripherin are required to direct expression to DRG neurons and for expression in response to neuronal injury (Belecky-Adams, et al., 1993). Similarly, intronic sequences are required for expression of a GAP-43 β galactosidase transgene in DRG neurons of transgenic mice following sciatic nerve crush (Vanselow, et al., 1994). Introns from the GAP-43 gene are also required to direct expression to neurons (Vanselow, et al., 1994). Transgenic mice containing up to 5.2kb of human VIP 5' flanking sequence, but with no introns, fused to a reporter gene fail to show expression of VIP in the CNS. As yet no transgenic mice

have been constructed containing sequences from the rat VIP promoter or introns of the VIP gene.

If constructs containing larger regions of the VIP 5' flanking region can be shown to mimic the expression of the endogenous VIP gene when microinjected into DRG neurons, mutation of the CRE in these constructs will provide confirmation of its role in VIP expression. Footprinting studies performed on such larger constructs would also help to identify sites that are required, together with the CRE, for VIP expression. Experiments could then be performed to show that these same sequences are capable of mediating analogous expression *in vivo* following nerve injury. This could be accomplished by making transgenic mice containing these same VIP sequences linked to a reporter gene. Regulatory DNA sequences necessary for this response could be identified by creating transgenic mice containing the same sequences but with mutations of elements thought to play a role in injury induced VIP expression, for example the CRE.

It is possible that VIP expression is controlled at alternative levels to transcription. Since the increase in VIP expression following axotomy is paralleled by a similar increase in VIP mRNA (Noguchi, et al., 1989), it would seem unlikely that regulation occurs at the level of translation as has been shown to occur for VIP in neurons of the human gastrointestinal tract (Bredkjaer, et al., 1991). However, regulation could occur through changes in mRNA stability or in the processing of the primary RNA transcript. It is known that the degradation rates of different eukaryotic mRNAs are determined by sequences within the RNA molecule itself and can vary by more than 2 orders of magnitude. The polyadenylation of certain mRNAs, and hence their stability, has been shown to vary following physiological stimuli (Sachs and Wahle, 1993, Carter and Murphy, 1989, Carter, et al., 1993). In addition untranslated sequences from a number of mRNA

species have been shown to contain destabilizing sequences (Hamilton, et al., 1993). At least two different mRNAs coding for VIP are transcribed in the rat anterior pituitary, differing in their degree of polyadenylation and stability (Chew, et al., 1994). It is therefore possible that differences in the stability of VIP mRNA between adult and neonatal rats could account for the spontaneous expression of VIP seen in adult but not neonatal rats. Experiments analysing the structure and stability of VIP RNA in both adult and neonatal rats are required to determine if this is so.

Previous experiments have shown that alternative splicing of introns can influence the production of stable mRNA (Buchman and Berg, 1988). Alternative splicing can be developmentally regulated (Breitbart, et al., 1985) and could account for developmental differences in VIP expression. It is unlikely that this is the case for developmental differences in the response of VIP to cAMP and calcium dependent signalling pathways as these were shown to be mediated by the CRE in Chapter 3. Northern blots would be able to show if splicing variants of VIP occur in either neonatal or adult rat DRG neurons.

NGF can act on untranslated regions to increase mRNA stability (Nishizawa, 1994). Therefore, developmental differences in splicing could account for regulation of VIP by NGF in cultured neonatal but not adult rat DRG neurons (Mulderry, 1994).

It will be important to determine whether mechanisms leading to the spontaneous expression of VIP in response to injury are also relevant to expression of other genes induced by injury. For instance it will be interesting to determine the role that a CRE-like sequence in the galanin gene, identified by Anouar et al (Anouar, et al., 1994), plays in galanin expression following axotomy. This may be performed by methods similar to those used in Chapter 2 in which antisense oligonucleotides,

corresponding to transcription factors, or dummy CREs are microinjected into DRG neurons. The effects of reduced transcription factor expression or transcription factor binding on other genes expressed in response to axotomy will provide an indication of the importance of these transcription factors in the control of the axotomy response.

APPENDIX

MATERIALS AND METHODS

APPENDIX

MATERIALS AND METHODS

A.1 Solutions

1 x 100mM buffer D:	20mM HEPES, 20% glycerol, 0.1M KCl, 0.2mM EDTA
10 x TBE:	pH8.3; 108.9 g/l Tris base, 55.7 g/l boric acid, 4.7 g/l EDTA. pH8.8; 162 g/l Tris base, 27.5 g/l boric acid, 9.5 g/l EDTA
Ampicillin:	A stock of ampicillin was made up at 50mg/ml and stored at -20°C.
B-agarose:	B-broth to which 1% agarose was added.
B-broth:	10g/l tryptone, 8g/l NaCl.
GTE:	50mM glucose, 25mM Tris HCl pH8.0, 10mM EDTA.
L-agar:	L-broth to which 1% bacto-agar was added.

L-Agar plates: L-agar was melted in a microwave oven and left to cool to 30-40°C. Ampicillin was added to the agar to a final concentration of 0.05mg/ml before pouring into plates and leaving to set at room temperature. Once set plates were dried at 37°C for 1-2 h before being used or stored at 4°C.

L-agarose:	L-broth to which 1% agarose was added.
L-broth:	10g/l tryptone, 5g/l yeast extract, 5g/l NaCl.
MOPS:	41.8g/l MOPS, 4.1g/l NaOAc, 3.4g/l EDTA
PBS:	137mM NaCl, 2.7mM KCl, 10mM phosphate buffer pH7.3.
SM Phage dilution buffer:	0.1M NaCl, 0.015M MgSO ₄ , 0.05M Tris HCl pH 7.5, 0.01% gelatine (w/v)
TE:	10mM Tris HCl (pH8), 1mM EDTA.

Tris saturated phenol: Frozen phenol was thawed by bringing to room temperature and then heating in a water bath to 68°C. The phenol was extracted with an equal volume of 0.5M Tris HCl (pH8.0) and then extracted with equal volumes of 0.1M Tris HCl (pH 8.0) until buffer pH was >7.0.

Phenol was finally stored at 4°C under a layer of 0.1M Tris HCl (pH8.0). 0.1% hydroxyquinoline was added as a preservative.

A.2 Lambda DNA preparations from plate lysates

Escherichia coli (*E.coli*) LE 392 were grown overnight in L-broth containing 0.2% maltose and 15mM MgSO₄. The following day 170µl of the appropriate dilution of bacteriophage was mixed with 600µl of LE 392 cells and incubated at 37°C for 20min. Following incubation the cells and bacteriophage mix was added to 7ml of melted soft top agarose at 37°C. This was mixed and immediately poured on top of a 145mm B-agarose plate. After setting, the plates were incubated at 37°C for 6-10h until confluent lysis had occurred. 10ml of SM buffer was then applied to each plate and plates were left gently shaking overnight at 4°C. Phage DNA was prepared from the plate lysates using the Qiagen lambda kit Midi columns according to the manufacturers instructions.

100µl of buffer L1 (20mg/ml RNase A, 6mg/ml DNase 1, 0.2mg/ml BSA, 10mM EDTA, 100mM Tris/HCl, 300mM NaCl, pH7.5) was added to 50ml plate lysate and incubated at 37°C for 30min. 10ml of ice cold buffer L2 (30% polyethylene glycol (PEG 6000), 3M NaCl) was then added and the reaction was incubated on ice for 60min. Phage particles were then pelleted by centrifugation at 10000rpm for 10min at room temperature in a Sorvall SS-34 rotor. The pellet was then resuspended in 3ml of buffer L3 (100mM Tris/HCl, 100mM NaCl, 25mM EDTA, pH7.5), followed by the addition of 3ml of buffer L4 (4% sodium dodecylsulfate). The solution was incubated at 70°C for 10min and then cooled on ice. 3ml of buffer L5 was then added (2.55M potassium acetate, pH 4.8) and the solution spun at 12,000rpm in an SS-34 rotor to obtain a particle free clear lysate. The supernatant was applied onto a Qiagen-tip 100, previously equilibrated

with 3ml buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH7.0) and allowed to enter the resin by gravity flow. The tip was washed with 10ml of buffer QC (1.0 M NaCl, 50mM Tris, 15% ethanol, pH7.0) and then the DNA was eluted with 5ml of buffer QF (1.25 M NaCl, 50mM Tris, 15% ethanol, pH8.5). The DNA was precipitated with 0.7 volumes of isopropanol and pelleted by centrifugation in an eppendorf centrifuge at 4°C. The DNA was washed with 70% ethanol, air-dried for 5min and resuspended in dH₂O

A.3 Minipreparation of plasmid DNA

Mini-prep DNA was prepared by the alkaline lysis method. Colonies were picked from agar plates into 3ml of L-broth containing 0.05mg/ml ampicillin and grown overnight at 37°C in a rotator. The following day 1.5ml of overnight culture was pelleted in an eppendorf tube by centrifugation (bench top centrifuge) 30 seconds 14,000rpm. The remaining culture was stored at 4°C for use in a large scale preparation of plasmid DNA (appendix A.4). Pellets were resuspended in 100ml of ice cold GTE buffer and mixed by vortexing. 20µl of freshly prepared alkaline-SDS solution (0.2M NaOH, 1%(v/v) SDS) was then added, gently mixed and tubes were placed on ice. 150ml of ice cold potassium acetate (3.0M KAc, 11.5% (v/v) glacial acetic acid) was added followed by a 5 min incubation on ice. Samples were then centrifuged for 5min and supernatants were added to fresh eppendorf tubes. Samples were extracted with 0.5 volumes of Tris-saturated phenol and 0.5 volumes of chloroform:isoamyl alcohol (24:1 v/v). The aqueous phases were precipitated with 0.7 volumes of propan-2-ol at room temperature for 5min. After centrifugation for 5min in an Eppendorf bench top centrifuge, the pellets were dried at 37°C and resuspended in 50µl of TE containing

20 μ g/ml of DNase-free pancreatic RNase (Sigma) (prepared by boiling a stock of 1mg/ml for 10min and storing at -20°C). Mini-prep DNA was stored at -20°C and 5-10 μ l were used for restriction analysis.

A.4 Large scale preparation of plasmid DNA

2ml of overnight cultures, containing individual colonies picked from agar plates, were diluted into 300ml-500ml of L-broth, containing 0.05mg/ml ampicillin and grown overnight in an orbital shaker at 37°C. The following day cultures were pelleted by centrifugation at 6000 rpm for 5min at 4°C in a Sorvall RS-5B centrifuge using a GSA rotor. After decanting the supernatant, bacterial pellets were resuspended in 12ml of ice-cold GTE buffer. 24ml of freshly prepared alkaline-SDS (0.2M NaOH, 1% (v/v) SDS) was then added and suspensions were left on ice for 5min. 16ml of ice cold potassium acetate (3.0M KAc, 11.5% (v/v) glacial acetic acid) was added and suspensions were incubated for a further 10min on ice. This was followed by centrifugation at 4000rpm for 15min in a Sorvall GSA rotor at 4°C to pellet out cell debris and cellular DNA. Supernatants containing the plasmid DNA were strained through butter muslin into 250 ml centrifuge pots and precipitated with 0.6 volumes of isopropanol at room temperature for 30min. Plamid DNA was recovered by centrifugation at 5000rpm for 15min at room temperature in a Sorvall GSA rotor. Supernatants were drained away and the pellets were washed with 70% ethanol, dried and resuspended in 1ml of TE. The DNA samples were then brought to a total volume of 4ml with TE after which 400 μ l of ethidium bromide (10mg/ml) was added. Exactly 4.4g of CsCl was then dissolved into the solutions followed by a 5-10 min incubation on ice. The solutions were centrifuged in 15ml polypropylene tubes (Corning) at 5000rpm for 5min at room temperature. Supernatants were transferred to 6ml polyallamer

ultracentrifuge tubes (Du Pont) and topped up with a stock solution containing 1.1g of CsCl in 1.0ml of H₂O. Ultracentrifuge tubes were then sealed with an ultra crimp tool and centrifuged in a Sorval TV-1665 ultracentrifuge rotor at 45000rpm for 16h at 20°C. Supercoiled plasmid DNA bands were visualised by daylight and recovered with a large bore needle and syringe. Ethidium bromide was removed by extracting several times with equal volumes of butan-1-ol. The plasmid preparations were then dialysed for 24 h against three 2 litre changes of H₂O at 4°C. The concentration of recovered plasmid was determined by spectrophotometry (OD₂₆₀ 1.0 = 50mg DNA/ml)

A.5 Restriction enzyme digests of DNA

DNA was digested using 1 unit of restriction enzyme per 1µg of DNA. For DNA that contained multiple restriction sites corresponding to the enzyme being used, the amount of enzyme was adjusted accordingly. 1 x reaction buffer, as specified by the manufacturer and dH₂O were added to a final volume large enough to give a final enzyme dilution of 1:10. This reduced the glycerol content, in which the enzyme is stored, to a level where it would not inhibit digestion. Digests were electrophoresed through an agarose gel (appendix A.10)

A.6 Recovery of DNA from agarose gels

DNA fragments greater than 500bp were electrophoresed through a 1% TBE agarose gel and recovered using a Geanclean Kit (BIO 101, inc), according to manufacturers instructions.

Briefly, DNA was visualised using a UV transilluminator and gel slices containing the required DNA band were excised using a clean scalpel blade. Slices were weighed and 0.5ml of TBE modifier (supplied with kit)

together with 4.5ml of NaI were added per gram of agarose. The mixture was incubated at 55°C until the gel was completely dissolved. 1µl of glass milk suspension (a silica matrix supplied with the kit) was added per µg of DNA and the mixture incubated on ice for 15min to allow binding of DNA to the glass. The glass beads were pelleted by centrifugation and washed 3 x with 10 to 50 volumes of New wash. The DNA was eluted from the glass beads by resuspending pelleted beads in an equal volume of dH₂O at 55°C for 2-3min.

A.7 Generating blunt ends following restriction enzyme digest

DNA with 3' recessed ends were filled in using the klenow proteolytic fragment of DNA polymerase 1. DNA from a restriction digest was precipitated by adding 1/10 the volume of 3M NaAc and 3 volumes of EtOH, incubating in a dry ice/ethanol bath for 15min and centrifuging at full speed in an eppendorf bench top centrifuge. The DNA was washed in 3 volumes of 70% EtOH before resuspending at 50µg/ml in 10mM Tris-HCL (pH7.5), 5mM MgCL₂, 7.5mM dithiothreitol and 33µM dNTPs. 1unit of Klenow was added per µg DNA (1 unit is the amount of enzyme required to convert 10 nmoles of dNTPs into an acid-insoluble form in 30 minutes at 37°C). The reaction was left at 25°C for 30min and terminated by heating to 75°C for 10min.

A.8 DNA ligation

Ligation reactions were performed in a total volume of 10µl, containing 1µl of 10 x ligation buffer (0.5M Tris HCl pH7.6, 100mM MgCl₂, 10mM DTT), 1mM ATP, 1unit of T4 DNA ligase (BCL) and a 1:1-1:5 molar ratio of vector DNA:insert DNA. Typically approximately 50-100ng of vector DNA was used. Ligation reactions were incubated between 10°C and 25°C for 3h -

overnight. Ligations were then transformed into *E.coli* as described in appendix A.9

A.9 Preparation and transformation of competent *E. coli*

A single colony of *E.coli* strain DH5 α was grown overnight in 3ml of L-broth, diluted into 40ml of L-broth and grown in an orbital shaker at 37°C to an OD₅₄₀ of 0.3-0.5. Cells were harvested by centrifugation at 6000rpm in a Sorvall SS34 rotor for 5min at 4°C. Cell pellets were placed on ice, resuspended in 0.25vol of ice cold 0.1M MgCl₂ and pelleted as before. The pellet was then resuspended in 0.05vol of ice cold 0.1M CaCl₂ and left on ice for 45min. Cells were pelleted and resuspended in the same volume of a buffer containing 0.1M MOPS pH6.5, 50mM CaCl₂ and 20% glycerol and left on ice for a further 20min to acquire full competence. Competent cells were aliquoted into 1ml eppendorf tubes, snap frozen in a dry ice bath and stored at -70°C.

For transformation of competent cells, 1-5 μ l of ligation mix (appendix A.8) or 10ng of plasmid DNA was added to 100-200 μ l of competent cells. The mixture was left on ice for 30min before being heat shocked at 45°C for 2min and returned to ice for 2min. The cells were added to 1ml of L-broth and incubated at 37°C for 1h. They were then pelleted, resuspended in 100 μ l of L-broth and subsequently spread on L-agar plates containing 0.1mg/ml of Ampicillin. The plates were incubated overnight at 37°C. Colonies that grew on the selective medium were picked for mini-prep analysis as described in appendix A.3.

A.10 Horizontal agarose gel electrophoresis

The percentage of agarose used depended on the size range of the linear DNA to be separated. 1% (w/v) agarose (Agarose MP, Boehringer)

was used to separate DNA fragments of $\approx 7\text{kb}$ to 0.56kb , 1.5% to 2% (w/v) agarose was used to separate DNA fragments of $\approx 1\text{kb}$ to 0.1kb and 5% (w/v) agarose (NuSeive GTG) was used to separate DNA fragments of oligonucleotides of $<0.1\text{kb}$.

100ml horizontal gel slabs (140mm x 110mm x 30mm) or 25ml mini agarose gels (83mm x 57mm x 30mm) containing the correct percentage of agarose were placed in gel electrophoresis apparatus (Gibco BRL, Model HS or Gibco BRL, Model Horizon 58 respectively) containing sufficient 1 x TBE buffer to cover the gel. Samples were loaded in 0.2 volumes of loading buffer and gels were usually electrophoresed at 100 V for as long as required to see sufficient band separation. After electrophoresis gels were stained in 1 x TBE buffer containing ethidium bromide ($0.5\mu\text{g/ml}$) for 5min and DNA fragments were visualised using a long wave UV transilluminator. DNA was photographed using Asa Polaroid film type 667 in a Polaroid Cu-5 camera.

A.11 Vertical gel electrophoresis

a) Non-denaturing (native)

6% native polyacrylamide gels were prepared by mixing 10ml of 30% 33:1 acrylamide:bisacrylamide (BDH, premixed) with 2.5ml of 10 x TBE in a final volume of 50ml. Gel polymerisation was catalysed by the addition of $50\mu\text{l}$ of TEMED (N, N, N', N',-tetramethylethylenediamine) and 0.5ml of 10% ammonium persulfate. The mixture was then immediately poured into 150mm x 170mm x 1.5mm glass sandwiches for use in electrophoretic mobility shift assays (appendix A.15). 20 tooth (1.5mm) combs were placed in the top of the gels and the gels were then left to polymerise. Polymerised gels were attached to vertical gel electrophoresis apparatus (Gibco BRL, Model V16-2) containing 0.5 x TBE buffer and pre-electrophoresed for up

to 4 h at 100V. Samples were loaded while still running and electrophoresed for a further 2-3h at 150V. Following electrophoresis, gels were transferred to Whatman No. 1 paper and dried using a heated vacuum gel drier (Biorad Model S83). Radiolabelled samples were visualised by exposing the dried gels to autoradiographic film for 1-24 h (appendix A.12).

b) Denaturing

6% denaturing gels were prepared by dissolving 42g of urea (Aristar, BDH), 20ml of 30% (33:1) acrylamide:bisacrylamide (BDH, premixed) and 10ml of 10 x TBE pH 8.8, in a final volume of 100ml. The solution was filtered through Whatman membrane filters (0.45 μ) under vacuum and degassed before adding 0.55ml of 10% APS and 55 μ l of TEMED. Solutions were immediately poured into 300mm x 400mm glass sandwiches previously sealed with 5% agarose. 20-tooth combs (0.4mm) were inserted and the gels were left to polymerise. After polymerisation, gels were attached to vertical gel electrophoresis apparatus (Gibco BRL, Model S2) containing 1 x TBE buffer. Gels were pre-run at approximately 2000V (less than 50W) for 30min prior to loading. Following loading, gels were run at 2000V for 3-4h before transferring to Whatman No 1. Gels were covered with Saran wrap and dried using a heated vacuum gel drier (Biorad Model 583). Saran wrap was removed from the dried gels and radiolabelled samples were visualised by autoradiography (appendix A.12).

A.12 Autoradiography

Detection of radiolabelled material was carried out using Fuji RX X-ray film. For ³²P, X-ray film was placed against dried gels inside cassettes. An

intensifying screen was placed on the opposite side of the film to the gel and the film was exposed at -70°C . For ^{35}S containing gels no intensifying screen was used and gels were exposed at room temperature. Following the required amount of exposure time films were developed for 2min in Kodak LX-24 developer (1:4 dilution), fixed for 1min in Kodak FX-40 fixer (1:4 dilution), rinsed in water and dried.

A.13 Multimerisation of DNA oligonucleotides

Two separate phosphorylation reactions were performed, each containing one out of a pair of complimentary oligonucleotides, as follows. $2\mu\text{g}$ of the oligonucleotide was mixed with $5\mu\text{l}$ of 10 x kinase/ligase buffer (500mM Tris Cl pH7.6, 100mM MgCl_2), $2.5\mu\text{l}$ 100mM dithiothreitol, 10mM ATP and $2\mu\text{l}$ of bacteriophage T4 polynucleotide kinase (8 units/ μl). The volume was made up to $50\mu\text{l}$ with dH_2O and the reactions were left at 37°C for 1h to allow phosphorylation of the 5' termini of the two oligonucleotides. At the end of 1h the two reactions were mixed and the oligonucleotides annealed together by incubating for 2min at 85°C , 15min at 65°C , 15min at room temperature and 15min on ice. The resulting 5' phosphorylated double stranded oligonucleotide was then ligated into a vector as described in appendix A.8 except that a series of ligation reactions were performed containing different molar ratios of vector:insert. For example, 1:0.01, 1:0.1, 1:1, 1:10, 1:100.

A.14 *In vitro*-transcription/translation

Genes cloned downstream from either the SP6 or T7 promoters were transcribed and translated using the Promega TNT coupled Reticulocyte Lysate System according to the manufactures instructions. Typically $2\mu\text{g}$ of a single DNA expression construct or $1\mu\text{g}$ of each of two constructs to be

co-translated were mixed with 25 μ l of TNT rabbit Reticulocyte Lysate, 2 μ l TNT Reaction buffer, 1 μ l TNT polymerase (SP6 or T7 or 1 μ l of each if necessary), 1 μ l of amino acid mixture minus leucine (1mM), 1 μ l of amino acid mixture minus methionine (1mM), 1 μ l of RNasin ribonuclease inhibitor (40u/ μ l) and nuclease free water to make the total volume up to 50 μ l. The reaction was incubated at 30°C for 120min and immediately used for an EMSA assay (appendix A.15) or frozen at -70°C.

A.15 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays, as outlined below were based on the method of Singh, et al., 1986 with a few modifications.

A typical electrophoretic mobility shift assay contained approximately 1ng of double stranded end filled oligonucleotide (appendix A.16), 0.5-2.5 μ g (depending on batch lot) of non specific competitor poly d(I-C) (Boehringer), 10-30 μ g of protein extract and sufficient 0mM or 100mM buffer D to bring the final reaction concentration to 100mM salt, in a volume 10-15 μ l.

In vitro translated protein extracts (typically 3 μ l out of 50 μ l, see appendix A.14) were mixed with buffer D and poly d(I-C) and left on ice for up to 20min. Where appropriate 1 μ l of anti-c-Jun antibody (1 μ g/ μ l, Santa Cruz Biotechnology, Inc) or 1 μ l of anti c-Fos and fos-related antigens (generous gift from Dr. M. Iadarola, NIH) were added before the 20min incubation. Following incubation 1 μ l labelled oligonucleotide probe (1ng see appendix A.16) and 2 μ l of dye mix (2% (w/v) bromophenol blue, 2% (w/v) xylene cyanol) were added and the reaction mixtures were incubated at room temperature for 20min. Reactions were then loaded onto 6% native polyacrylamide gels (appendix A.11) and protein/DNA complexes were resolved from unbound DNA by electrophoresing at 150V for 1.5-2.5h.

Antibody bound to protein/DNA complexes resulted in greater retardation of the complexes within the gel, resulting in "supershifts". Dried gels were exposed to autoradiographic film for 16-72h (appendix A.12).

A.16 Labelling of double stranded DNA oligonucleotide probes

Double stranded oligonucleotides containing 5' overhangs were radioactively labelled with ^{32}P dATP by incubation with $1\mu\text{l}$ of 0.1M DTT, $2\mu\text{l}$ of labelling mix (7.5mM dCTP, 7.5mM dGTP, 7.5mM dTTP, diluted 1:5 in dH_2O), 5mCi of (^{32}P) dATP αP (NEN, 3000Ci/mmol) and $2\mu\text{l}$ of Sequenase Version 2.0 (diluted 1:8 in enzyme dilution buffer: 10mM Tris HCl pH7.5, 5mM DTT, 0.5mg/ml BSA) at room temperature for 20-30min. $50\mu\text{l}$ of dH_2O was then added and the reactions terminated by extraction with an equal volume of Tris saturated phenol (appendix A.1). Labelled oligonucleotide was separated from unincorporated label by centrifugation through Nu-Clean sephadex D25 disposable spun columns (IBI) and the total volume was brought up to $100\mu\text{l}$ with dH_2O .

A.17 Dideoxy sequencing

Plasmid DNA was sequenced using Sequenase version 2.0 according to manufacturers instructions (USB).

DNA samples (5-10 μg) were denatured in a final volume of $75\mu\text{l}$ containing $15\mu\text{l}$ of 2M NaOH, at 37°C for 10min. DNA samples were then precipitated by adding $15\mu\text{l}$ of 3M NaAc and $300\mu\text{l}$ of EtOH, incubating in a dry ice/ethanol bath for 15min and centrifuging at full speed in an eppendorf bench top centrifuge. Pellets were resuspended in $7\mu\text{l}$ of dH_2O . The DNA samples were then annealed to the appropriate primer in a final volume of $10\mu\text{l}$, containing $2\mu\text{l}$ of 5 x sequenase buffer (5 x; 200mM Tris HCl pH7.5, 100mM MgCl_2 , 250mM NaCl), $7\mu\text{l}$ of denatured DNA and 10ng

of primer. Reactions were heated at 80°C for 2 min and slowly cooled to room temperature. Annealed DNA samples (10µl) were then radioactively labelled with ³⁵S dATP by incubation with 1µl of 0.1M DTT, 2µl of labelling mix (7.5mM dCTP, 7.5mM dGTP, 7.5mM dTTP, diluted 1:5 in dH₂O), 5mCi of (³⁵S) dATPαS (Amersham, 1000Ci/mmol) and 2µl of Sequenase Version 2.0 (diluted 1:8 in enzyme dilution buffer: 10mM Tris HCl pH7.5, 5mM DTT, 0.5mg/ml BSA) at room temperature for 2-5min. Reactions were terminated by the incubation of 3.5µl of each labelled DNA sample with 2.5µl aliquots of one of the four ddNTPs at 37°C for 10-20min. 4µl of stop solution was then added and samples were boiled for 2min before loading onto 6% denaturing polyacrylamide/urea gels (appendix A.11). Gels were electrophoresed at 1500V for 3-4 h and fixed for 30min in 5% methanol, 15% acetic acid before drying and exposing to autoradiographic film (appendix A.12), typically for 16 h.

A.18 PC12 cells maintenance and electroporation

PC12 cells were maintained in Dulbecco's modification of eagle medium (ICN biomedical inc.) containing 10% horse serum, 5% foetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). Cells were incubated at 37°C in 5% CO₂ 95% air and differentiated following transfection with 50ng/ml NGF. Consistent transfection of PC12 cells, as measured by comparing CAT expression between parallel transfections, was obtained by electroporation of 3x10⁶ cells at 200V in the presence of 5µg of reporter construct DNA using a Biorad gene pulser™. 1.5x10⁶ undifferentiated cells were then plated out per petri-dish and left overnight in the presence of 50ng/ml NGF to settle down.

A.19 Preparation of Dorsal Root Ganglion Neurons

DRG with attached roots were dissected aseptically from the cervical, thoracic and lumbar regions (40-48/animal) of adult (200-250g) or neonatal (first-second postnatal day) Wistar rats and collected in Ham's F-14 growth medium (Imperial Laboratories) containing 4% Ultrosor G serum substitute (Gibco). Ganglia were carefully separated from roots and capsular connective tissue before being enzymatically treated with 0.125% collagenase (Worthington) for 3h (adults) or 30min (neonates). Cells were washed in serum free F-14 medium and treated with 0.25% trypsin (Worthington) in serum free F-14 for 30min. Cells were dispersed by mechanical trituration through a glass Pasteur pipette and suspended in F14 containing Ultrosor G. Neuronal enrichment was achieved by plating neurons from 1 adult rat on a 60mm diameter petri dish, previously coated with polyornithine (500 μ g/ml). After leaving overnight to allow non-neuronal cells to firmly attach to the dish, most of the dead cells and axonal and myelin debris were discarded prior to selectively removing weakly adherent neuronal cells with a stream of medium from a Pasteur pipette. Neurons from neonatal rats or enriched cells from adult rats were plated at a density of 4,000 to 10,000 neurons per well on shallow 10mm diameter polystyrene wells (Cel-Cult, Sterilin) previously coated with polyornithine (500 μ g/ml) and laminin (5 μ g/ml) and maintained in Ham's F14 medium (Imperial Laboratories) containing 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco), 4% Ultrosor G serum substitute (Gibco) and 50 ng/ml mouse salivary gland NGF when required (generous gift from Dr. J. Winter, Sandoz Institute, London, UK.). Cells were incubated at 36.5°C in 97% air: 3% CO₂.

A.20 Microinjection

Plasmid DNA was diluted to 200 $\mu\text{g/ml}$ in 10mM Tris-HCl buffer, pH7.5 and loaded into Eppendorf femtotip injection pipettes, tip diameter $0.5\pm 0.2\mu\text{m}$. Microinjection was performed using an Eppendorf microinjector 5242 and micromanipulator 5170 system. Plasmid constructs were microinjected at 200 $\mu\text{g/ml}$ 1-4 days after plating with a standard injection time of 0.5s.

DRG neurons were easily distinguished from the non-neuronal cells present in the cultures by morphological criteria alone. These include a relatively large rounded cell body with distinct nucleus and nucleolus, together with a phase bright plasma membrane.

Although attempts have been made to position the pipette for microinjection into the neuronal nucleus, cell movement in response to the injection pipette together with the relatively large volume of perinuclear cytoplasm in DRG neurons meant that most injections were actually cytoplasmic.

It has previously been estimated (Mulderry, et al., 1993) using a standard injection time of 0.5s and injection pressure of 100-400hPa that the average volume delivered per injection is in the order of 10^{-13} - 10^{-12} litres. At a DNA concentration of 200 $\mu\text{g/ml}$ this would correspond to approximately 10^3 - 10^4 plasmid copies. However due to differences in tip diameter, injection pressure and characteristics of individual cells it is likely that the volume of individual injections and hence the number of introduced plasmid copies per cell is highly variable. The efficacy of this system of neuronal transfection has been assayed previously (Mulderry, et al., 1993), by injecting Cascade Blue-labelled dextran. After 2 days, the number of neurons of healthy appearance labelled by Cascade Blue fluorescence, expressed as a proportion of the number of injections, was fairly consistent

from one well to another. This efficiency was not significantly different between cultures grown in the presence or absence of NGF.

A.21 Depolarisation and forskolin treatment of DRG neurons

For depolarisation experiments F14 medium was diluted to 75% of normal strength by addition of 11.25g/l KCl. This produced isotonic medium containing 40mM K⁺. For control cells medium was diluted to 75% of normal strength by addition of 7.6g/l NaCl. Calcium content was not adjusted and the diluted medium therefore contained 1.5mM Ca²⁺. Forskolin (Sigma) was dissolved in ethanol to a concentration of 10mM. This was then diluted into the above medium at 1:1000 to give a final concentration of 10 μ M.

A.22 Chloramphenicol acetyl transferase assay

Cells transfected with reporter constructs capable of expressing chloramphenicol acetyl transferase (CAT) were lysed and CAT expression was quantified using a CAT ELISA kit (Boehringer Mannheim), according to the manufacturers instructions.

Briefly, cells were washed twice with 100 μ l of PBS before lysis in 200 μ l buffer (kit lysis buffer) for 30 min at room temperature. The cell extract was centrifuged for 15 min at full speed (14,000rpm) in an eppendorf bench top centrifuge to remove cellular debris. The supernatant was transferred to a microtitre plate module (MTP) which had been coated previously with anti-CAT antibodies (polyclonal sheep antibody). After incubation at 37°C for 2h the solution was discarded and the modules were washed 4 x with 250 μ l of washing buffer. 200 μ l of a digoxigenin labelled anti-CAT antibody (2 μ g/ml) was added to each well and incubated at 37°C for 1h. The solution was discarded and the modules were washed 4 x with 250 μ l of

washing buffer. 200 μ l of a peroxidase-conjugated antibody to digoxigenin (150mU/ml) was added to each well and incubated at 37°C for 1h. The solution was discarded and the modules were washed 4 x with 250 μ l of washing buffer. After the last wash 200 μ l of peroxidase substrate was added to each well and the samples were incubated at room temperature until a green colour was obtained resulting from cleavage of the peroxidase substrate. The absorbance of the samples was measured at 410nm using a Dynatech MR600 microplate reader with the resulting absorbance being directly proportional to actual CAT concentration. Calibration curves were obtained for each reaction by using CAT standards of known concentration.

A.23 β Galactosidase assay

Cells transfected with reporter constructs capable of expressing β -galactosidase were lysed and expression was quantified using a 4-methylumbelliferyl β -D-galactosidase (MUG) assay for β -galactosidase. Cells were rinsed in PBS before transferring to 400 μ l of MUG reaction solution (60mM Na₂HPO₄, 40mM NaH₂PO₄, 10mM KCl, 1mM MgSO₄, 0.1% Triton X-100, 0.2mg/ml MUG, pH7.0). Cells were incubated with shaking overnight at 4°C followed by addition of 200 μ l of stop buffer (300mM glycine, 15mM EDTA, pH11.2). 100 μ l aliquots were diluted into 4ml of distilled water in a quartz cuvette and methylumbelliferone fluorescence was measured using a spectrophotofluorometer (with an excitation wavelength of 360nm and an emission wavelength of 450nm. Assays were calibrated against dilutions of purified *E. coli* β -galactosidase (Sigma).

A.24 Immunocytochemistry

For immunocytochemistry cells were cultured and maintained on glass coverslips to allow easy visualisation of staining. Cultures were rinsed briefly in PBS and fixed for 30min at room temperature with 4% paraformaldehyde in PBS. They were then rinsed in PBS containing 0.1% Triton X-100 and incubated overnight at 4°C with antisera diluted in PBS containing 0.1% Triton X-100, 10% horse serum and 0.004% sodium azide. VIP immunoreactivity was stained using a rabbit antiserum purchased from Immuno-Nuclear Corporation (Stillwater, Minnesota) at 1:3000 and substance P and CGRP immunoreactivities were stained using antisera from Peter Mulderry, rabbit anti-substance P 7/0310, diluted 1:5000 and rabbit anti-rat α -CGRP 18/2305, diluted 1:15,000. Cultures were rinsed briefly in PBS containing 0.1% Triton X-100 and incubated in 50 μ l of biotinylated anti-rabbit second antibody for 45min. Following a further rinse in PBS containing 0.1% Triton X-100 the cultures were stained with streptavidin-linked fluorescein isothiocyanate. In all cases specificity of immunostaining was checked by preincubation of antisera with the relevant synthetic peptide antigens (porcine VIP, substance P and rat α -CGRP at a concentration of 150nM) preventing any labelling of cultured cells.

A.25 Confocal microscopy

Fluorescent and phase contrast images of immunostained cells were taken using an MRC-600 confocal laser scanning optical microscope with the detection aperture set at maximum to prevent any optical sectioning. Epi-illumination was used (sample illuminated and detected from the same side) to provide a positive correlation between sample concentration and fluorescent intensity (Rost, 1991). Samples stained with fluorescein conjugated antibodies were excited and visualised through a standard

FITC filter set. The excitation and emission spectra for fluorescein do not overlap preventing emitted light from being reabsorbed (Pearse, 1980). This prevents a negative correlation between antibody concentration and fluorescence intensity. The images obtained are composed of pixels each of which can take a value of 0-255 depending on fluorescence intensity. NIH Image software was used to calculate the average pixel value and the number of pixels per cell. These two values were multiplied together to provide a total pixel intensity, used as an index of VIP content of the cell. Average pixel intensity per cell was not used as it is not known whether a small strongly stained cell contains the same amount of VIP as a large weakly stained cell.

A.26 Statistics

For all parametric statistics, results were analysed first using a 2-way analysis of variance for all combinations of injected construct and treatment of cells after injection. Statistical significance of difference between treatments or constructs was determined by either a post-hoc Student Newman-Keuls one way analysis of variance or by a Students T test using an F distribution. For the non-parametric results obtained in chapter 2, a Mann-Whitney comparison by rank test was performed. For all tests the critical value for significance was taken as $P < 0.05$.

- Adams, S.R., Harootunian, A.T., Buechler, T.J., Taylor, S.S. & Tsien, R.Y. (1991). Fluorescent ratio imaging of cyclic AMP in single cells. *Nature* **349**, 694-697.
- Agoston, D.V., Colburn, S., Krajniak, K.G. & Waschek, J.A. (1992). Distinct regulation of vasoactive intestinal peptide (VIP) expression at mRNA and peptide levels in human neuroblastoma cells. *Neuroscience Letters* **139**, 213-216.
- Aldskogius, H., Arvidsson, J. & Grant, G. (1985). The reaction of primary sensory neurons to peripheral nerve injury with particular emphasis on transganglionic changes. *Brain Research Reviews* **10**, 27-46.
- Alessi, D.R., Smythe, C. & Keyse, S.M. (1993). The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in *Xenopus* oocyte extracts. *Oncogene* **8**, 2015-2020.
- Anand, P., Gibson, S.J., Scaravilli, F., Blank, M.A., McGregor, G.P., Appenzeller, O., Dhital, K., Polak, J.M. & Bloom, S.R. (1990). Studies of vasoactive intestinal polypeptide expression in injured peripheral neurons using capsaicin, sympathectomy and mf mutant rats. *Neuroscience Letters* **118**, 61-66.
- Andersen, B., Milstead, A., Kennedy, G. & Nilson, J.H. (1988). Cyclic AMP and phorbol esters interact synergistically to regulate expression of the chorionic gonadotropin genes. *Journal of biological chemistry* **262**, 15578-15583.
- Angel, P., Hattori, K., Smeal, T. & Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**, 875-885.
- Anouar, Y., MacArthur, L., Cohen, J., Iacangelo, A.L. & Eiden, L.E. (1994). Identification of a TPA-responsive element mediating preferential transactivation of the galanin gene promoter in chromaffin cells. *The Journal of Biological Chemistry* **269**, 6823-6831.

- Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J. & Montminy, M. (1994). Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**, 226-229.
- Aronow, B., Lattier, D., Silbiger, R., Dusing, M., Hutton, J., Jones, G., Stock, J., McNeish, J., Potter, S., Witte, D. & Wiginton, D. (1989). Evidence for a complex regulatory array in the first intron of the human adenosine deaminase gene. *Genes and Development* **3**, 1384-1400.
- Artalejo, C.R., Ariano, M.A., Perlman, R.L. & Fox, A.P. (1990). Activation of facilitation by calcium channels in chromaffin cells by D₁ dopamine receptors through a cAMP/protein kinase A-dependent mechanism. *Nature* **348**, 239-242.
- Arvidsson, J., Ygge, J. & Grant, G. (1986). Cell loss in lumbar dorsal root ganglia and transganglionic degeneration after sciatic nerve resection in the rat. *Brain Research* **373**, 15-21.
- Aszodi, A., Muller, U., Friedrich, P. & Spatz, H.C. (1991). Signal convergence on protein kinase A as a molecular model of learning. *Proceedings of the National Academy of Sciences, USA* **88**, 5832-5836.
- Barbin, G., Manthorpe, M. & Varon, S. (1984). Purification of chick eye neurotrophic factor. *Journal of Neurochemistry* **43**, 1468-1478.
- Baumann, H. & Wong, G.G. (1989). Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *Journal of Immunology* **143**, 1163-1167.
- Begley, C.G., Lipkowitz, S., Gobel, V., Mahon, K.A., Bertness, V., Green, A.R., Gough, N.M. & Kirsch, I.R. (1992). Molecular characterisation of NSCL, a gene encoding a helix-loop-helix protein expressed in the developing nervous system. *Proceedings of the national academy of science (USA)* **89**, 38-42.

- Belecky-Adams, T., Wight, D.C., Kopchick, J.J. & Parysek, L.M. (1993). Intragenic sequences are required for cell type-specific and injury-induced expression of the rat peripherin gene. *Journal of Neuroscience* **13**, 5056-5065.
- Benbrook, D.M. & Jones, N.C. (1990). Heterodimer formation between CREB and JUN proteins. *Oncogene* **5**, 295-302.
- Benowitz, L.I. & Routtenberg, A. (1987). A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism and synaptic plasticity. *Trends in Neuroscience* **10**, 527-532.
- Bielinska, A., Shivdasani, R.A., Zhang, L. & Nabel, G.J. (1990). Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science* **250**, 997-1000.
- Bisby, M.A. (1988). Dependence of GAP-43 transport on axonal regeneration in rat dorsal root ganglion cells. *Brain Research* **458**, 157-161.
- Bloom, G.S., Richards, B.W., Leopold, P.L., Ritchey, D.M. & Brady, S.T. (1993). GTP γ S Inhibits organelle transport along axonal microtubules. *The Journal of Cell Biology* **120**, 467-476.
- Bloom, S.R., Christofides, N.D., Delamarter, J., Kavoshima, E. & Polak, J.M. (1983). Diarrhoea in VIPoma patients associated with cosecretion of a second active peptide (peptide histidine isoleucine) explained by a single coding gene. *Lancet* **2**, 1163-1165.
- Bourne, H.R., Sanders, D.A. & McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell types. *Nature* **348**, 125-132.
- Brasier, A.R., Ron, D., Tate, J.E. & Habener, J.F. (1990). A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1 a

induced, NFB mediated transactivation of the angiotensinogen gene acute-phase response element. *EMBO Journal* **9**, 3933-3944.

Bredkjaer, H.E., Ronnov-Jessen, D., Fahrenkrug, L., Ekblad, E. & Fahrenkrug, J. (1991). Expression of prepro VIP-derived peptides in the human gastrointestinal tract: A biochemical and immunocytochemical study. *Regulatory Peptides* **33**, 145-164.

Breitbart, R.E., Nguyen, H.T., Medford, R.M., Destree, A.T., Mahdavi, V. & Nadal-Girard, B. (1985). Intricate combinatorial patterns of exon splicing generate multiple regulated troponin T isoforms from a single gene. *Cell* **41**, 67-82.

Brenneman, D.E. & Eiden, L.E. (1986). Vasoactive intestinal polypeptide and electrical activity influence neuronal survival. *Proceedings of the National Academy of Sciences, USA* **83**, 1159-1162.

Brenneman, D.E., Neale, E.A., Foster, G.A., d'Autremont, S.W. & Westbrook, G.L. (1987). Non-neuronal cells mediate neurotrophic action of vasoactive intestinal peptide. *Journal of Cell Biology* **104**, 1603-1610.

Brenneman, D.E., Nicol, T., Warren, D. & Bowers, L.M. (1990). Vasoactive intestinal peptide: a neurotrophic releasing agent and an astroglial mitogen. *Journal of Neuroscience Research* **25**, 386-394.

Brenneman, D.E., Schultzberg, M., Bartfai, T. & Gozes, I. (1992). Cytokine regulation of neuronal survival. *Journal of Neurochemistry* **58**, 454-460.

Brindle, P.K. & Montminy, M.R. (1992). The CREB family of transcription activators. *Current Opinion in Genetics and Development* **2**, 199-204.

Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E. & Palmiter, R.D. (1988). Introns increase transcriptional efficiency in transgenic mice. *Proceedings of the National Academy of Sciences, USA*. **85**, 836-840.

- Brown, R.S., Sander, C. & Argos, P. (1985). The primary structure of transcription factor TFIIIA has 12 consecutive repeats. *FEBS Letters* **186**, 271-247.
- Buchman, A.R. & Berg, P. (1988). Comparison of intron-dependent and intron-independent gene expression. *Molecular and Cellular Biology* **8**, 4395-4404.
- Cameron, A.A., Leah, J.D. & Snow, P.J. (1988). The coexistence of neuropeptides in feline sensory neurons. *Neuroscience* **27**, 969-979.
- Carter, D.A. & Murphy, D. (1989). Independent regulation of neuropeptide mRNA level and poly (A) tail length. *Journal of Biological Chemistry* **264**, 6601-6603.
- Carter, D.A., Li-Jin, C. & Murphy, D. (1993). In vitro regulation of rat prolactin messenger ribonucleic acid poly (A) tail length: Modulation by bromocriptine. *Journal of Neuroendocrinology* **5**, 201-204.
- Cheah, T.B. & Geffen, L.B. (1973). Effects of axonal injury on norepinephrine, tyrosine hydroxylase and monoamine oxidase levels in sympathetic ganglia. *Journal of Neurobiology* **4**, 443-452.
- Cheema, S.S., Richards, L., Murphy, M. & Bartlett, P.F. (1994). Leukemia inhibitory factor prevents the death of axotomised sensory neurons in the dorsal root ganglia of the neonatal rat. *Journal of Neuroscience Research* **37**, 213-218.
- Chetkovich, D.M., Gray, R., Johnson, D. & Sweatt, J.D. (1991). N-methyl-D-aspartate receptor activation increases cAMP levels and voltage gated calcium channel activity in area CA1 of hippocampus. *Proceedings of the National Academy of Sciences, USA* **88**, 6467-6471.
- Chew, L.J., Murphy, D. & Carter, D.A. (1994). Alternatively Polyadenylated Vasoactive Intestinal Peptide mRNA's are differentially regulated at the level of stability. *Molecular endocrinology* **8**, 603-613.

- Chong, M.S., Fitzgerald, M., Winter, J., Hu-Tsai, M., Emson, P.C., Wiese, U. & Woolf, C.J. (1992). GAP-43 mRNA in rat spinal cord and dorsal root ganglia neurons: developmental changes and re-expression following peripheral nerve injury. *European Journal of Neuroscience* **4**, 883-895.
- Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. & Goodman, R.H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.
- Cohen, D. & Curran, T. (1988). Fra-1; A serum inducible, cellular immediate early gene that encodes a fos-related antigen. *Molecular and Cellular Biology* **8**, 2063-2069.
- Collart, M.A., Tourkine, N., Belin, D., Vassalli, P., Jeanteur, P. & Blanchard, J.M. (1991). c-fos Gene Transcription in Murine Macrophages is Modulated by a Calcium Dependent Block to Elongation in Intron 1. *Molecular and Cellular Biology* **11**, 2826-2831.
- Collins, F., Schmidt, M.F., Guthrie, P.B. & Kater, S.B. (1991). Sustained increase in intracellular calcium promotes neuronal survival. *Journal of Neuroscience* **11**, 2528-2587.
- Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. & Goodman, H.M. (1986). A cyclic AMP- and phorbol ester-inducible DNA element. *Nature* **323**, 353-356.
- Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. & Goodman, H.M. (1988). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. *EMBO Journal* **7**, 3793-3805.
- Conaway, R.C. & Conaway, J.W. (1993). General initiation factors for RNA polymerase II. *Annual Review of Biochemistry* **62**, 161-190.

- Costa, R.H., Grayson, D.R., Xanthopoulos, K.G. & Darnell, J.E. (1988). A liver-specific DNA-binding protein recognises multiple nucleotide sites in regulatory regions of transthyretin, α_1 -antitrypsin, albumin, and simian virus 40 genes. *Proceedings of the National Academy of Sciences* **85**, 3840-3844.
- Curran, T. & Franzen, B.R.J. (1988). Fos and Jun: the AP1 connection. *Cell* **55**, 395-397.
- Curtis, R., Adryan, K.M., Zhu, Y., Harkness, P.J., Lindsay, R.M. & DiStefano, P.S. (1993). Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* **365**, 253-255.
- Curtis, R., Scherer, S.S., Somogyi, R., Adryan, K.M., Ip, N.Y., Zhu, Y., Lindsay, R.M. & DiStefano, P.S. (1994). Retrograde axonal transport of LIF is increased by peripheral nerve injury: Correlation with increased LIF expression in distal nerve. *Neuron* **12**, 191-204.
- Dash, P.K., Hochner, B. & Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. *Nature* **345**, 718-721.
- Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R. & Kandel, E.R. (1991). cAMP response element-binding protein is activated by Ca^{2+} /calmodulin- as well as cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences, USA* **88**, 5061-5065.
- de Felipe, C. & Hunt, S.P. (1994). The Differential Control of c-Jun Expression in Regenerating Sensory Neurons and Their Associated Glial Cells. *The Journal of Neuroscience* **14**, 2911-2923.
- de Felipe, C., Jenkins, R., O'Shea, R., Williams, T.S.C. & Hunt, S.P. (1993). The role of immediate early genes in the regeneration of the central nervous system. *Advances in Neurology* **59**, 263-271.

- DeFranco, C., Damon, D.H., Endoh, M. & Wagner, J.A. (1993). Nerve growth factor induces transcription of NGFIA through complex regulatory elements that are also sensitive to serum and phorbol 12-myristate 13-acetate. *Molecular Endocrinology* **7**, 365-379.
- Delegeane, A.M., Ferland, L.H. & Mellon, P.L. (1987). The enhancer of the human glycoprotein hormone shows dependence on cyclic AMP-inducible elements. *Molecular and Cellular Biology* **7**, 3994-4002.
- Deutsch, P.J., Hoeffler, J.P., Jameson, L., Lin, J.C. & Habener, J.F. (1988). Structural determinants for transcriptional activation by cAMP-responsive DNA elements. *Journal of Biological Chemistry* **263**, 18466-18472.
- Deutsch, P.J., Jameson, J.L. & Habener, J.F. (1987). Cyclic AMP responsiveness of human gonadotropin-alpha gene transcription is directed by a repeated 18-base pair enhancer. *Journal of Biological Chemistry* **262**, 12169-12174.
- Devor, M. (1991). Sensory basis of autotomy in rats. *Pain* **45**, 109-110.
- DiStefano, P.S., Friedman, B., Radziejewski, C., Alexander, C., Boland, P., Schick, C.M., Lindsay, R.M. & Wiegand, S.J. (1992). The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron* **8**, 983-993.
- Dobson, S.P., Quinn, J.P., Morrow, J.A. & Mulderry, P.K. (1994). The rat vasoactive intestinal polypeptide cyclic AMP response element regulates gene transcriptional responses differently in neonatal and adult rat sensory neurons. *Neuroscience Letters* **167**, 19-23.
- Doughty, S.E., Atkinson, M.E. & Shehab, S.A.S. (1991). A quantitative study of neuropeptide cell bodies of primary afferent sensory neurons following rat sciatic nerve peripheral axotomy. *Regulatory Peptides* **35**, 59-72.

- Dynlacht, B.D., Hoey, T. & Tjian, R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66**, 563-576.
- Fedulova, S.A., Kostyuk, P.G. & Veselovsky, N.S. (1991). Ionic mechanisms of electrical excitability in rat sensory neurons during postnatal ontogenesis. *Neuroscience* **41**, 303-309.
- Fink, J.S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G. & Goodman, R.H. (1988). The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. *Proceedings of the National Academy of Sciences, USA* **85**, 6662-6666.
- Fink, J.S., Verhave, M., Walton, K., Mandel, G. & Goodman, R.H. (1991). Cyclic AMP- and phorbol ester-induced transcriptional activation are mediated by the same enhancer element in the human vasoactive intestinal peptide gene. *Journal of Biological Chemistry* **266**, 3882-3887.
- Fitzgerald, M., Wall, P.D., Goedert, M. & Emson, P.C. (1985). Nerve growth factor counteracts the morphological and neurochemical effects of chronic nerve section. *Brain Research* **332**, 131-141.
- Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., El-Yassir, N., Molony, V. & Bladon, C.M. (1990). The involvement of neurokinin receptor subtypes in somatosensory processing in the superficial dorsal horn of the cat. *Brain Research* **519**, 169-182.
- Forman, D.S., McQuarrie, I.G., Grafstein, B. & Edwards, D.L. (1981). *Effect of a conditioning lesion on axonal regeneration and recovery of function*. Springer Verlag, Berlin.
- Foulkes, N.S. & Sassone-Corsi, P. (1992). More is better: activators and repressors from the same gene. *Cell* **68**, 411-414.

- Frisén, J., Risling, M., Theodorsson, E. & Fried, K. (1992). NPY-like immunoreactivity in sensory nerve fibers in rat sciatic neuroma. *Brain Research* **577**, 142-146.
- Gauldie, J., Richards, C., Harnish, D., Lansdorf, P. & Baumann, H. (1987). Interferon β /B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Science: USA*. **84**, 7251-7255.
- Gentz, R., Rauscher III, F.J., Abate, C. & Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243**, 1695-1699.
- Giladi, E., Shani, Y. & Gozes, I. (1990). The complete structure of the rat VIP gene. *Molecular Brain Research* **7**, 261-267.
- Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry* **56**, 615-649.
- Gilman, M.Z., Wilson, R.N. & Weinberg, R.A. (1986). Multiple protein binding sites in the 5'-flanking region regulate c-fos expression. *Molecular and Cellular Biology* **6**, 4305-4316.
- Giniger, E. & Ptashne, M. (1988). Cooperative DNA binding of the yeast transcriptional activator GAL4. *Proceedings of the National Academy of Sciences, USA* **85**, 382-386.
- Glowa, J.R., Panlilio, L.V., Brenneman, D.E., Gozes, I., Fridkin, M. & Hill, J.M. (1992). Learning impairment following intracerebral administration of the HIV envelope protein GP120 or a VIP antagonist. *Brain Research* **570**, 49-53.
- Gonzalez, G.A. & Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675-680.

- Gozes, I., Hill, J.M., Mervis, R.F., Fridkin, M. & Brenneman, D.E. (1990). VIP antagonist produces neuronal damage and retardation of behavioral development in neonatal rats. *Soc. Neuroscience Abstracts* **16**, 1292.
- Gozes, I., Shacter, P., Shani, Y. & Giladi, E. (1988). Vasoactive Intestinal Peptide gene expression from embryos to ageing rats. *Neuroendocrinology* **47**, 27-31.
- Gozes, I., Shani, Y. & Rostene, W.H. (1987). Developmental expression of the VIP gene in brain and intestine. *Molecular Brain Research* **2**, 137-148.
- Graves, B.J., Johnson, P.F. & McKnight, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**, 565-576.
- Greenberg, M.E., Ziff, E.B. & Greene, L.A. (1986). Stimulation of Neuronal Acetylcholine Receptors Induces Rapid Gene Transcription. *Science* **234**, 80-83.
- Greene, L.A. & Tischler, A. (1976). Establishment of a nonadrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proceedings of the national academy of sciences: USA* **73**, 2424-2428.
- Grove, J.R., Price, D.J., Goodman, H.M. & Avruch, J. (1987). Recombinant fragment of protein kinase inhibitor blocks cyclic AMP dependent gene transcription. *Science* **238**, 530-533.
- Haegerstrand, A., Jonzon, B., Dalsgaard, C.J. & Nilsson, J. (1989). Vasoactive intestinal polypeptide stimulates cell proliferation and adenylate cyclase activity of cultured human keratinocytes. *Proceedings of the National Academy of Sciences, USA* **86**, 5993-5996.
- Hai, T. & Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity.

- Hai, T., Liu, F., Coukos, W.J. & Green, M.R. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes and Development* **3**, 2083-2090.
- Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E. & Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* **55**, 917-924.
- Hall, G.F. & Kosik, K.S. (1993). Axotomy-Induced neurofilament phosphorylation is inhibited in situ by microinjection of PKA and PKC inhibitors into identified lamprey neurons. *Neuron* **10**, 613-625.
- Hamilton, B.J., Nagy, E., Malter, J.S., Arrick, B.A. & Rigby, W.F.C. (1993). Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. *Journal of Biological Chemistry* **268**, 8881-8887.
- Hartung, H.P., Wolters, K. & Tokya, K.V. (1986). Substance P binding properties and studies on cellular responses in guinea-pig macrophages. *Journal of Immunology* **136**, 3856-3863.
- Hayakawa, Y., Obata, K.I., Itoh, N., Yanaihara, N. & Okamoto, H. (1984). Cyclic AMP regulation of pro-vasoactive intestinal polypeptide/PHM synthesis in human neuroblastoma cells. *Journal of Biological Chemistry* **259**, 9207-9211.
- Hendry, I.A., Murphy, M., Hilton, D.J., Nicola, N.A. & Bartlett, P.F. (1992). Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. *Journal of Neuroscience* **12**, 3427-3434.
- Henry, J.L. (1978). Effects of substance P on functionally-identified units in cat spinal cord. *Brain Research* **114**, 439-451.

- Herdegen, T., Bastmeyer, M., Bahr, M., Stuermer, C., Rodrigo, B. & Zimmermann, M. (1993). Expression of JUN, KROX, and CREB transcription factors in goldfish and rat retinal ganglion cells following optic nerve lesion is related to axonal sprouting. *Journal of Neurobiology* **24**, 528-543.
- Herdegen, T., Brecht, S., Mayer, B., Leah, J., Kummer, W., Bravo, R. & Zimmermann, M. (1993). Long-lasting expression of JUN and KROX transcription factors and nitric oxide synthase in intrinsic neurons of the rat brain following axotomy. *The Journal of Neuroscience* **13**, 4130-4145.
- Herdegen, T., Fiallos-Estrada, C.E., Schmid, W., Bravo, R. & Zimmermann, M. (1992). The transcription factors c-JUN, JUN D and CREB, but not FOS and KROX-24, are differentially regulated in axotomized neurons following transection of rat sciatic nerve. *Molecular Brain Research* **14**, 155-165.
- Heumann, R., Korsching, S., Bandtlow, C. & Thoenen, H. (1987). Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *Journal of Cell Biology* **104**, 1623-1631.
- Hill, J.M., Gozes, I., Hill, J.L., Fridkin, M. & Brenneman, D.E. (1991). Vasoactive intestinal peptide antagonist retards the development of neonatal behaviours in the rat. *Peptides* **12**, 187-192.
- Hill, J.M., Mervis, R.F., Politi, J., Mccune, S.K., Gozes, I., Fridkin, M. & Brenneman, D.E. (1994). Blockade of VIP during neonatal development induces neuronal damage and increases VIP and VIP receptors in brain. *Annals of the New York Academy of Sciences* **739**, 211-225.
- Hirai, S.I., Ryseck, P., Mechta, F., Bravo, R. & Yaniv, M. (1989). Characterisation of jun D: A new member of the jun protooncogene family. *EMBO Journal* **8**, 1433-1439.

- Hoeffler, J.P., Lustbader, J.W. & Chen, C.-Y. (1991). Identification of multiple nuclear factors that interact with cyclic adenosine 3',5'-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions. *Molecular Endocrinology* **5**, 256-266.
- Hökfelt, T., Wiesenfeld-Hallin, Z., Villar, M. & Melander, T. (1987). Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neuroscience Letters* **83**, 217-220.
- Hökfelt, T., Zhang, X. & Wiesenfeld-Hallin, Z. (1994). Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends in Neurosciences* **17**, 22-30.
- Hope, I.A. & Struhl, K. (1987). GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. *EMBO Journal* **6**, 2781-2784.
- Hyman, S.E., Comb, M., Pearlberg, J. & Goodman, H.M. (1989). An AP-2 Element acts Synergistically with the Cyclic AMP and Phorbol Ester Inducible Enhancer of the Human Proenkephalin Gene. *Molecular and Cellular Biology* **9**, 321-324.
- Inaishi, Y., Kashihara, Y., Sakaguchi, M., Nawa, H. & Kuno, M. (1992). Cooperative regulation of calcitonin gene-related peptide levels in rat sensory neurons via their central and peripheral processes. *Journal of Neuroscience* **12**, 518-524.
- Ip, N.Y., McClain, J., Barrezueta, N.X., Aldrich, T.H., Pan, L., Li, Y., Wiegand, S.J., Friedman, B., Davis, S. & Yancopoulos, G.D. (1993). The α component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. *Neuron* **10**, 89-102.
- Itoh, N., Obata, K., Yanaihara, N. & Okamoto, H. (1983). Human preprovasoactive intestinal polypeptide contains a novel PHI-27 like peptide PHM-27. *Nature* **304**, 547-549.

- Jenkins, R. & Hunt, S.P. (1991). Long-term increase in the levels of *c-jun* mRNA and Jun protein-like immunoreactivity in motor and sensory neurons following axon damage. *Neuroscience Letters* **129**, 107-110.
- Ji, R., Zhang, Q., Bedecs, K., Arvidsson, J., Zhang, X., Xu, X., Wiesenfeld-Hallin, Z., Bartfai, T. & Hokfelt, T. (1994). Galanin antisense oligonucleotides reduce galanin levels in dorsal root ganglia and induce autotomy in rats after axotomy. *Proceeding of the National Academy of Sciences; USA*. **91**, 12540-12543.
- Johnson, A.D. & Herskowitz, I. (1985). A repressor (MATa2 product) and its operator control expression of a set of cell type specific genes in yeast. *Cell* **42**, 237-247.
- Johnson, E.M.J., Rich, K.M. & Yip, H.K. (1986). The role of NGF in sensory neurons in vivo. *Trends in Neuroscience* **9**, 33-37.
- Johnson, F.B. & Krashnow, M.A. (1992). Differential regulation of transcription preinitiation complex assembly by activator and repressor homeo domain proteins. *Genes and Development* **6**, 2177-2189.
- Johnson, P.F. & McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. *Annual Review of Biochemistry* **58**, 799-839.
- Kalcheim, C., Barde, Y.-A., Thoenen, H. & Le Douarin, N.M. (1987). *In vivo* effect of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells. *EMBO Journal* **6**, 2871-2873.
- Karin, M. & Smeal, T. (1992). Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends in Biochemical Sciences* **17**, 418-422.
- Karins, C. & Said, S.I. (1973). Hyperglycemic and glycogenolytic effects of vasoactive intestinal polypeptide. *Proceedings for the society of experimental and biological medicine* **142**, 1014-1017.

- Karpinski, B.A., Morle, G.D., Huggenvik, J., Uhler, M.D. & Leiden, J.M. (1992). Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. *Proceedings of the National Academy of Sciences, USA* **89**, 4820-4824.
- Kashiba, H., Senba, E., Kawai, Y., Ueda, Y. & Tohyama, M. (1992). Axonal blockade induces the expression of vasoactive intestinal polypeptide and galanin in rat dorsal root ganglion neurons. *Brain Research* **577**, 19-28.
- Keen, P., Nielsch, U. & Watts, P.A. (1989). An investigation into the mechanism of induction of vasoactive intestinal peptide gene expression in rat sensory neurones following nerve injury. *Journal of Physiology* **410**, 62P.
- Knyihár-Csillik, E., Kreutzberg, G.W., Raivich, G. & Csillik, B. (1991). A case for neurotransmitter plasticity at the molecular level: axotomy-induced VIP increase in the upper spinal dorsal horn is related to blockade of retrograde axoplasmic transport of nerve growth factor in the peripheral nerve. *Acta Histochemica* **91**, 77-83.
- Kobierski, L.A., Chu, H.-M., Tan, Y. & Comb, M.J. (1991). cAMP-dependent regulation of proenkephalin by JunD and JunB: positive and negative effects of AP-1 proteins. *Proceedings of the National Academy of Sciences, USA* **88**, 10222-10226.
- Koo, E.H., Hoffman, P.N. & Price, D.L. (1988). Levels of neurotransmitter and cytoskeletal protein mRNAs during nerve regeneration in sympathetic ganglia. *Brain Research* **449**, 361-363.
- Kreutzberg, G.W. (1982). *Repair and regeneration of the nervous system*. Springer,
- Kwok, R.P.S., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R. & Goodman, R.H. (1994).

Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**, 223-226.

Kyriakis, J.M., Banerjee, P., Nikolaki, E., Dai, T., Rubie, E.A., Ahmad, M.F., Avruh, J., Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**, 156-160.

Lamb, N.J.C., Fernandez, A., Tourkine, N., Jeanteur, P. & Blanchard, J.M. (1990). Demonstration in living cells of an intragenic negative regulatory element within the rodent c-fos gene. *Cell* **61**, 485-496.

Lamperti, E.D., Rosen, K.M. & Villa-Komaroff, L. (1991). Characterization of the gene and messages for vasoactive intestinal polypeptide (VIP) in rat and mouse. *Molecular Brain Research* **9**, 217-231.

Landschulz, W.H., Johnson, P.F. & Mcknight, S.L. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP. *Science* **243**, 1682-1688.

Lasek, R.J. & Hoffman, P.N. (1976). *The neuronal cytoskeleton, axonal transport and axonal growth*. Cold Spring Harbour Laboratory,

Leah, J.D., Herdegen, T. & Bravo, R. (1991). Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for a role in the regeneration process. *Brain Research* **566**, 198-207.

Lee, W., Mitchell, P. & Tjian, R. (1987). Purified transcription factor AP1 interaction with TPA inducible enhancer elements. *Cell* **49**, 741-752.

Lemaigre, F.P., Ace, C.I. & Green, M.R. (1993). The cAMP response element binding protein, CREB is a potent inhibitor of diverse transcriptional activators. *Nucleic Acids Research* **21**, 2907-2911.

Levine, J.D., Fields, H.L. & Basbaum, A.I. (1993). Peptides and the primary afferent nociceptor. *Journal of Neuroscience* **13**, 2273-2286.

- Lieberman, A.R. (1971). The axon reaction. A review of the principle features of the perikayal response to nerve injury. *International Review of Neurobiology* **14**, 49-124.
- Lindsay, R.M., Shooter, E.M., Radeke, M.J., Misko, T.P., Dechant, G., Thoenen, H. & Lindholm, D. (1990). Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. *European Journal of Neuroscience* **2**, 389-396.
- Lindsay, R.M., Thoenen, H. & Barde, Y.-A. (1985). Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Developmental Biology* **112**, 319-328.
- Lopez-Cabrera, M., Letovsky, J., Hu, K. & Siddiqui, A. (1990). Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: Trans-activation and repression by CCAAT/enhancer binding protein. *Proceedings of the National Academy of Sciences* **87**, 4895-6476.
- Lu, S.C. & Bensadoun, A. (1993). Identification of the 5' regulatory elements of avian lipoprotein lipase gene: Synergistic effect of multiple factors. *Biochimica et Biophysica Acta* **1216**, 375-384.
- Magistretti, P.J. & Schorderet, M. (1984). VIP and noradrenaline act synergistically to increase cyclic AMP in cerebral cortex. *Nature* **308**, 280-282.
- Magistretti, P.J., Morrison, J.H., Shoemaker, W.J., Vivica, S. & Bloom, F.E. (1981). Vasoactive intestinal polypeptide induces glycogenolysis in mouse cortical slices: A possible regulatory mechanism for the local control of energy metabolism. *Proceedings of the National Academy of Sciences* **78**, 6535-6539.
- Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. & Yancopoulos, G.D. (1990). Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. *Science* **247**, 1446-1451.

- Maruta, H. & Burgess, A.W. (1994). Regulation of the RAS signaling network. *Bioessays* **16**, 489-496.
- McCulloch, J. & Kelly, P.A.J. (1983). A functional role for vasoactive intestinal polypeptide in anterior cingulate gyrus. *Nature* **304**, 438-440.
- McKnight, G.S. (1991). Cyclic AMP Second Messenger Systems. *Current Opinion in Cell Biology* **3**, 213-217.
- McMurray, C.T., Wilson, W.D. & Douglass, J.O. (1991). Hairpin formation within the enhancer region of the human enkephalin gene. *Proceedings of the National Academy of Sciences, USA* **88**, 666-670.
- Mechti, N., Piechaczyk, M., Blanchard, J.M., Jeanteur, P. & Lebleu, B. (1991). Sequence Requirements for Premature Transcription Arrest Within the First Intron of the Mouse c-fos Gene. *Molecular and Cellular Biology* **11**, 2832-2841.
- Meinkoth, J.L., Ji, Y., Taylor, S.S. & Feramisco, J.R. (1990). Dynamics and distribution of cyclic AMP dependant protein kinase in living cells. *Proceedings of the National Academy of Sciences, USA* **87**, 9595-9599.
- Mendelson, S.C. & Quinn, J.P. (1995). Characterisation of potential regulatory elements within the rat preprotachykinin-A promoter. *Neuroscience Letters* **184**, 125-128.
- Meri, H., Dormann, A. & Spira, M.E. (1983). Comparison of ultrastructural changes in proximal and distal segments of transected giant fibers of the cockroach *Periplaneta americana*. *Brain Research* **263**, 1-14.
- Mitchell, P.J. & Wang, C. (1987). Positive and negative regulation of transcription in vitro: Enhancer-binding protein AP2 is inhibited by SV40 antigen. *Cell* **50**, 847-861.

- Mitchell, P.J., Timmons, P.M., Hebert, J.M., Rigby, P.W.J. & Tjian, R. (1991). Transcription factor AP-2 is expressed in neuronal crest cell lineages during mouse embryogenesis. *Genes and development* **5**, 105-119.
- Moncada, S., Palmer, R.M.J. & Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacology Review* **43**, 109-142.
- Montminy, M.R. & Bilezikjian, L.M. (1987). Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175-178.
- Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. & Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proceedings of the National Academy of Sciences, USA* **83**, 6682-6686.
- Moody, T.W., Zia, F., Goldstein, A.L., Naylor, P.H., Sarin, E., Brenneman, D.E., Koros, A.M.C., Reubi, J.C., Korman, L.Y., Fridkin, M. & Gozes, I. (1992). VIP analogues inhibit small cell lung cancer growth. *Biomedical Research* **13**, 131-135.
- Morgan, J.I. & Curran, T. (1986). Role of ion flux in the control of c-fos expression. *Nature* **322**, 522-555.
- Morris, R., Southam, E., Braid, D.J. & Garthwaite, J. (1992). Nitric oxide may act as a messenger between dorsal root ganglion neurones and their satellite cells. *Nature* **357**, 29-32.
- Morrison, C.F., McAllister, J., Dobson, S.P., Mulderry, P.K. & Quinn, J.P. (1994). An activator element within the preprotachykinin-A promoter. *Molecular and Cellular Neurosciences* **5**, 165-175.
- Mu, X., Silos-Santiago, I., Carroll, S.L. & Snider, W.D. (1993). Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. *Journal of Neuroscience* **13**, 4029-4041.

- Mulderry, P.K & .Dobson, S.P. (1995). Regulation of VIP and other neuropeptides by c-Jun in sensory neurons: Implications for the neuropeptide response to axotomy. *European Journal of Neuroscience* **submitted**,
- Mulderry, P.K. & Lindsay, R.M. (1990). Rat dorsal root ganglion neurons in culture express vasoactive intestinal polypeptide (VIP) independently of nerve growth factor. *Neuroscience Letters* **108**, 314-320.
- Mulderry, P.K. (1993). Synergistic regulation of VIP expression by cyclic AMP and calcium in newborn but not adult rat sensory neurons in culture. *Neuroscience* **53**, 229-238.
- Mulderry, P.K. (1994). Neuropeptide expression in sensory neurons in culture: Effects of nerve growth factor and other neurotrophic factors. *Neuroscience* **59**, 673-688.
- Mulderry, P.K., Chapman, K.E., Lyons, V. & Harmar, A.J. (1993). 5'-Flanking Sequences from the Rat Preprotachykinin Gene Direct High-Level Expression of a Reporter Gene in Adult Rat Sensory Neurons Transfected in Culture by Microinjection. *Molecular and Cellular Neurosciences* **4**, 164-172.
- Murphy, M., Reid, K., Brown, M.A. & Bartlett, P.F. (1993). Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* **117**, 1173-1182.
- Murphy, M., Reid, K., Hilton, D.J. & Bartlett, P.F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proceedings of the National Academy of Sciences, USA* **88**, 3498-3501.
- Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., Weintraub, H. & Baltimore, D. (1989). Interactions between heterologous helix-

loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Mutt, V. (1988). Vasoactive intestinal polypeptide and related peptides: isolation and chemistry. *Annals of the New York Academy of Sciences* **527**, 130-142.

Nakabeppu, Y., Ryder, K. & Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. *Cell* **55**, 907-915.

Nielsch, U. & Keen, P. (1989). Reciprocal regulation of tachykinin- and vasoactive intestinal peptide-gene expression in rat sensory neurones following cut and crush injury. *Brain Research* **481**, 25-30.

Nishizawa, K. (1994). NGF-induced stabilisation of GAP-43 mRNA is mediated by both 3' untranslated region and a segment encoding the carboxy-terminus peptide. *Biochemical and biophysical research communications* **200**, 789-796.

Nobou, F., Besson, J., Rostene, W. & Rosselin, G. (1985). Ontogeny of vasoactive intestinal peptide and somatostatin in different structures of the rat brain: effects of hypo- and hypercorticism. *Developmental Brain Research* **20**, 296-301.

Noguchi, K., Deleon, M., Nohin, R.L., Senba, E. & Ruda, M.A. (1993). Quantitation of axotomy-induced alteration of neuropeptide messenger-RNAs in dorsal root ganglion neurons with special reference to neuropeptide-Y messenger-RNA and the effects of neonatal capsaicin treatment. *Journal of Neuroscience Research* **35**, 54-66.

Noguchi, K., Morita, Y., Kiyama, H., Ono, K. & Tohyama, M. (1988). A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using *in situ* hybridization histochemistry. *Molecular Brain Research* **4**, 31-35.

- Noguchi, K., Senba, E., Morita, Y., Sato, M. & Tohyama, M. (1989). Prepro-VIP and prepro-tachykinin mRNAs in the rat dorsal root ganglion cells following peripheral axotomy. *Molecular Brain Research* **6**, 327-330.
- Noguchi, K., Senba, E., Morita, Y., Sato, M. & Tohyama, M. (1990). α -CGRP and β -CGRP are differentially regulated in the rat spinal cord and dorsal root ganglia. *Molecular Brain Research* **7**, 299-304.
- Oblinger, M.M. & Lasek, R.J. (1984). A conditioning lesion of the peripheral axons of dorsal ganglion cells accelerates regeneration of only their peripheral axons. *Journal of Neuroscience* **4**, 1736-1744.
- Oblinger, M.M., Szumlas, R.A., Wong, J. & Liuzzi, F.J. (1989). Changes in cytoskeletal gene expression affect the composition of regenerating axonal sprouts elaborated by dorsal root ganglion neurons in vivo. *Journal of Neuroscience* **9**, 2645-2653.
- Okamoto, S., Okamura, H., Miyake, M., Takahashi, Y., Takagai, S., Akagi, Y., Okamoto, H. & Iyata, Y. (1991). A diurnal variation of vasoactive intestinal peptide (VIP) mRNA under a daily light dark cycle in the rat suprachiasmatic nucleus. *Histochemistry* **95**, 525-528.
- Otto, D., Unsicker, K. & Grothe, C. (1987). Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transected ganglia. *Neuroscience Letters* **83**, 156-160.
- Panlilio, L.V., Hill, J.M., Brenneman, D.E., Fridkin, M., Gozes, I. & Glowa, J.R. (1990). GP120 and a VIP antagonist impair Morris water maze performance in rats. *Society for Neuroscience Abstracts* **16**, 1330.
- Paterson, J.M., Morrison, C.F., Dobson, S.P., McAllister, J. & Quinn, J.P. (1995). Characterisation of a functional E box motif in the proximal rat preprotachykinin-A promoter. *Neuroscience Letters* **191**, 185-188.
- Payan, D.G., Levine, J.D. & Goetzl, E.J. (1984). Modulation of immunity and hypersensitivity by sensory neuropeptides. *Journal of Immunology* **132**, 1601-1604.

- Pearse, A.G.E. (1980). *Histochemistry theoretical and applied*. Churchill Livingstone,
- Pincus, D.W., DiCicco-Bloom, E.M. & Black, I. (1990). Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. *Nature* **343**, 564-567.
- Piper, P.J., Said, S.I. & Vane, J.R. (1970). Effects on smooth muscle preparation of identified vasoactive peptides from intestine and lung. *Nature* **225**, 1144-1146.
- Pleasure, D.E. (1994). *Biochemistry of Neuropathy*. Raven Press, New York.
- Prestridge, D.S. (1991). Signal scan: A computer program that scans DNA sequences for eukaryotic transcriptional elements. *CABIOS* **7**, 203-206.
- Pruss, R.M., Moskal, J.R., Eiden, L.E. & Beinfeld, M.C. (1985). Specific regulation of vasoactive intestinal polypeptide biosynthesis by phorbol ester in bovine chromaffin cells. *Endocrinology* **117**, 1020-1026.
- Quinn, J.P. (1991). Variation in the composition of the AP1 complex in PC12 cells following induction by NGF and TPA. *Molecular and Cellular Neurosciences* **2**, 253-258.
- Reid, A.M., Schules, A. & Titchen, D.A. (1988). Effects of the vagus nerve on gastric motility and release of vasoactive intestinal polypeptide in the anaesthetised lamb. *Journal of Physiology* **396**, 11-24.
- Reinberg, D. & Roeder, R.G. (1987). Factors involved in specific transcription by mammalian RNA polymerase II. Purification and functional analysis of initiation factors IIB and IIE. *Journal of Biological Chemistry* **262**, 3310-3321.

- Richardson, P.M. & Riopelle, R.J. (1984). Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *Journal of Neuroscience* **4**, 1683-1689.
- Robinson, G.A. (1994). Immediate early gene expression in axotomized and regenerating retinal ganglion cells of the adult rat. *Molecular Brain Research* **24**, 43-54.
- Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramée, G.R., Nikolics, K. & Winslow, J.W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**, 767-773.
- Ross, R.A., Spengler, B.A. & Biedler, J.L. (1983). Coordinate morphological and biochemical interconversion of human neuroblastoma cells. *Journal of the National Cancer Institute* **71**, 741-749.
- Rost, F.W.D. (1991). *Quantitative fluorescence microscopy*. Cambridge University Press, Cambridge.
- Ryder, K., Lanahan, A., Perez-Albuérne, E. & Nathans, D. (1989). JunD: A third member of the jun gene family. *Proceedings of the National Academy of Sciences, USA* **86**, 1500-1503.
- Ryder, K., Lau, L.F. & Nathans, D. (1988). A gene activated by growth factors is related to the oncogene v-jun. *Proceedings of the National Academy of Sciences, USA* **85**, 1487-1491.
- Sachs, A. & Wahle, E. (1993). Poly (A) tail metabolism and function in eukaryotes. *Journal of Biological Chemistry* **268**, 22955-22958.
- Said, S.I. & Mutt, V. (1970). Polypeptide with broad biological activity: isolation from small intestine. *Science* **169**, 1217-1218.
- Said, S.I. & Mutt, V. (1988). Vasoactive intestinal peptide and related peptides. *Annals of the New York Academy of Sciences* **527**, 1-11.

- Said, S.I. (1984). Isolation, localization and characterization of gastrointestinal peptides. *Peptides* **5**, 143-150.
- Sastry, B.R. (1979). Substance P effects on spinal nociceptive neurons. *Life Science* **24**, 2169-2178.
- Sawadogo, M. & Roeder, R.G. (1985). Interaction of a gene specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43**, 165-175.
- Schlaepfer, W.W. (1974). Calcium induced degeneration of axoplasm in isolated segments of rat peripheral nerve. *Brain Research* **69**, 203-215.
- Schlaepfer, W.W. (1977). Structural alterations of peripheral nerve induced by the calcium ionophore A23187. *Brain Research* **136**, 1-9.
- Seamon, K.I., Padgett, W. & Daly, J.W. (1981). Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proceedings of the National Academy of Sciences, USA* **78**, 3363.
- Searle, P.F., Davison, B.L., Stuart, G.W., Wilkie, T.H., Norstedt, G. & Palmiter, R.D. (1984). Regulation, linkage and sequence of mouse metallothionein I and II genes. *Molecular and Cellular Biology* **4**, 1221-1230.
- Shehab, S.A.S. & Atkinson, M.E. (1986). Vasoactive intestinal polypeptide (VIP) increases in the spinal cord after peripheral axotomy of the sciatic nerve originate from primary afferent neurons. *Brain Research* **372**, 37-44.
- Shehab, S.A.S., Atkinson, M.E. & Payne, J.N. (1986). The origins of the sciatic nerve and changes in neuropeptides after axotomy: a double labelling study using retrograde transport of True blue and vasoactive intestinal polypeptide immunohistochemistry. *Brain Research* **376**, 180-185.

- Sheng, M., McFadden, G. & Greenberg, M.E. (1990). Membrane depolarization and calcium induce *c-fos* transcription via phosphorylation of transcription factor CREB. *Neuron* **4**, 571-582.
- Sheng, M.E., Thompson, M.A. & Greenberg, M.E. (1991). CREB: a Ca²⁺ Regulated Transcription Factor Phosphorylated by CaM Kinases. *Science* **252**, 1427-1430.
- Silver, B.J., Bokar, J.A., Virgin, J.B., Vallen, E.A., Milsted, A. & Nilson, J.H. (1987). Cyclic AMP regulation of the human glycoprotein hormone alpha-subunit gene is mediated by an 18-base-pair element. *Proceedings of the National Academy of Sciences, USA* **84**, 2198-2202.
- Singh, H.R., Sen, R., Baltimore, D. & Sharp, P.A. (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature* **319**, 154-158.
- Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miao, G.G., Schilling, K., Robertson, L.M., Curran, T. & Morgan, J.I. (1993). Continuous *c-fos* expression precedes programmed cell death *in vivo*. *Nature* **363**, 166-169.
- Smith, A.G. (1991). Culture and differentiation of embryonic stem cells. *Journal of Tissue Culture Methods* **13**, 89-94.
- Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M. & Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-692.
- Smith, G.D., Harmer, A.J., McQueen, D.S. & Seckl, J.R. (1992). Increase in substance P, CGRP, but not somatostatin content of innervating dorsal root ganglia in adjuvant monoarthritis in the rat. *Neuroscience Letters* **137**, 257-260.

- Strittmatter, S.M., Vartanian, T. & Fishman, M.C. (1992). GAP-43 as a plasticity protein in neuronal form and repair. *Journal of Neurobiology* **23**, 507-520.
- Sun, Y., Rao, M.S., Landis, S.C. & Zigmond, R.E. (1992). Depolarization increases vasoactive intestinal peptide- and substance P-like immunoreactivities in cultured neonatal and adult sympathetic neurons. *Journal of Neuroscience* **12**, 3717-3728.
- Sun, Y., Rao, M.S., Zigmond, R.E. & Landis, S.C. (1994). Regulation of vasoactive intestinal peptide expression in sympathetic neurons in culture and after axotomy: The role of cholinergic differentiation factor/leukemia inhibitory factor. *Journal of Neurobiology* **25**, 415-430.
- Symes, A., Lewis, S., Corpus, L., Rajan, P., Hyman, S.E. & Fink, J.S. (1994). STAT proteins participate in the regulation of the vasoactive intestinal peptide gene by the ciliary neurotrophic factor family of cytokines. *Molecular Endocrinology* **8**, 1750-1763.
- Symes, A.J., Rao, M.S., Lewis, S.E., Landis, S.C., Hyman, S.E. & Fink, J.S. (1993). Ciliary neurotrophic factor coordinately activates transcription of neuropeptide genes in a neuroblastoma cell line. *Proceedings of the National Academy of Sciences, USA* **90**, 572-576.
- Tanaka, H., Vickart, P., Bertrand, J., Rayner, B., Morvan, F., Imbach, J., Paulin, D. & Malvy, C. (1994). Sequence-specific interaction of α - β -anomeric double-stranded DNA with the p50 subunit of NF κ B: application to the decoy approach. *Nucleic Acids Research* **22**, 3069-3074.
- Taylor, S.S. (1989). cAMP-Dependant Protein Kinase. *Journal of Biological Chemistry* **264**, 8443-8446.
- Troy, C.M., Muma, N.A., Greene, L.A., Price, D.L. & Shelanski, M. (1990). Regulation of peripherin and neurofilament expression in regenerating rat motor neurons. *Brain Research* **529**, 232-238.

- Tsukada, T., Fink, J.S., Mandel, G. & Goodman, R.H. (1987). Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. *Journal of Biological Chemistry* **262**, 8743-8747.
- Tsukada, T., Fukushima, M., Takebe, H. & Nakai, Y. (1994). Vasoactive intestinal peptide gene expression in the rat pheochromocytoma cell line PC12. *Molecular and Cellular Endocrinology* **107**, 231-239.
- Vallejo, M., Miller, C.P. & Habener, J.F. (1992). Somatostatin gene transcription regulated by a bipartite pancreatic islet D-cell-specific enhancer coupled synergistically to a cAMP response element. *Journal of Biological Chemistry* **267**, 12868-12875.
- Vallejo, M., Ron, D., Miller, C.P. & Habener, J.F. (1993). C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP responsive elements. *Proceedings of the National Academy of Sciences, USA* **90**, 4679-4683.
- Van Nguyen, T., Kobierski, L., Comb, M. & Hyman, S.E. (1990). The effect of depolarization on expression of the human proenkephalin gene is synergistic with cAMP and dependent upon a cAMP-inducible enhancer. *Journal of Neuroscience* **10**, 2825-2833.
- Vanselow, J., Grabezyk, E., Ping, J., Baetscher, M., Teng, S. & Fishman, M.C. (1994). GAP-43 Transgenic mice: dispersed genomic sequences confer a GAP-43 like expression pattern during development and regeneration. *The Journal of Neuroscience* **14**, 499-510.
- Verge, V.M.K., Xu, X.-J., Langel, Ü., Hökfelt, T., Wiesenfeld-Hallin, Z. & Bartfai, T. (1993). Evidence for endogenous inhibition of autotomy by galanin in the rat after sciatic nerve section: demonstrated by chronic intrathecal infusion of a high affinity galanin receptor antagonist. *Neuroscience Letters* **149**, 193-197.

- Verge, V.M.K., Xu, Z., Xu, X.-J., Wiesenfeld-Hallin, Z. & Hökfelt, T. (1992). Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: *in situ* hybridization and functional studies. *Proceedings of the National Academy of Sciences, USA* **89**, 11617-11621.
- Villar, M.J., Cortés, R., Theodorsson, E., Wiesenfeld-Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P.C. & Hökfelt, T. (1989). Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience* **33**, 587-604.
- Wakisaka, S., Kajander, K.C. & Bennet, G.J. (1991). Increased neuropeptide Y (NPY)-like immunoreactivity in rat sensory neurons following peripheral axotomy. *Neuroscience Letters* **124**, 200-203.
- Waschek, J.A., Hsu, C.-M. & Eiden, L.E. (1988). Lineage-specific regulation of the vasoactive intestinal peptide gene in neuroblastoma cells is conferred by 5.2 kilobases of 5'-flanking sequence. *Proceedings of the National Academy of Sciences, USA* **85**, 9547-9551.
- Waschek, J.A., Krajniak, K.G., Bravo, D.T. & Agoston, D.V. (1992). Regulation of the human and mouse vasoactive intestinal peptide (VIP) genes. *Biomedical Research* **13**, 1-6.
- Watson & Latchman (1995). The cyclic AMP response element in the calcitonin/calcitonin gene-related peptide gene promoter is necessary but not sufficient for its activation by nerve growth factor. *Journal of Biological Chemistry* **270**, 9655-9660.
- Weaver, C.A., Gordon, D.F., Kissil, M.S., Mead, D.A. & Kemper, B. (1984). Isolation and complete nucleotide sequence of the gene for bovine parathyroid hormone. *Gene* **29**, 319-329.

- White, J.D. & Gall, C.M. (1987). Differential regulation of neuropeptide and proto-oncogene mRNA content in the hippocampus following recurrent seizures. *Molecular Brain Research* **3**, 21-29.
- Whitson, P.A., Hsieh, W.T., Wells, R.D. & Matthews, K.S. (1987). Influence of supercoiling and sequence context on operator DNA binding with lac repressor. *Journal of Biological Chemistry* **262**, 14592-14599.
- Wiesenfeld-Hallin, Z., Hokfelt, T., Lundberg, J.M., Forssmann, W.G., Reinecke, M., Tschopp, F.A. & Fischer, J.A. (1984). Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioural responses of the rat. *Neuroscience Letters* **52**, 199-204.
- Wiesenfeld-Hallin, Z., Xu, X.-J., Håkanson, R., Feng, D.-M. & Folkers, K. (1990). Plasticity of the peptidergic mediation of spinal reflex facilitation after peripheral nerve section in the rat. *Neuroscience Letters* **116**, 293-298.
- Winter, J. (1987). Characterization of capsaicin-sensitive neurones in adult rat dorsal root ganglion cultures. *Neuroscience Letters* **80**, 134-140.
- Wong, J. & Oblinger, M.M. (1990). A comparison of peripheral and central axotomy effects on neurofilament and tubulin gene expression in rat dorsal root ganglion neurons. *Journal of Neuroscience* **10**, 2215-2222.
- Wong, J. & Oblinger, M.M. (1990). Differential regulation of peripherin and neurofilament gene expression in regenerating rat DRG neurons. *Journal of Neuroscience Research* **27**, 332-341.
- Wong, J. & Oblinger, M.M. (1991). NGF rescues substance P expression but not neurofilament or tubulin gene expression in axotomized sensory neurons. *Journal of Neuroscience* **11**, 543-552.

- Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J. & Bevan, S. (1988). Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *Journal of Neuroscience* **8**, 3208-3220.
- Woolf, C. & Wiesenfeld-Hallin, Z. (1986). Substance P and calcitonin gene related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. *Neuroscience Letters* **66**, 226-230.
- Woolf, C.J., Reynolds, M.L., Molander, C., O'Brien, C., Lindsay, R.M. & Benowitz, L.I. (1990). The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience* **34**, 465-478.
- Wu, W. & Li, L. (1993). Inhibition of nitric oxide synthase reduces motorneuron death due to spinal root avulsion. *Neuroscience Letters* **153**, 121-124.
- Xia, z., Refsdal, C.D., Merchant, K.M., Dorsa, D.M. & Storm, D.R. (1991). Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory. *Neuron* **1991**, 431-443.
- Xu, X.J., Hao, J.X., Hokfelt, I. & Wiesenfeld-Hallin, Z. (1994). The effects of intrathecal neuropeptide Y on the spinal nociceptive flexor reflex in rats with intact sciatic nerves and after peripheral axotomy. *Neuroscience* **63**, 817-826.
- Yamagami, T., Ohsawa, K., Nishizawa, M., Inoue, C., Gotoh, E., Yanaihara, N., Yamamoto, H. & Okamoto, H. (1988). Complete nucleotide sequence of human vasoactive intestinal peptide/PHM-27 gene and its inducible promoter-a. *Annals of New York Academy of Sciences* **527**, 87-102.
- Yip, H.K., Rich, K.M., Lampe, P.A. & Johnson, E.M. (1984). The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. *Journal of Neuroscience* **4**, 2986-2992.

Zawel, L. & Reinberg, D. (1993). Initiation of transcription by RNA polymerase II: A multi-step process. *Progress in Nucleic Acid Research and Molecular Biology* **44**, 67-108.

RAT VIP CYCLIC AMP RESPONSIVE ELEMENT MEDIATES EXPRESSION IN NEONATAL RAT DORSAL ROOT GANGLION NEURONS. S.P. Dobson, P.K. Mulderry, J.P. Quinn, J.A. Morrow, I. Gozes*, A.J. Harmar, SPON; Brain Research Association. MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Edinburgh U.K. *Sackler school of medicine, Tel Aviv University, Israel.

Cyclic AMP and calcium influx caused by depolarization act synergistically to stimulate high levels of VIP expression in neonatal rat dorsal root ganglion (DRG) neurons in culture (Mulderry, *Neuroscience* **53**: 229, 1993). The aim of this study was to investigate the role of a putative cyclic AMP responsive element (CRE), 5' to the rat VIP transcription start site, in regulating VIP gene expression in response to potassium (K⁺) evoked depolarisation and a forskolin-stimulated increase in cyclic AMP. Neonatal rat DRG neurons grown in culture wells were transfected by microinjection (Mulderry *et al*, *Mol.Cell. Neurosci.* **4**: 164, 1993) with a plasmid containing 3 tandem copies of the rat VIP CRE, in the form of a 33 bp synthetic oligonucleotide, preceding the c-fos promoter and CAT reporter gene. After injection of plasmid into 350 neurons/well, expression of CAT was undetectable (<3pg) when cells were grown in medium alone or medium plus forskolin (10µM). Elevated K⁺ (40mM) resulted in expression of 3.5-10pg/well whilst K⁺ combined with forskolin resulted in 35-49 pg/well. A control plasmid containing the c-fos promoter and CAT reporter gene but no CRE expressed 3-9 pg CAT/well in response to combined forskolin and K⁺ whilst expression was undetectable when forskolin or K⁺ were used alone. Thus, the CRE can mediate synergistic transcriptional responses to combined stimulation with forskolin and depolarization in DRG neurons. DRG neurons were also microinjected with a plasmid containing 1.5kb of 5' flanking sequence (including the CRE) from the rat VIP gene preceding a *LacZ* reporter gene. This construct showed 10-13 fold higher levels of expression in the presence of forskolin and K⁺ than under control conditions. Our results indicate that the rat VIP CRE could mediate the large increase in VIP expression induced by cyclic AMP and depolarization in DRG neurons.

NSL 10192

The rat vasoactive intestinal polypeptide cyclic AMP response element regulates gene transcriptional responses differently in neonatal and adult rat sensory neurons

S.P. Dobson, J.P. Quinn, J.A. Morrow and P.K. Mulderry*

MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Morningside Park, Edinburgh EH10 5HF, UK

(Received 1 October 1993; Accepted 22 November 1993)

Key words: Microinjection; Forskolin; Depolarisation; Reporter gene; Vasoactive intestinal polypeptide; Cyclic AMP responsive element; Transfection; c-fos

We studied the ability of the rat vasoactive intestinal polypeptide (VIP) cyclic AMP responsive element (CRE) to regulate reporter gene expression through a *c-fos* promoter in rat sensory neurons transfected in culture by plasmid microinjection. The CRE enhanced the synergistic response of the promoter to combined potassium-evoked depolarisation and forskolin treatment in neonatal but not adult rat neurons. This corresponds to endogenous VIP expression which is induced synergistically by the same stimuli in neonatal but not adult rat neurons. We conclude that VIP expression in sensory neurons, which is induced by axotomy *in vivo*, could be regulated through the CRE.

Vasoactive intestinal peptide (VIP) is one of several neuropeptides whose expression is increased in sensory neurons of the dorsal root ganglia (DRG) in response to peripheral axotomy [1–4]. There is pharmacological evidence that VIP functions as a neurotransmitter of primary afferent terminals in the spinal cord specifically after nerve injury [5] suggesting that plasticity of neuropeptide expression may be an important feature of primary afferent function. It is therefore of interest to know the mechanisms responsible for this plasticity.

We have used DRG neurons in culture to investigate mechanisms regulating VIP expression. Our previous studies have shown that adult rat neurons grown in culture exhibit a spontaneous induction of VIP similar to that seen after peripheral nerve injury [6]. Unlike their counterparts from adult animals, neonatal rat DRG neurons supported in culture with NGF do not spontaneously express VIP. However, VIP expression can be induced in these cells by the synergistic effects of combined potassium-evoked depolarisation (resulting in Ca^{2+} influx) and drugs that increase intracellular cyclic AMP (cAMP) concentrations [7]. Although forskolin, an activator of adenylate cyclase, and depolarisation each individually have modest effects on VIP concentrations in adult rat DRG cultures, there is no synergistic interaction of the kind seen in neonatal rat DRG neurons [7].

These observations led us to hypothesise that spontaneous VIP expression in adult rat neurons and synergistically induced expression in neonates might depend on a common regulatory mechanism which, being spontaneously activated in the mature cells, is no longer capable of mediating a response to combined activation of cAMP- and Ca^{2+} -dependent signalling pathways.

The effects of cAMP and Ca^{2+} influx on VIP levels in neonatal rat DRG cultures were of interest in view of the reported presence of a consensus cAMP responsive element (CRE) DNA sequence in the 5'-flanking region of the rat VIP gene [8,9]. Similar DNA sequence elements from other genes have been shown to mediate synergistic transcriptional responses to cAMP and Ca^{2+} -influx [10], indicating that VIP expression in neonatal rat DRG cultures could result from increased VIP gene transcription following specific binding of cellular transcription factors to the CRE. Furthermore, CREs have been shown to participate in driving constitutive gene expression in certain cell types [11], suggesting that the CRE might also be a site through which spontaneous VIP expression in mature DRG neurons is regulated. Although the rat VIP CRE has not been shown to be functional, a similar, but not identical, DNA sequence element associated with the human VIP gene is known to function as an enhancer of gene transcription [12–14]. The aim of the present study was to examine the extent to which the rat VIP CRE could mediate patterns of gene expression analo-

*Corresponding author. Fax: (44) (31) 447-2081.

gous to those seen for endogenous VIP in rat DRG neurons in culture, with the goal of assessing the likelihood that this element mediates VIP induction in response to axotomy in vivo.

We have taken the approach of using a reporter plasmid containing a synthetic oligonucleotide sequence comprising the rat VIP CRE inserted upstream of the previously characterised *c-fos* minimal promoter driving expression of a chloramphenicol acetyl transferase (CAT) reporter gene. A reporter gene vector, FOS, containing the mouse *c-fos* promoter (nucleotides -56 to +72 relative to the transcription start site) [15] linked to the CAT gene was used as the basis for reporter plasmid construction. Synthetic double-stranded oligonucleotides corresponding to the rat VIP CRE (nucleotides -94 to -67) and the rat somatostatin CRE (nucleotides -37 to -45) (Fig. 1) were synthesised (Oswel DNA Services, University of Edinburgh) to allow insertion into the unique *Sal*I restriction site immediately upstream of the *c-fos* promoter. All clones were confirmed by sequence analysis using a primer corresponding to a sequence within the *c-fos* promoter. The somatostatin CRE is probably the best-characterised element of this type and is known to be capable of mediating a response to forskolin, when in the context of specific promoters [16,17]. Thus, it was used as a positive control with which to compare the rat VIP CRE results.

Rat DRG cultures were prepared as described previously [18] and maintained in Ham's F14 medium (Imperial Laboratories) containing 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco), 4% Ultrosor G serum substitute (Gibco), 50 ng/ml mouse salivary gland NGF (generous gift from Dr. J. Winter, Sandoz Institute, London, UK). Prior to microinjection, media contained cytosine- β -D-arabinoiside (10 μ M) to counteract proliferation of non-neuronal cells. Plasmid constructs were microinjected at 200 μ g/ml 1-4 days after plating. Microinjection was carried out as described previously [19] except that instead of glass coverslips, cultures were

plated on shallow 10-mm diameter polystyrene wells at a density of 4,000-10,000 neurons per well. Three hundred and fifty neurons per well were injected with plasmid. Following injections, neurons were left overnight in normal medium before addition of medium containing K^+ or forskolin. For depolarisation experiments, medium containing 40 mM K^+ was prepared as described previously [7]. Forskolin (Sigma), stock solution dissolved in ethanol at 10 mM was added at 1:1000 to yield a final concentration of 10 μ M. Cells were left in the presence of stimuli for 72 h before extraction in lysis buffer (Boehringer-Mannheim) for 30 min at room temperature. Lysates were assayed for CAT immunoreactivity using an enzyme-linked immunosorbent assay kit calibrated with purified CAT (Boehringer-Mannheim) at 3-200 pg per assay well. Statistical analysis of CAT immunoreactivity results were carried out using a 2-way analysis of variance for all combinations of injected construct and treatment of cells after injection. A post-hoc Student Newman-Keuls test was used to detect significance of individual comparisons. The critical value for significance of differences between group means was taken as $P < 0.05$.

After injection of the control plasmid, FOS, into neonatal rat DRG neurons, there was no significant increase in CAT immunoreactivity over the low levels detected in uninjected cultures under basal conditions, or in the presence of forskolin or under depolarising conditions alone. Under conditions of combined forskolin and depolarisation, however, significant concentrations of specific CAT immunoreactivity could be detected (Fig. 2). Injection of the plasmid containing the VIP CRE resulted in CAT expression significantly different from those seen with FOS only under conditions of combined forskolin and depolarisation, when CAT levels were approximately 4-fold higher than those in cultures injected with FOS (Fig. 2). Unlike the VIP CRE, injection of the plasmid containing the somatostatin CRE resulted in significant levels of CAT expression under basal conditions.

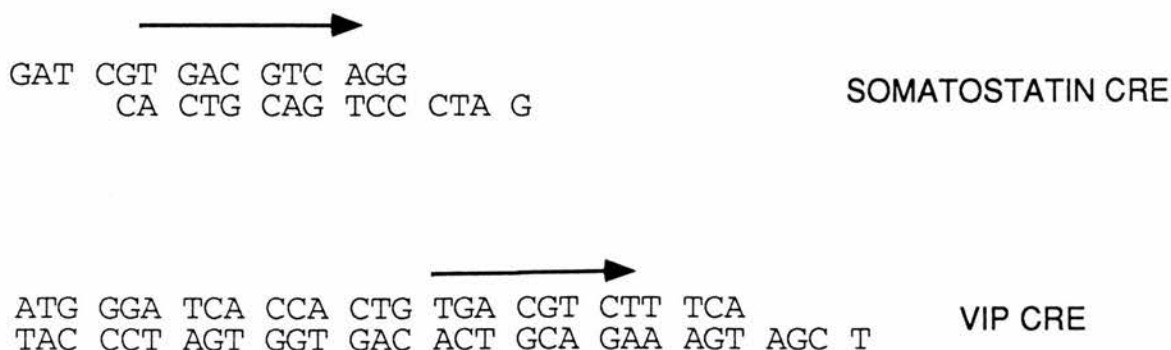


Fig. 1. Oligonucleotide sequences cloned into the *Sal*I restriction site 5' of the *c-fos* minimal promoter. Arrows indicate site and direction of the consensus CRE sequences.

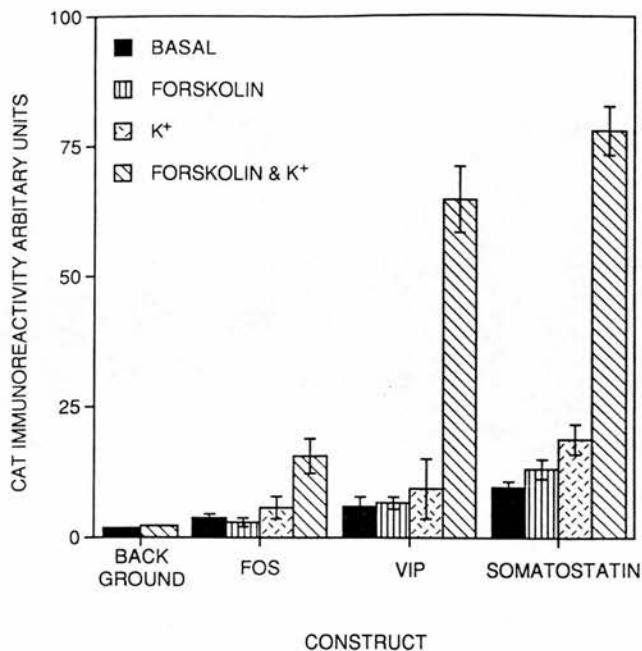


Fig. 2. Effects of 10 μ M forskolin and 40 mM K⁺ on reporter gene expression in neonatal rat DRG neurons. Each value is mean \pm standard error for at least 4 separate culture wells. As absolute levels of CAT immunoreactivity varied between experiments, results were normalised against values obtained with an internal control (construct containing three copies of the VIP CRE stimulated with forskolin and K⁺). Background = cultures that had received no injections.

This was not significantly altered by forskolin or depolarisation alone, but was increased to levels similar to those seen with the VIP CRE construct under conditions of combined forskolin and depolarisation (Fig. 2).

Injection of FOS into adult rat neurons produced similar results to those seen in neonates: there was no significant CAT under basal conditions but combined depolarisation and forskolin treatment resulted in the appearance of detectable CAT immunoreactivity. Results obtained from adult rat cells injected with the VIP CRE construct differed from those obtained in neonates, however, in that CAT concentrations were not significantly different from those seen with the FOS plasmid under basal or stimulated conditions (Fig. 3). Adult rat neurons injected with the somatostatin CRE construct did not express significant levels of CAT under basal conditions but did express CAT at significantly higher levels (approximately 1.6-fold) than FOS under conditions of combined depolarisation and forskolin treatment (Fig. 3).

If the absolute levels of expression of the FOS plasmid were much higher in DRG neurons from adult rats than in neonates, then this might obscure any effect of the VIP CRE and hence lead to the apparently different result. To test this, expression levels of the FOS plasmid were compared as directly as possible using adult and neona-

tal rat DRG cultures prepared, injected and assayed at the same time (Table I). The results of this experiment showed that absolute levels of FOS expression differed by no more than 2-fold between neonatal and adult rat neurons, indicating that the different results obtained with the CRE-containing constructs are indeed due to a change in CRE function.

Our results show that the rat VIP CRE is capable of enhancing the response of the *c-fos* promoter to forskolin and depolarisation in neonatal rat DRG neurons. This is the first demonstration that this particular element can act as a functional enhancer. No such effect was seen in adult rat neurons showing that the ability of the CRE to enhance synergistic responses to cAMP and depolarisation declines as the neurons mature, just as the response of endogenous VIP does. Because FOS itself exhibited a synergistic response to combined depolarisation and forskolin, it is not possible to say from our results whether the effect of the VIP CRE on CAT expression in neonatal rat neurons was due to a response on the part of the CRE itself to cAMP and depolarisation or only an enhancement of the FOS response. The cause of the FOS response is unknown. This construct does not respond to combined depolarisation and forskolin treatment in transfected PC12 cells even though associated CREs do confer responsiveness to these stimuli (S.P. Dobson and J.P. Quinn, unpublished data). Nonetheless, the magnitude of the FOS response is virtually identical in neonatal and adult rat DRG neurons, showing that the differ-

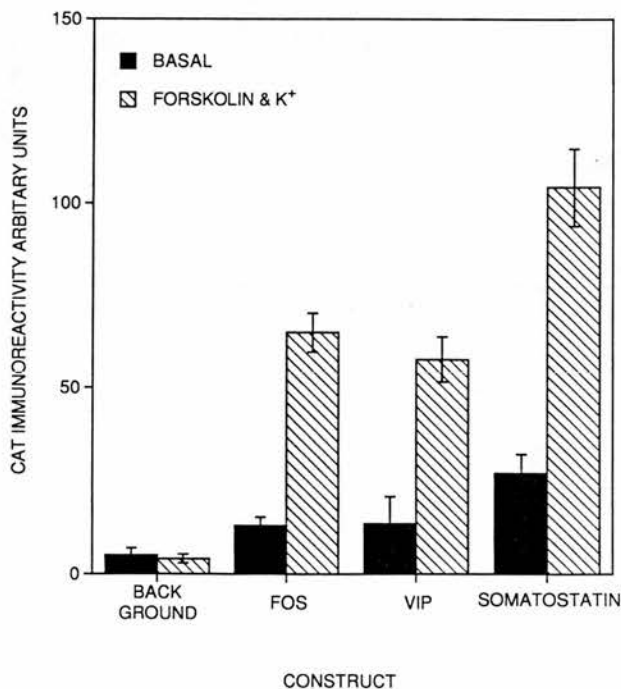


Fig. 3. Effects of 10 μ M forskolin and 40 mM depolarisation on reporter gene expression in adult rat DRG neurons. Each value is mean \pm standard error for at least four separate culture wells. Other details as for Fig. 2.

ences seen with the VIP CRE construct can be attributed to properties of the CRE. Our results therefore support the view that the VIP CRE is likely to participate in regulating the endogenous VIP response to combined depolarisation and forskolin seen in neonatal rat neurons [7].

In both neonatal and adult rat neurons, our results obtained with the somatostatin CRE were slightly but significantly different from those obtained with the VIP CRE, suggesting that different CREs can confer specific patterns of gene expression. The rat VIP CRE identified by Giladi et al. [8] (TGACGTCT) bears a 7/8 homology with the somatostatin consensus CRE sequence (TGACGTCA) [17]. However, it has been shown that mutating the final adenosine nucleotide of the somatostatin CRE into a thymidine, as in the VIP CRE, can affect its enhancer activity [16]. The different results obtained with the two CREs may therefore reflect this slight sequence difference. On the other hand, the human VIP CRE has been shown to contain two adjacent CRE-like sequences, one identical to that in the rat and another upstream of it, that are both required for full enhancer activity [12]. Our rat VIP CRE oligonucleotides were designed to include not only the consensus CRE identified by Giladi et al. [8] but also a second sequence (TGGGATCA) upstream of it that has 5/8 homology with the consensus CRE and whose position corresponds approximately to the second element in the human VIP gene. It is possible that this sequence also contributes to the enhancer function of the CRE.

Although the lack of effect of the VIP CRE on CAT expression in adult rat neurons corresponds to the absence of synergistic response to cAMP and depolarisation by endogenous VIP in these neurons [7], the CRE

did not direct spontaneous expression as is seen for endogenous VIP. The idea that spontaneous VIP expression in adult neurons and induced expression in neonates might both be mediated through the CRE is therefore not supported by the present results. However, it is still possible that the CRE participates in regulating spontaneous VIP expression in adults in association with other regulatory elements. We are currently performing further experiments with regions of 5'-flanking DNA from a rat VIP genomic clone [8] to investigate this.

Another problem with the interpretation of our results is that endogenous VIP expression is seen in only approximately 30% of DRG neurons in culture [7]. Since our microinjection technique is not selective for specific neuronal subpopulations [19] we would expect that the majority of injected neurons in our experiments were not expressing endogenous VIP. Since we have shown a difference in CRE function in DRG neurons at different stages of maturity, it is conceivable that its function also differs in different subpopulations of DRG neurons. Further studies involving localisation of reporter gene and endogenous VIP expression will be needed to determine whether this is so.

The mechanisms responsible for the synergistic response to cAMP and depolarisation and the reasons for its disappearance in mature neurons are unknown. One possibility lies in cAMP-sensitive Ca^{2+} channels whose abundance in rat DRG neurons declines as the neurons mature [20]. However, the uniform response of the FOS plasmid in neonatal and adult rat neurons argues against this possibility and suggests that the different responses mediated by the VIP CRE may reflect differences in the cellular transcription factor proteins that bind to the CRE. What these factors may be remains to be determined, but the human VIP CRE has been shown to bind Fos, Jun and CREB *in vitro* [13]. In this context it is of interest that peripheral axotomy of DRG neurons has been shown to lead to altered expression of Jun and CREB [21–23] and further, that adult rat DRG neurons spontaneously express Jun in culture (see Jenkins et al. [23] and references therein). Taken together, these observations suggest that interactions between these factors and the CRE could play a role in regulating the VIP response to nerve injury *in vivo*.

We are grateful to Dr. Janet Winter for supplying us with NGF and to Norma Brearley for help with preparation of the manuscript. S.P.D. is a Medical Research Council student.

TABLE I

COMPARISON OF ABSOLUTE LEVELS OF CAT IMMUNOREACTIVITY (pg/WELL), BETWEEN ADULT AND NEONATAL RAT NEURONS, FOLLOWING MICROINJECTION WITH FOS PLASMID

Adult and neonatal rats DRG neurons were plated out at similar densities on alternate days and 350 neurons/culture were microinjected with FOS plasmid. Following stimulation with combined 10 μ M forskolin and 40 mM K^+ for identical periods of time, extracts were assayed simultaneously for CAT immunoreactivity. Each value is mean \pm standard error for four separate culture wells.

	Neonatal rat neurons	Adult rat neurons
Uninjected basal conditions	3.83 \pm 1.82	8.40 \pm 0.79
Neurons injected with FOS basal conditions	13.06 \pm 4.14	28.74 \pm 5.26
Neurons injected with FOS 10 μ M forskolin + 40 mM K^+	59.48 \pm 18.20	109.86 \pm 18.52

1 Nielsch, U. and Keen, P., Reciprocal regulation of tachykinin- and vasoactive intestinal peptide-gene expression in rat sensory neurons following cut and crush injury, *Brain Res.*, 481 (1989) 25–30.

- 2 Noguchi, K., Senba, E., Morita, Y., Sato, M. and Tohyama, M., Prepro-VIP and prepro-tachykinin mRNAs in the rat dorsal root ganglion cells following peripheral axotomy, *Mol. Brain Res.*, 6 (1989) 327-330.
- 3 Shehab, S.A.S. and Atkinson, M.E., Vasoactive intestinal polypeptide (VIP) increases in the spinal cord after peripheral axotomy of the sciatic nerve originate from primary afferent neurons, *Brain Res.*, 372 (1986) 37-44.
- 4 Villar, M.J., Cortés, R., Theodorsson, E., Wiesenfeld-Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P.C. and Hökfelt, T., Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin, *Neuroscience*, 33 (1989) 587-604.
- 5 Wiesenfeld-Hallin, Z., Xu, X.-J., Häkanson, R., Feng, D.-M. and Folkers, K., Plasticity of the peptidergic mediation of spinal reflex facilitation after peripheral nerve section in the rat, *Neurosci. Lett.*, 116 (1990) 293-298.
- 6 Mulderry, P.K. and Lindsay, R.M., Rat dorsal root ganglion neurons in culture express vasoactive intestinal polypeptide (VIP) independently of nerve growth factor, *Neurosci. Lett.*, 108 (1990) 314-320.
- 7 Mulderry, P.K., Synergistic regulation of VIP expression by cyclic AMP and calcium in newborn but not adult rat sensory neurons in culture, *Neuroscience*, 53 (1993) 229-238.
- 8 Giladi, E., Shani, Y. and Gozes, I., The complete structure of the rat VIP gene, *Mol. Brain Res.*, 7 (1990) 261-267.
- 9 Lamperti, E.D., Rosen, K.M. and Villa-Komaroff, L., Characterization of the gene and messages for vasoactive intestinal polypeptide (VIP) in rat and mouse, *Mol. Brain Res.*, 9 (1991) 217-231.
- 10 Van Nguyen, T., Kobierski, L., Comb, M. and Hyman, S.E., The effect of depolarization on expression of the human proenkephalin gene is synergistic with cAMP and dependent upon a cAMP-inducible enhancer, *J. Neurosci.*, 10 (1990) 2825-2833.
- 11 Vallejo, M., Miller, C.P. and Habener, J.F., Somatostatin gene transcription regulated by a bipartite pancreatic islet D-cell-specific enhancer coupled synergistically to a cAMP response element, *J. Biol. Chem.*, 267 (1992) 12868-12875.
- 12 Fink, J.S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G. and Goodman, R.H., The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 6662-6666.
- 13 Fink, J.S., Verhave, M., Walton, K., Mandel, G. and Goodman, R.H., Cyclic AMP- and phorbol ester-induced transcriptional activation are mediated by the same enhancer element in the human vasoactive intestinal peptide gene, *J. Biol. Chem.*, 266 (1991) 3882-3887.
- 14 Tsukada, T., Fink, J.S., Mandel, G. and Goodman, R.H., Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP, *J. Biol. Chem.*, 262 (1987) 8743-8747.
- 15 Gilman, M.Z., Wilson, R.N. and Weinberg, R.A., Multiple protein-binding sites in the 5'-flanking region regulate *c-fos* expression, *Mol. Cell. Biol.*, 6 (1986) 4305-4316.
- 16 Deutsch, P.J., Hoeffler, J.P., Jameson, L., Lin, J.C. and Habener, J.F., Structural determinants for transcriptional activation by cAMP-responsive DNA elements, *J. Biol. Chem.*, 263 (1988) 18466-18472.
- 17 Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H., Identification of a cyclic-AMP-responsive element within the rat somatostatin gene, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 6682-6686.
- 18 Lindsay, R.M., Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons, *J. Neurosci.*, 8 (1988) 2394-2405.
- 19 Mulderry, P.K., Chapman, K.E., Lyons, V. and Harmar, A.J., 5'-Flanking sequences from the rat preprotachykinin gene direct high-level expression of a reporter gene in adult rat sensory neurons transfected in culture by microinjection, *Mol. Cell. Neurosci.*, 4 (1993) 164-172.
- 20 Fedulova, S.A., Kostyuk, P.G. and Veselovsky, N.S., Ionic mechanisms of electrical excitability in rat sensory neurons during postnatal ontogenesis, *Neuroscience*, 41 (1991) 303-309.
- 21 Herdegen, T., Fiallos-Estrada, C.E., Schmid, W., Bravo, R. and Zimmermann, M., The transcription factors c-JUN, JUN D and CREB, but not FOS and KROX-24, are differentially regulated in axotomized neurons following transection of rat sciatic nerve, *Mol. Brain Res.*, 14 (1992) 155-165.
- 22 Jenkins, R. and Hunt, S.P., Long-term increase in the levels of *c-jun* mRNA and Jun protein-like immunoreactivity in motor and sensory neurons following axon damage, *Neurosci. Lett.*, 129 (1991) 107-110.
- 23 Jenkins, R., McMahon, S.B., Bond, A.B. and Hunt, S.P., Expression of *c-jun* as a response to dorsal root and peripheral nerve section in damaged and adjacent intact primary sensory neurons in the rat, *Eur. J. Neurosci.*, 5 (1993) 751-759.