

Identification & analysis of a novel cysteine rich protein

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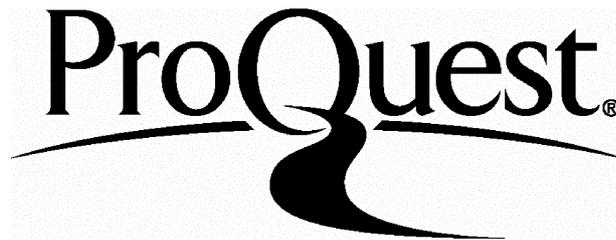
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## ABSTRACT

During neurulation the hindbrain forms a series of transient segments termed rhombomeres (r) that underlie subsequent head development, but gaps remain in understanding of this process. This thesis describes a whole mount in situ hybridisation screen of a subtracted chick cDNA hindbrain library to identify further genes with potential roles during hindbrain development. The expression patterns of 445 randomly chosen clones have been analysed and 39 (9 %) of the cDNA clones were found to exhibit restricted expression within developing hindbrain tissue and/or adjacent tissue. Of these, 3 clones were identified that have previously characterised roles during hindbrain development. Nine clones identified in this screen encode molecules that are identical to or closely related to proteins with previously unknown roles in early embryonic neural development and 27 encode for proteins of unknown functions.

One of the clones, 0B7/cE13, was identified during the in situ screen due to its expression pattern in the dorsal neural tube during the period of neural crest emergence. Analysis of the predicted peptide sequence suggests that cE13 is a secreted protein, that has a number of cysteine-rich repeat motifs found in bone morphogenetic protein (BMP) binding proteins. Detailed analysis of the expression pattern of E13 in both the chick and mouse shows that it is co-expressed or expressed in adjacent tissues compared with some members of the BMP signalling family in a number of embryonic tissues. Biochemical experiments demonstrate that chick cE13 binds BMP-4 and functional analysis in *Xenopus* embryos shows that cE13 promotes the induction of *Xhox3* expression by BMP-4. These findings implicate cE13 with a BMP modulating activity. Since BMPs have been implicated in neural crest cell induction, initiation of migration, and apoptosis, this suggests a role for E13 in regulating aspects of neural crest development by BMPs.

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# CONTENTS

<b>ABSTRACT.....</b>	<b>2</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>3</b>
<b>CONTENTS.....</b>	<b>4</b>
<b>LIST OF FIGURES .....</b>	<b>11</b>
<b>LIST OF TABLES .....</b>	<b>13</b>
<b>ABBREVIATIONS .....</b>	<b>14</b>

## CHAPTER ONE

### INTRODUCTION

<b>1.1</b>	<b>BACKGROUND .....</b>	<b>17</b>
<b>1.2</b>	<b>NEURAL INDUCTION.....</b>	<b>18</b>
1.2.1	Role of BMP in neural induction .....	18
1.2.2	Modulation of BMP signalling .....	21
1.2.3	Regulation of the BMP-signalling pathway .....	22
1.2.4	The BMP signalling pathway is an evolutionarily conserved mechanism .....	24
<b>1.3</b>	<b>PATTERNING THE CENTRAL NERVOUS SYSTEM .....</b>	<b>29</b>
1.3.1	Segmentation of the hindbrain .....	29
1.3.2	Establishment of axial identity in the hindbrain .....	30
1.3.3	Transcription factors .....	31
1.3.4	Restriction of cell movements .....	32
1.3.5	Other molecules exhibiting restricted expression patterns in the developing hindbrain.....	33
<b>1.4</b>	<b>NEURAL CREST .....</b>	<b>34</b>
1.4.1	Neural crest induction .....	34
	(i) Multiple roles for BMPs during neural crest induction .....	35
	(ii) Slug is a neural crest inducing molecule.....	36

1.4.2	Initiation of neural crest migration.....	36
1.4.3	Migratory pathways of neural crest cells.....	38
1.4.4	Segmented migration of neural crest cells from the hindbrain.....	39
1.4.5	Segmental migration of trunk neural crest cells .....	42
<b>1.5</b>	<b>AIMS OF THE PROJECT .....</b>	<b>43</b>

## CHAPTER TWO

### MATERIALS & METHODS

<b>2.1</b>	<b>BACTERIAL STRAINS.....</b>	<b>44</b>
<b>2.2</b>	<b>MAINTENANCE &amp; MANIPULATION OF BACTERIAL STRAINS..</b> .....	<b>44</b>
2.2.1	Bacterial growth media and antibiotics .....	44
2.2.2	Growth of bacterial cultures .....	45
2.2.3	Maintenance and storage of bacterial cultures.....	45
2.2.4	Heat-shock transformation of competent cells .....	45
<b>2.3</b>	<b>ISOLATION, MANIPULATION &amp; MODIFICATION OF DNA.....</b>	<b>46</b>
2.3.1	Isolation of plasmid DNA.....	46
2.3.2	Excision of phagemid DNA from Lambda ZAP II.....	46
2.3.3	Restriction enzyme digestion of DNA .....	46
2.3.4	Phenol/chloroform extraction .....	46
2.3.5	Ethanol precipitation of DNA.....	46
2.3.6	Dephosphorylation of DNA.....	47
2.3.7	Ligation of DNA fragments.....	47
<b>2.4</b>	<b>ANALYSIS OF DNA .....</b>	<b>48</b>
2.4.1	Agarose gel electrophoresis.....	48
2.4.2	Determination of nucleic acid concentration.....	48
2.4.3	DNA sequencing & analysis.....	48
<b>2.5</b>	<b>SYNTHESIS OF mRNA .....</b>	<b>48</b>
<b>2.6</b>	<b>COLONY SCREENING USING RADIOACTIVE LABELED</b> <b>PROBES.....</b>	<b>49</b>
2.6.1	Colony blot .....	49
2.6.2	Hybridisation .....	49
<b>2.7</b>	<b>DETECTION OF TRANSCRIPTS &amp; EMBRYO MANIPULATION</b>	<b>50</b>
2.7.1	Generation of DIG labeled probes; <i>in vitro</i> transcription.....	50
2.7.2	Whole mount in situ hybridisation.....	50
2.7.3	Flat-mounting and photography.....	50
<b>2.8</b>	<b>IN OVO ELECTROPORATION .....</b>	<b>51</b>

<b>2.9</b>	<b>PRODUCTION &amp; DETECTION OF RECOMBINANT PROTEIN .51</b>	
2.9.1	Construction of pMT/BIP/cE13/V5-HisA expression vector.....	51
2.9.2	Generation & purification of recombinant protein .....	52
<b>2.10</b>	<b>PROTEIN ANALYSIS .....</b>	<b>53</b>
2.10.1	Electrophoresis of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).....	53
2.10.2	Western blotting of proteins .....	53
<b>2.11</b>	<b>CO-IMMUNOPRECIPITATION ANALYSIS.....</b>	<b>54</b>
<b>2.12</b>	<b>XENOPUS EMBRYO CULTURE &amp; MANIPULATIONS .....</b>	<b>55</b>
2.12.1	Isolation & injection of <i>Xenopus</i> embryos .....	55
2.12.2	Isolation of <i>Xenopus</i> ectodermal explants .....	55
2.12.3	RNA isolation and RT-PCR of animal caps .....	56
<b>2.13</b>	<b>CLONING OF MURINE E13 BY RT-PCR .....</b>	<b>57</b>
<b>2.14</b>	<b>ANIMALS .....</b>	<b>57</b>

## CHAPTER THREE

### IDENTIFICATION OF GENES EXPRESSED DURING HINDBRAIN, MIDBRAIN & CRANIAL NEURAL CREST DEVELOPMENT

<b>3.1</b>	<b>INTRODUCTION.....</b>	<b>58</b>
3.1.1	Rationale of screen .....	60
<b>3.2</b>	<b>COMPLEXITY OF THE SUBTRACTED cDNA LIBRARY .....</b>	<b>61</b>
<b>3.3</b>	<b>IDENTIFICATION OF CLONES WITH RESTRICTED EXPRESSION PATTERNS IN DEVELOPING CRANIAL NEURAL TISSUE .....</b>	<b>62</b>
<b>3.4</b>	<b>SEQUENCE ANALYSIS OF CLONES EXHIBITING RESTRICTED DOMAINS OF EXPRESSION .....</b>	<b>63</b>
3.4.1	Genes with previously characterised roles in neural development....	63
3.4.2	Identification of known molecules with previously uncharacterised expression in cranial neural tissues .....	70
	(i) Segmentally expressed enzymes.....	70
	(ii) Components of intracellular signal transduction cascades.....	72
	(iii) Transmembrane proteins.....	73
	(iv) Cytoskeleton associated proteins .....	73
	(v) Specific antagonists of other proteins.....	73
3.4.3	Clones encoding for proteins of unknown function.....	74
<b>3.5</b>	<b>DISCUSSION.....</b>	<b>76</b>

3.5.1	Use of an in situ hybridisation screen to identify transcripts with spatially restricted expression patterns within developing cranial neural tissue .....	76
3.5.2	Identification and sequence analysis of genes exhibiting restricted domains of expression within embryonic cranial neural tissue .....	77
3.5.3	Enzymes exhibiting segmental expression patterns in the developing chick hindbrain.....	78
3.5.4	Central cannabinoid receptor-1 maybe expressed in early reticular neurones.....	79
3.5.5	Co-expression of apoptotic regulatory genes in rhombomere 5 .....	79
3.5.6	Genes expressed in neural crest migrating from rhombomere 4 .....	84

## CHAPTER FOUR

### ISOLATION & SEQUENCE ANALYSIS OF E13

<b>4.1</b>	<b>INTRODUCTION.....</b>	<b>87</b>
<b>4.2</b>	<b>MOLECULAR CHARACTERISATION OF CHICK E13 .....</b>	<b>88</b>
4.2.1	Isolation of chick E13.....	88
4.2.2	Structure of the chick E13 cDNA .....	88
4.2.3	Predicted structure of the chick E13 protein.....	91
<b>4.3</b>	<b>CHICK E13 IS SIMILAR TO <i>DROSOPHILA</i> CROSSVEINLESS 2.....</b>	<b>96</b>
<b>4.4</b>	<b>IDENTIFICATION AND SEQUENCE ANALYSIS OF MURINE E13 .....</b>	<b>99</b>
<b>4.5</b>	<b>IDENTIFICATION AND SEQUENCE ANALYSIS OF ZEBRAFISH E13 .....</b>	<b>100</b>
<b>4.6</b>	<b>DISCUSSION.....</b>	<b>105</b>
4.6.1	Chick E13 is an extracellular protein comprising a number of cysteine-rich repeat motifs.....	105
4.6.2	Chick E13 is the homologue of <i>Drosophila</i> Crossveinless-2 (Cv-2).....	106
4.6.3	Identification of vertebrate E13 homologues .....	107

## CHAPTER FIVE

### EXPRESSION OF E13 DURING DEVELOPMENT

<b>5.1</b>	<b>INTRODUCTION.....</b>	<b>108</b>
<b>5.2</b>	<b>SPATIAL ANALYSIS OF E13 EXPRESSION DURING CHICK DEVELOPMENT.....</b>	<b>109</b>
5.2.1	Dorsal neural tube .....	109
(i)	Expression pattern of cE13 in the dorsal neural tube .....	109
(ii)	Expression profile of known neural crest cell markers.....	113
(iii)	Comparative expression profile of cE13 with known neural crest markers .....	117
5.2.2	Other sites of chick E13 expression .....	118
(i)	Surrounding regressing primitive streak .....	118
(ii)	Developing inner ear.....	118
(iii)	Developing heart.....	118
(iv)	Sites of sympathetic ganglia differentiation .....	119
(v)	Developing head.....	119
(vi)	Nephrogenous mesenchyme .....	120
(vii)	Intersomitic region .....	120
(viii)	Developing limb.....	121
(ix)	Developing eye .....	121
<b>5.3</b>	<b>SPATIAL ANALYSIS OF E13 DURING MURINE EMBRYOGENESIS .....</b>	<b>125</b>
5.3.1	Isolation of a murine E13 cDNA .....	125
5.3.2	Murine E13 expression profile.....	125
<b>5.4</b>	<b>DISCUSSION.....</b>	<b>129</b>

## CHAPTER SIX

### FUNCTIONAL ANALYSES OF E13

<b>6.1</b>	<b>INTRODUCTION.....</b>	<b>132</b>
<b>6.2</b>	<b>GENERATION OF CELLS EXPRESSING RECOMBINANT CHICK E13 PROTEIN.....</b>	<b>133</b>
<b>6.3</b>	<b><i>IN VITRO</i> BINDING OF cE13 &amp; BMP-4 PROTEIN.....</b>	<b>136</b>
<b>6.4</b>	<b>OVEREXPRESSION OF cE13 IN <i>XENOPUS</i> EMBRYOS.....</b>	<b>138</b>
<b>6.5</b>	<b><i>XENOPUS</i> ANIMAL CAP ASSAYS.....</b>	<b>142</b>
6.5.1	Injection of cE13 mRNA.....	144

6.5.2	Co-injection of cE13 and <i>BMP-4</i> mRNA .....	145
<b>6.6</b>	<b>OVEREXPRESSION OF CHICK E13 IN THE DORSAL NEURAL TUBE.....</b>	<b>148</b>
<b>6.7</b>	<b>DISCUSSION .....</b>	<b>152</b>
6.7.1	Chick E13 protein interacts with human BMP-4 protein.....	152
6.7.2	Whole <i>Xenopus</i> embryo injections suggest that chick E13 has a similar activity to BMP signalling antagonists.....	153
6.7.3	The antagonistic function of Chick E13 on BMP signalling is indirect .....	154
6.7.4	Chick E13 mRNA can promote the expression of downstream BMP targets.....	154
6.7.5	Function of Chick E13 in the dorsal neural tube.....	155

## CHAPTER SEVEN

### GENERAL DISCUSSION

<b>7.1</b>	<b>PROPOSED MODEL OF E13 FUNCTION .....</b>	<b>158</b>
7.1.1	Expression during neural induction .....	159
7.1.2	Chick E13 is expressed in the dorsal neural tube during the initiation of neural crest cell migration .....	160
	(i) Possible relationship of cE13 with BMP-4.....	161
	(ii) Murine E13 is not expressed at sites of pre-migratory neural crest.....	162
	(iii) Molecular cues that govern neural crest migration differ between chick & mouse .....	167
7.1.3	Expression in the outflow tract of the developing heart .....	167
7.1.4	E13 expression coincides with BMP family members that are required for inner ear development .....	168
7.1.5	Chick E13 is expressed adjacent to the BMP expressing dorsal aorta at sites of sympathetic ganglia formation .....	169
7.1.6	Potential role of E13 in the development of the chick respiratory system .....	170
7.1.7	E13 is expressed in a number of domains during chick and mouse cephalic development.....	170
7.1.8	Chick E13 is co-expressed with <i>BMP-4</i> in nephrogenic mesenchyme .....	171
7.1.9	Expression of E13 is restricted to the intersomitic region .....	172
7.1.10	Chick E13 is expressed in the lens and retina of the developing eye .....	173
7.1.11	E13 is expressed in developing limbs.....	173
<b>7.2</b>	<b>EXPLANATION OF APPARENT ANTAGONISTIC EFFECT OF cE13.....</b>	<b>175</b>
<b>7.3</b>	<b>FINAL PERSPECTIVES.....</b>	<b>176</b>

7.3.1	Identification of further genes expressed in the developing chick hindbrain.....	176
7.3.2	Further investigation into the function of E13.....	177
	(i) Does E13 associate with the extracellular matrix and localise BMP molecules? .....	177
	(ii) Is E13 protein cleaved <i>in vivo</i> ?.....	178
	(iii) Functions of cE13 <i>in vivo</i> .....	179
	(iv) Does E13 affect the specification of dorsal neuronal cell types in the neural tube? .....	180
<b>APPENDIX A .....</b>		<b>181</b>
<b>APPENDIX B .....</b>		<b>184</b>
<b>APPENDIX C .....</b>		<b>186</b>
<b>REFERENCES.....</b>		<b>191</b>

## LIST OF FIGURES

Figure 1.1: The neural default model in <i>Xenopus</i> .....	19
Figure 1.2: Neural tube axial identity and neural crest migration in the chick embryo .....	25
Figure 1.3: Summary of the correlation between gene expression and rhombomere boundary .....	27
Figure 3.1: Patterns and sequence similarity of screened clones exhibiting restricted expression domains within cranial neural tissue of the developing chick embryo .....	64
Figure 3.2: Functional classification of clones identified during the screen with expression restricted to cranial neural tissues .....	75
Figure 3.3: Anti-apoptotic role of ch-IAP1 in the TNF $\alpha$ signalling pathway .....	81
Figure 3.4: Inhibition of protein translation and cell death by eIF2 $\alpha$ kinase .....	82
Figure 3.5: Proposed relationship between EIF2 $\alpha$ kinase and ch-IAP1 in r5 .....	83
Figure 4.1: Nucleotide sequence and predicted amino acid sequence of chick E13 .	89
Figure 4.2: Amino acid alignments of cE13 with VWFC & VWFD domains.....	92
Figure 4.3: Amino acid alignment of the five CR coded by cE13 with the four CR motifs coded by <i>Xenopus</i> Chordin .....	94
Figure 4.4: Schematic comparison of CR motif containing proteins.....	95
Figure 4.5: Alignment of <i>Drosophila</i> Cv-2 with chick E13 protein.....	97
Figure 4.6: Partial amino acid sequence alignment of chick, mouse and zebrafish E13 .....	101
Figure 4.7: Multiple amino acid sequence alignment of chick E13, mouse E13, zebrafish E13 and <i>Drosophila</i> Cv-2 .....	103
Figure 5.1: Expression pattern of cE13 during cranial neural crest maturation .....	110
Figure 5.2: Comparative expression profile of cE13 with known neural crest markers .....	114
Figure 5.3: Expression profile of chick E13.....	123
Figure 5.4: Murine E13 expression profile.....	127

Figure 6.1: Construction and expression of recombinant chick E13 protein .....	134
Figure 6.2: Direct interaction of cE13 with BMP-4.....	137
Figure 6.3: Chick E13 mRNA dorsalises <i>Xenopus</i> embryos when injected ventrally .....	140
Figure 6.4: Chick E13 mRNA does not induce neural markers in <i>Xenopus</i> ectoderm explants .....	143
Figure 6.5: Chick E13 mRNA increases the expression of a downstream BMP target .....	146
Figure 6.6: Overexpression of cE13 in the hindbrain region of the chick neural tube .....	150
Figure 7.1: Cartoon of chick E13, <i>BMP-2</i> , <i>BMP-4</i> & <i>BMP-7</i> transcript distribution during a number of developmental stages .....	163
Figure 7.2: Cartoon of murine E13, <i>BMP-4</i> & <i>BMP-7</i> transcript distribution during a number of developmental stages .....	165

## LIST OF TABLES

Table 3.1: Summary of the frequency of the 9 randomly picked clones used to screen 1.2x10 <sup>3</sup> clones from the subtracted DNA library by southern hybridisation.....	62
Table 5.1: Summary of cE13, <i>BMP-2</i> , <i>BMP-4</i> & <i>BMP-7</i> transcript distribution during chick development from HH stage 8 to 29 .....	130
Table 5.2: Summary of mE13, <i>BMP-2</i> , <i>BMP-4</i> & <i>BMP-7</i> transcript distribution during murine development from 7.25 dpc to 10.75 dpc .....	131

## ABBREVIATIONS

ADP	Adenosine diphosphate
AER	Apical Ectodermal Ridge
A-P	Antero-Posterior
ATP	Adenosine triphosphate
BA	Branchial Arch
BAMBI	BMP and Activin Membrane-Bound Inhibitor
B-CK	Brain-type Creatine Kinase
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenetic Protein
bp	base pairs
BSA	Bovine serum albumin
CB-1	cannabinoid receptor-1
cDNA	complementary DNA
cE13	chick E13
ch-IAP1	chick cellular inhibitor of apoptosis-1
CHAPS	3-[(3-cholamidopropyl)-dimethylammonin]-1-propane-sulfonate
CHL	Chordin like
CNS	Central Nervous System
CR	Cysteine-rich Repeat
CRABPs	Cellular Retinoic Acid Binding Proteins
CTP	Cytidine triphosphate
<i>Cv-2</i>	Crossveinless 2
Da	Dalton
DDW	Double Distilled Water
DEPC	Diethyl-pyrocabonate
DES	Drosophila Expression System
DIG	Digoxigenin
DiI	1,1'-diocatadecyl-3,3',3'-tetramethylindocarbonocyanine perchlorate
DNA	Deoxyribonucleic acid
dpc	days postcoitum
Dpp	Decapentaplegic
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic
EF-1 $\alpha$	Elongation Factor-1 alpha
EGTA	Ethyleneglycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
eIF-2 $\alpha$	eukaryotic initiation factor subunit-2 alpha
En-2	Engrailed 2

EST	Expressed sequence tag
FGF	Fibroblast Growth Factor
g	grams
GDP	guanosine diphosphate
GTP	Guanosine triphosphate
Hepes	N(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid
HH	Hamburger and Hamilton
HRI	Haem-regulated inhibitor
HRP	Horseradish peroxidase
I- $\kappa$ B	Inhibitor of $\kappa$ B
IAA	Isoamyl alcohol
IPTG	Isopropyl $\beta$ -D-thiogalactopyranoside
kb	kilobase
$K_d$	Equilibrium constant for the dissociation reaction
kDa	kiloDalton
LB	Luria-Bertani
M	Molar
MAD	Mothers Against Decapentaplegic
MAPK	Mitogen-activated protein kinase
mE13	murine E13
Met-tRNAi	initiator methionine-transfer RNA
ml	millilitre
mM	millimolar
mRNA	messenger RNA
NAM	Normal Amphibian Medium
NaOAc	Sodium acetate
NaPi	Sodium phosphate
NCAM	Neural Cell Adhesion Molecule
NCBI	National Center for Biotechnology Information
nm	nanometer
NP40	Nonidet P-40 (detergent)
nt	nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PBTx	Phosphate Buffered Saline/0.1% Triton-X-100
PBTw	Phosphate Buffered Saline/0.1% Tween20
PEK	Pancreatic eIF-2 $\alpha$ kinase
Pfam	Protein family searches
pg	picogram
PNA	Peanut agglutinin

PNS	Peripheral Nervous System
poly(A)	polyadenylic acid or polyadenylate
r	rhomomere
RA	all-trans Retinoic Acid
RAI	ribonuclease angiogenin inhibitor
RARs	Retinoic Acid Response elements
RI	ribonuclease inhibitor
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	revolutions per minute
RT-PCR	Reverse transcriptase PCR
S2	Schneider 2 insect cells
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sin1	Sty1 interacting
Sog	Short gastrulation
SPDSY	Spermidine Synthase
TBE	Tris-borate-EDTA buffer
TEMED	1 NNN'N'-tetramethylethylene-diamine
TGF- $\beta$	Transforming Growth Factor Beta
TNF $\alpha$	Tumour Necrosis Factor alpha
Tris	2-amino-2-(hydroxymethyl)-propane-1:3-diol
Tsg	Twisted gastrulation
$\mu$ g	microgram
$\mu$ l	microlitre
$\mu$ M	micromolar
ULIP	unc-33 Like Phosphoproteins
UTP	Uridine triphosphate
UTR	untranslated region
v/v	volume/volume
VWF	von Willebrand Factor
VWFD	von Willebrand Factor type D
w/v	weight/volume
<i>Xhox3</i>	<i>Xenopus</i> homeobox 3
$^{\circ}$ C	Degree Celsius

# CHAPTER ONE

## INTRODUCTION

### 1.1 BACKGROUND

The fusion of egg and sperm signals the onset of a remarkable chain of events that ultimately result in the formation of a mature organism. The research field of developmental biology is focused on understanding the events that occur in the formation of a multicellular organism from just one cell.

The formation of the nervous system has its origins in the initial steps of embryonic life. During gastrulation, dramatic cell movements occur that transform the blastula stage embryo from an apparently uniform mass of cells into the definitive body plan of the embryo, establishing three germ layers; the ectoderm, mesoderm and endoderm. During this period, interactions between the ectoderm and the underlying mesoderm are critical for many aspects of organogenesis including the development of the central nervous system (CNS). The precursor of the CNS, the neural tube, forms as a direct consequence of the events that occur during gastrulation. In vertebrates, a substantial part of the early developmental events that occur in an embryo are similar in even distantly related species.

Described in this chapter is an overview of the development of the vertebrate nervous system and the underlying molecular mechanisms that are required for its formation and elaboration.

## 1.2 NEURAL INDUCTION

During vertebrate development in the gastrulating embryo a specialised cluster of cells are formed at the dorsal marginal zone in the dorsal mesoderm layer that secrete many signalling molecules. In amphibia, this region is termed the Spemann's organiser (Grimlich and Cooke, 1983), and functional homologues have been described in other vertebrate species such as zebrafish (the embryonic shield: Shih and Fraser, 1996), chick (Hensen's node: Waddington and Schmidt, 1933) and mouse (the node: Beddington, 1994). The process of neural induction and the role of the organiser is best understood in the amphibian embryo, in particular the African claw-toed frog *Xenopus leavis* (reviews see: Harland and Gerhart, 1997; Smith and Schoenwolf, 1998; Weinstein and Hemmati-Brivanlou, 1999).

During *Xenopus* gastrulation, signals from the organiser are propagated vertically to the neural ectoderm apposed to it (vertical signalling) and horizontally within in the plane of the ectoderm (planar signalling: Figure 1.1; A). Signals from the organiser induce neural tissue to form the overlying neural ectoderm (presumptive neural plate) and regionalise it along the anterior-posterior axis (reviews see: Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). Studies in the past ten years have successfully identified molecules with the ability to induce neural tissue and these are reviewed below.

### 1.2.1 Role of BMP in neural induction

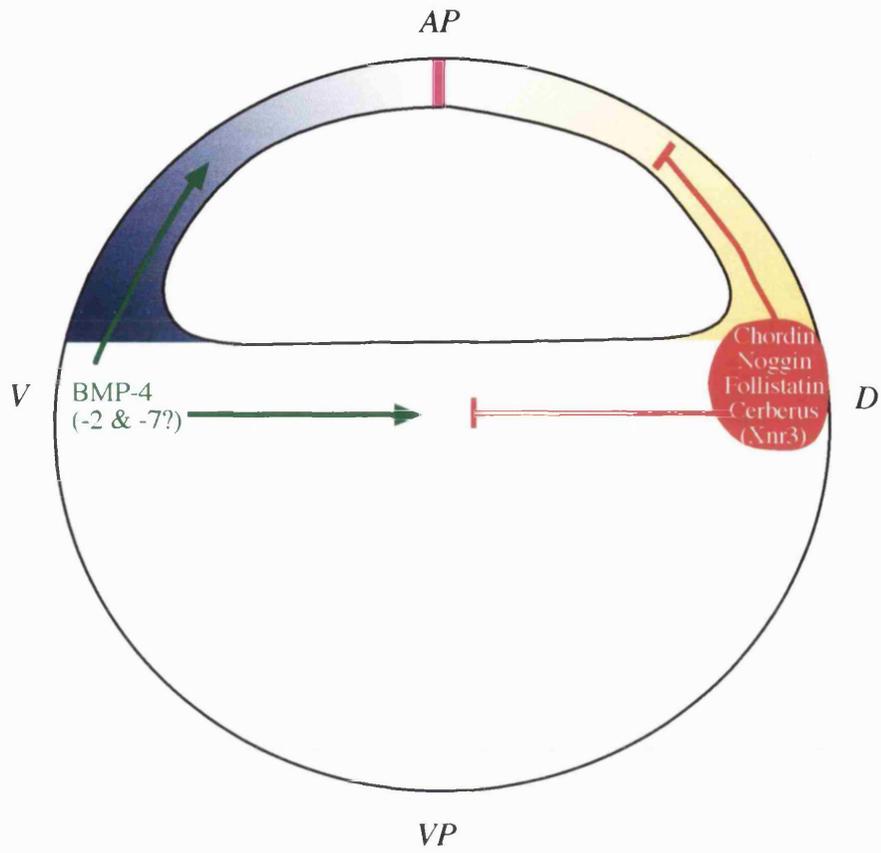
The Bone Morphogenetic Protein (BMP) protein family is a member of the Transforming Growth Factor-Beta (TGF- $\beta$ ) superfamily (Massague, 1990). In the developing *Xenopus* embryo *BMP-2*, *BMP-4* and *BMP-7* are widely expressed in the ectoderm during gastrulation and later become downregulated in the presumptive neural plate/dorsal regions, but are maintained in the future epidermal/ ventral areas as the organiser forms (Fainsod, *et al.*, 1994; Dale, *et al.*, 1992; Hemmati-Brivanlou and Thomsen, 1995). The expression patterns of these BMP molecules within non-neural territories suggests that they might function in the specification of ventral mesoderm and ventral ectoderm (epidermis). Experiments to address these issues demonstrate that ventral mesoderm is induced in animal caps cut from embryos injected with *BMP-4* mRNA (Dale, *et al.*, 1992; Jones, *et al.*, 1992). Moreover,



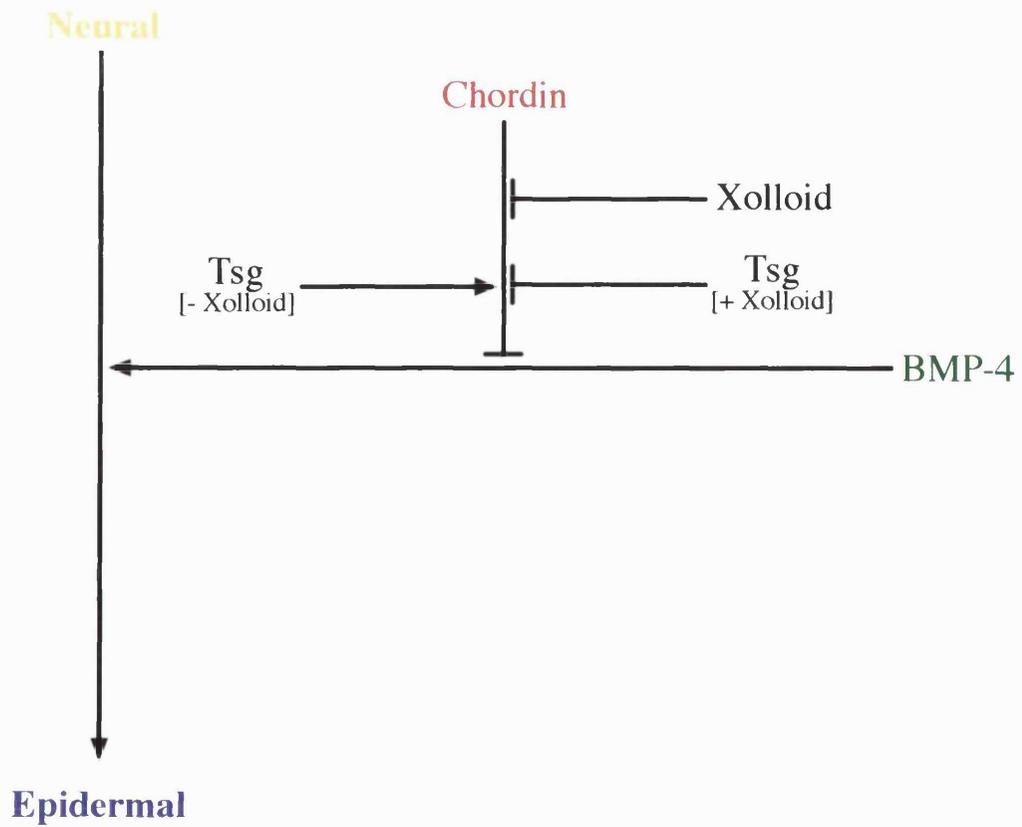
Figure 1.1: The neural default model in *Xenopus*

A: Pictorial representation of a late blastula/early gastrula stage *Xenopus* embryo and the spatial relationship of the signals that govern neural induction. The organiser region is shown in red, prospective neural tissue in yellow, epidermis in blue and the border region between the neural and epidermal regions in pink (position of future cement gland). Abbreviations: AP, animal pole; D, dorsal; V, ventral; VP, vegetal pole. B: Relationship between BMP-4, Chordin and associated extracellular molecules. (Adapted from; Streit and Stern, 1999).

**A**



**B**



inhibition of the BMP-4 signalling pathway by overexpressing a truncated *BMP-4* receptor (Hemmati-Brivanlou and Melton, 1994), or antisense *BMP-4* mRNA (Sasai, *et al.*, 1995) in *Xenopus* animal caps converts ventral mesoderm into dorsal mesoderm. Experiments show that BMPs are also responsible for inducing epidermis or non-neural ectoderm. Un-injected *Xenopus* ectodermal tissue will form epidermis due to endogenous BMPs, but dissociation and re-aggregation of ectodermal tissue that dilutes (removing) the soluble BMP signal, result in the formation of neural tissues (Wilson and Hemmati-Brivanlou, 1995). Indeed, if BMP-4 protein is added and incubated with the dissociated animal caps, neural tissue formation is inhibited and the formation of epidermis is re-established (Wilson and Hemmati-Brivanlou, 1995). Similar results are obtained when *BMP-2* or *BMP-7* are overexpressed in ectodermal explants (Suzuki, *et al.*, 1997a) and in ectoderm dissociation experiments (Suzuki, *et al.*, 1997b). These data suggest that BMP signalling is important in specifying both ventral mesoderm and epidermis (ventral ectoderm) (review see: Dale and Jones, 1999). These findings are supported by the identification and characterisation of several molecules that are secreted by the organiser that can modulate BMP activity.

### 1.2.2 Modulation of BMP signalling

Genes encoding Noggin (Smith and Harland, 1992), Chordin (Sasai, *et al.*, 1994), Follistatin (Hemmati-Brivanlou, *et al.*, 1994), Cerberus (Bouwmeester, *et al.*, 1996) and Xnr3 (Hansen, *et al.*, 1997) proteins are all expressed in the *Xenopus* organiser when neural induction is thought to occur. Ectopic expression of any of these secreted factors by mRNA or DNA injections into *Xenopus* ectoderm explants result in the induction of neural tissue (Sasai, *et al.*, 1995; Lamb, *et al.*, 1993; Hemmati-Brivanlou, *et al.*, 1994; Bouwmeester, *et al.*, 1996; Hansen, *et al.*, 1997). Noggin, Chordin, Follistatin and Cerberus have all been shown to directly bind to BMP molecules thereby blocking binding to their receptor and hence attenuating BMP signalling (Zimmerman, *et al.*, 1996; Piccolo, *et al.*, 1996; Fainsod, *et al.*, 1997; Piccolo, *et al.*, 1999). This has been demonstrated by determining the affinity of ligand to receptor and ligand to antagonist: for example, the  $K_d$  values for BMP-4 with Noggin ( $K_d = 20$  pM) and BMP-4 with Chordin ( $K_d = 300$  pM) are formed with a higher affinity than compared to that of BMP-4 and its receptor ( $K_d =$

approximately 900 pM) (Zimmerman, *et al.*, 1996; Piccolo, *et al.*, 1996). The mechanism of Follistatin's action is not known but has been suggested to form an inactive trimeric complex with a BMP-4 molecule and a BMP receptor, suggesting that Follistatin's antagonistic action on BMP signalling differs to that of Chordin and Noggin (Iemura, *et al.*, 1998). The mode of inhibition of Xnr3 on BMPs is not known, but it may compete with BMP molecules by binding to the BMP receptor (Hansen, *et al.*, 1997). From these data the 'default model' of neural induction has been widely accepted (Figure 1.1; A); ectodermal cells are predisposed to undergo neural differentiation forming the neural plate and that BMP signalling inhibits this tendency (review see: Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999).

Recent work suggests that BMPs and BMP signalling modulators are not required for the initiation of neural induction. Fibroblast growth factor (FGF) signalling is required for the induction of posterior neural tissues and, unlike the BMP signalling cascade, is not dependent on the formation of the organiser (Alvarez, *et al.*, 1998; Storey, *et al.*, 1998). Recently, FGF signalling properties have been identified in a population of cells that ultimately form part of the organiser, this signalling occurs prior to, and is required for organiser formation (Streit, *et al.*, 2000). These data suggest that the early events of neural induction occurs prior to gastrulation, and that FGF signalling acts upstream of the later BMP signalling cascade.

### 1.2.3 Regulation of the BMP-signalling pathway

The balance between BMPs and their antagonists regulates the formation of epidermal and neural cell fates. This balance is regulated by several mechanisms that act at the transcriptional, translational and post-translational levels.

Examples of BMP regulation at the transcriptional level are *Xenopus* Brain factor 2 (XBF-2: Mariani and Harland, 1998) and Gremlin (Kroll, *et al.*, 1998). Overexpression of XBF-2 in ectodermal explants results in a decrease in the level of BMP-4 transcription and neural induction, and by testing the effects of expressing XBF-2 fusions with either transcriptional activator or repressor domains has identified XBF-2 as a transcriptional repressor of the BMP-4 gene (Mariani and Harland, 1998). A *Xenopus* expression cloning screen identified Gremlin; a novel

nuclear protein that is co-expressed in an ectodermal domain with *BMP-4* during gastrulation (Kroll, *et al.*, 1998). Overexpression of Gremlin in ectodermal tissue results in neuralisation of the tissue and suppresses BMP-4 expression (Kroll, *et al.*, 1998). These results suggest that Gremlin can regulate BMP-4 expression presumably at the transcriptional level, but it is not reported whether Gremlin can bind to DNA or has the ability to directly repress transcription.

At the translational level, BMP signalling is regulated by two genes that are related to the RNA-helicases eukaryotic initiation factor 4A (*eIF-4A*), a component of the basal translation initiation complex. One of these genes, *eIF-4AII*, is expressed in the prospective neural plate during gastrula stages and can induce neural fates when overexpressed in ectodermal explants. The second *eIF-4A* related gene, *eIF-4AIII*, is expressed in a complementary domain to *eIF-4AII* in the ventral ectoderm, and can promote epidermal fate (Weinstein, *et al.*, 1997). These results suggest that the two related RNA-helicases have opposing effects on BMP signalling, possibly by selective mRNA transcription.

A post-translational control is placed on BMP signalling by a metalloprotease called Xolloid that is co-expressed in the early *Xenopus* embryo with BMP molecules (Goodman, *et al.*, 1998). Whole *Xenopus* embryos injected with *Xolloid* mRNA become ventralised (Goodman, *et al.*, 1998), suggesting that Xolloid may promote BMP signalling. Injection of *Chordin* mRNA into ventral blastomeres of developing *Xenopus* embryos induces a partial secondary axis (Sasai, *et al.*, 1994), but co-injections of *Xolloid* and *Chordin* mRNAs rescues this phenotype (Piccolo, *et al.*, 1997). The proposed model suggests that Xolloid can free inactivated BMP-4 molecule from a Chordin/BMP-4 complex by cleaving the Chordin protein into two fragments (Piccolo, *et al.*, 1997). Therefore, Xolloid limits the activity of Chordin's antagonistic effect on BMP signalling during *Xenopus* development (Piccolo, *et al.*, 1997).

Recently, another level of complexity in the extracellular modulation of BMP signalling has been identified in the developing *Xenopus* embryo. Twisted gastrulation (Tsg) is co-expressed with *BMP-4* and overexpression of Tsg in the developing *Xenopus* embryo results in a reduction in neural and dorsal structures, suggesting that Tsg promotes BMP signalling (Oelgeschläger, *et al.*, 2000). Tsg can bind to BMP-4, and its agonistic activity on BMP signalling is initiated once Chordin

has been cleaved by Xolloid; the Tsg molecule competes with the cleaved Chordin fragments that are still capable of binding and attenuating BMP-4 molecules, enabling the BMP-4 molecule in the BMP-4/Tsg complex to bind and activate its receptor (Oelgeschläger, *et al.*, 2000). However, Tsg can also antagonise BMP signalling, in the absence of Xolloid, by binding and stabilising the BMP-4/Chordin complex, preventing BMP-4 from interacting with its receptor (Figure 1.1; B: Ross, *et al.*, 2001; Chang, *et al.*, 2001).

#### 1.2.4 The BMP signalling pathway is an evolutionarily conserved mechanism

Dorsal-ventral patterning by the BMP signalling pathway appears to be an evolutionary conserved mechanism from insects to vertebrates, although this axis appears to have been inverted during evolution somewhere after the divergence of insects and vertebrates.

*Drosophila*'s BMP homologue, *Decapentaplegic* (Dpp), is expressed in dorsal embryonic ectoderm, providing dorsal-ventral patterning information by inhibiting development of the ventral nervous system and promoting dorsal cell fates (Ferguson and Anderson, 1992a; Ferguson and Anderson, 1992b; Podos and Ferguson, 1999). A number of molecules are employed by the embryo to establish and shape a Dpp morphogen gradient from high dorsal to low ventral; these include Short gastrulation (Sog) and Tolloid. Sog, like its vertebrate functional (Holley, *et al.*, 1995) and structural (Francois and Bier, 1995) homologue Chordin, directly binds to and inhibits Dpp signalling in the ventral portion of the ectoderm (Francois, *et al.*, 1994; Francois and Bier, 1995), allowing the formation of neural ectoderm.

The *Drosophila* Xolloid related metalloprotease Tolloid, is co-expressed dorsally with *Dpp*, thus imposing a level of control in the dorsal ectoderm (Shimell, *et al.*, 1991; Marques, *et al.*, 1997). Tolloid recognises Sog when it has formed the inactive Dpp/Sog complex, cleaving the bound Sog protein, releasing Sog fragments and an active form of Dpp capable of BMP-like signalling (Marques, *et al.*, 1997). *Drosophila* Twisted gastrulation, was identified as an extracellular protein required for the differentiation of the most dorsal cell type the amnioserosa (Mason, *et al.*, 1994; Mason, *et al.*, 1997), and has since been shown to both promote and inhibit Dpp/BMP-like signalling in a manner similar to that described in *Xenopus* (Oelgeschläger, *et al.*, 2000; Ross, *et al.*, 2001).



Figure 1.2: Neural tube axial identity and neural crest migration in the chick embryo

Cartoon of a developing chick embryo. The left-hand side shows the axial identity of the neural tube and the right-hand side illustrates the routes of neural crest cell migration and major derivatives from particular axial levels of origin. Abbreviations: BA; branchial arch; ov, otic vesicle; r, rhombomere.

## AXIAL IDENTITY

## NEURAL CREST

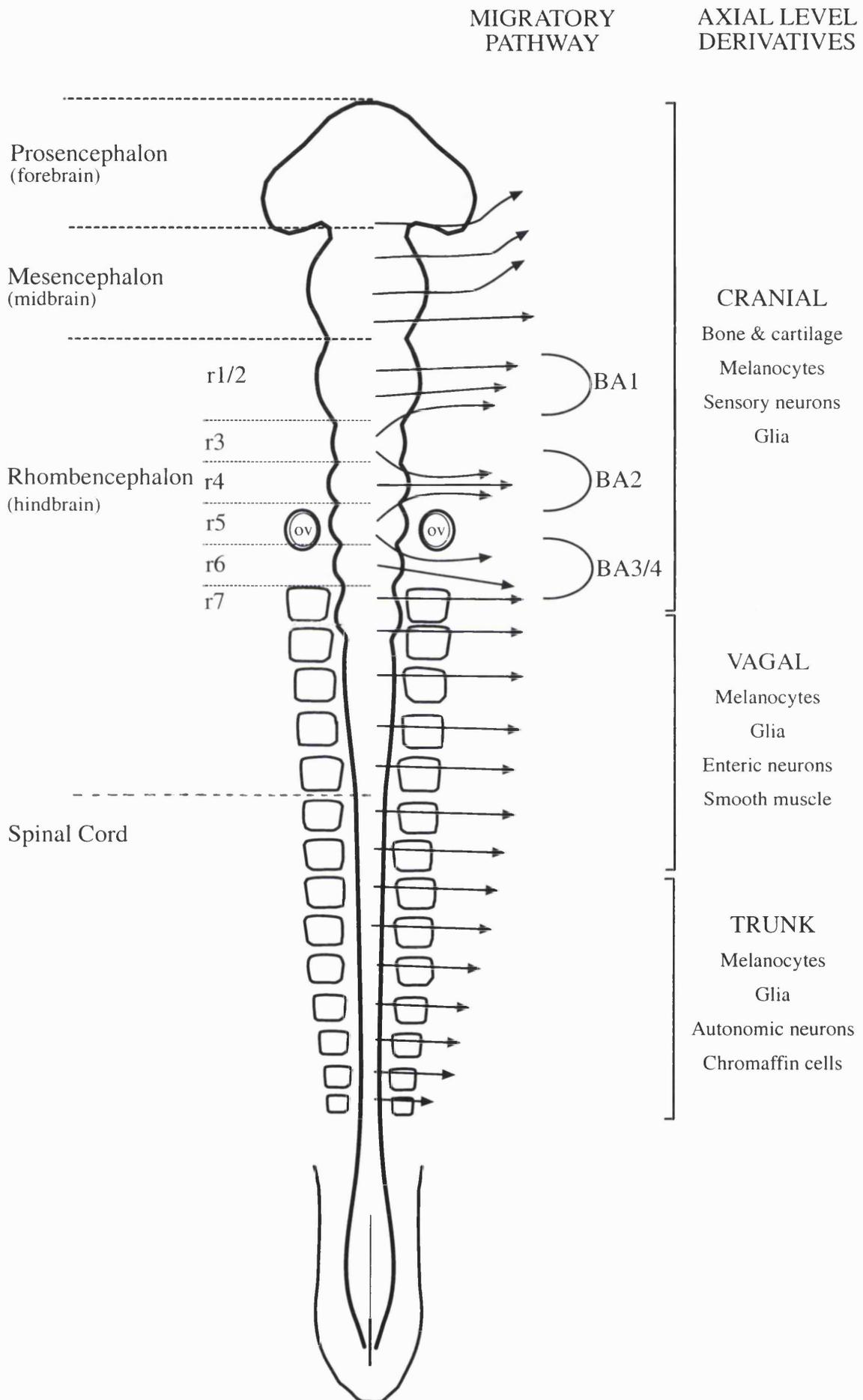
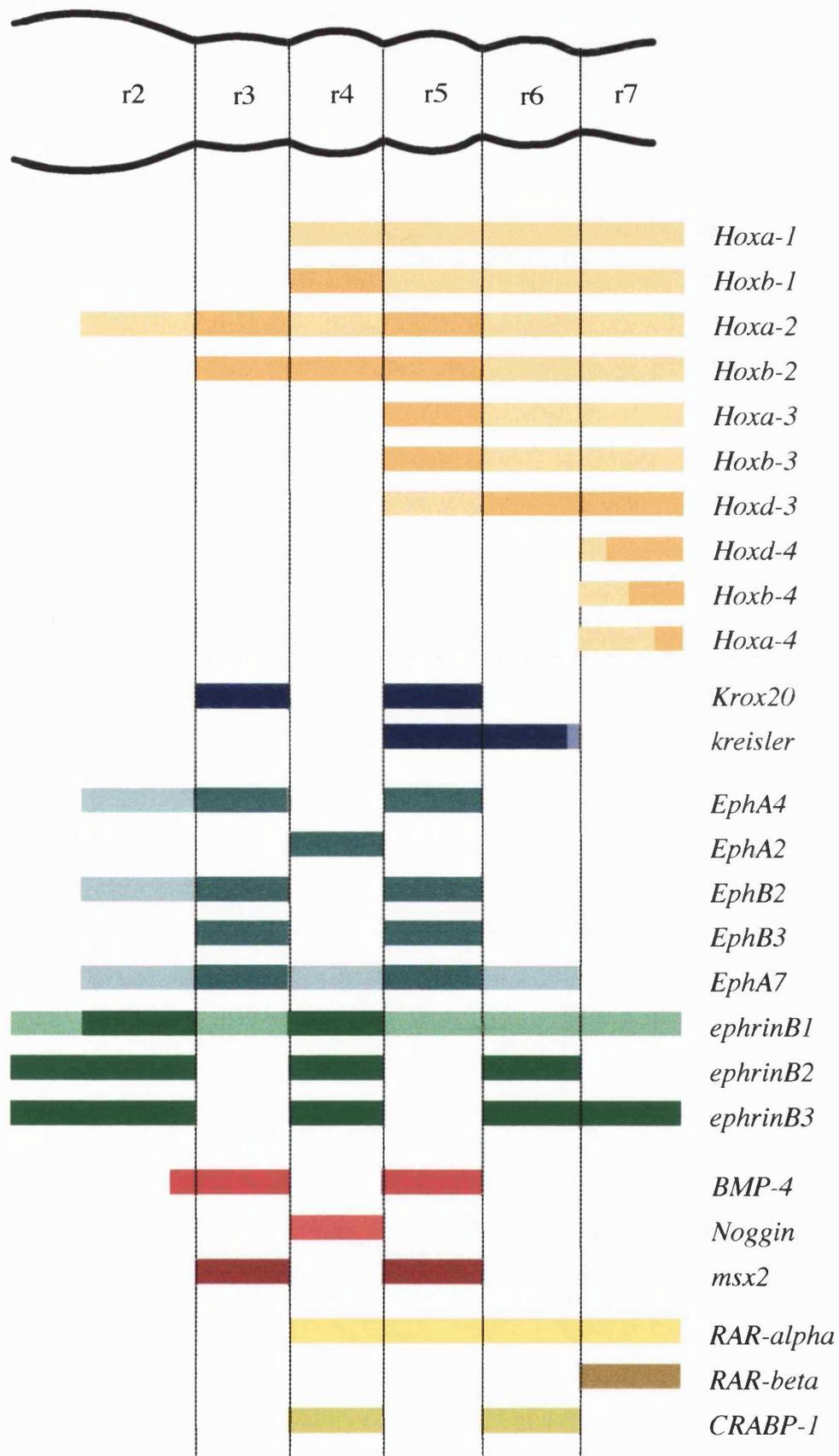




Figure 1.3: Summary of the correlation between gene expression and rhombomere boundary

Overview of some of the molecules that exhibit restricted domains of expression within specific rhombomeres of the developing hindbrain. Rhombomeres are designated r2 to r7. The dashed vertical lines indicate rhombomere boundaries. Gene expression patterns are depicted in arbitrary colours, with the darkest colour indicating the highest level of expression. Related genes are indicated by the same colour: Hox homeobox genes in orange, transcription factors in blue, Eph tyrosine kinase receptors in emerald green, ephrin ligands in green, components of the BMP signalling pathway in red, and retinoid signalling-related genes in yellow. (Modified from; Lumsden and Krumlauf, 1996).



### 1.3 PATTERNING THE CENTRAL NERVOUS SYSTEM

During neurulation the ectodermal layer of the gastrulating embryo becomes partitioned into the neural tube, epidermis and neural crest. The formation of the neural tube is accomplished by a series of tightly controlled cell movements and shape changes of cells of the neural plate, notochord and presumptive epidermis. These morphological changes cause the neural plate to bend and elevate at its lateral edges, and these raised edges eventually meet and fuse at the midline at their most dorsal point. The neural epithelium undergoes a process whereby a series of restrictions develop giving rise to a number of morphologically distinct domains. The trunk region of the neural tube remains as a simple tube, but the cephalic region becomes subdivided forming a series of bulges, termed neuromeres. The formation of these morphologically distinct units gives rise to various regions of the brain and these are termed the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain) (Figure 1.2).

#### 1.3.1 Segmentation of the hindbrain

The formation of the hindbrain represents an attractive and accessible model for the study of local CNS patterning, due to the relatively low number of cell types that are formed and the segmental nature of its formation. Segmentation is a process that is used widely in many developing organisms to generate homologous yet distinct repeated blocks of tissue along the body axis in a developing embryo. These lineage-restricted compartments do not intermingle with adjacent populations of cells and correspond with domains of gene expression (Lawrence and Struhl, 1996; Pasini and Wilkinson, 2002). These compartments exhibit regionalised gene expression domains (Figure 1.3) that provide cells within a particular compartment with a specific identity for example; the *Hox* genes provide positional information to individual rhombomere as to their position along the body axis (Krumlauf, 1994).

The developing hindbrain is transiently segmented into morphological units termed rhombomeres (r). Compartmentalisation of the chick hindbrain begins at Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) stage 9-, and is completed by HH stage 12. At HH stage 12, the neural tube has been partitioned into eight rhombomeres, but by HH stage 24, they are once again invisible. During this

period of development the blueprint for future head development is formed. The formation of the rhombomeres correlates with later cranial developmental processes and is suggestive of segmental specification. Branchial and visceral motor neurons display a rhombomere specific organisation (Lumsden and Keynes, 1989). The trigeminal (V<sup>th</sup>) ganglion arises in r2 and r3, the facial (VII<sup>th</sup>) nerve in r4 and r5, the glossopharyngeal (IX<sup>th</sup>) nerve in r6 and r7 and the somatic motor nucleus of the VI<sup>th</sup> nerve in r5 and r6 (Lumsden and Keynes, 1989). These neurons exhibit a two-segment periodicity, while their axons project laterally from even-numbered rhombomeres, ultimately innervating derivatives from a single branchial arch (Lumsden and Keynes, 1989). These data suggest that each rhombomere in the hindbrain has a precise and specific identity. To identify the molecular mechanisms that underlie these processes, research has focused on elucidating how each rhombomere can establish and maintain its individual identity.

Cell lineage studies have shown that neural epithelial cells labeled before the formation of the rhombomere boundary can contribute to more than one rhombomere, but labeling of cells after boundary formation reveals that there is a restriction of cells to the injected rhombomere (Fraser, *et al.*, 1990; Birgbauer and Fraser, 1994). Moreover, cell grafting and transplantation experiments show that cells from odd-numbered rhombomeres (r3 and r5) have a more limited capacity to mix with cells from neighbouring even-numbered rhombomeres (r2, r4 and r6) than they do with themselves (Guthrie and Lumsden, 1991; Guthrie, *et al.*, 1993; Wizenmann and Lumsden, 1997). These data suggest that there are intrinsic differences between rhombomeres preventing cells from mixing between adjacent rhombomeres; this effect is possibly due to alternate cell populations having different adhesive or repulsive properties.

### 1.3.2 Establishment of axial identity in the hindbrain

The vertebrate *Hox* genes are homologues of the *Drosophila HOM-C* genes play a central role in specifying and maintaining axial identity along the anterior-posterior (A-P) axis of the neural tube (McGinnis and Krumlauf, 1992; Lumsden and Krumlauf, 1996). The *Drosophila* homeotic genes are organised in to two clusters (Lewis, 1978), whereas the vertebrate *Hox* genes are arranged in four separate

clusters that have arisen through duplication of an ancestral complex (reviews see: Wilkinson and Krumlauf, 1990; Krumlauf, 1994).

As in *Drosophila*, the expression of the vertebrate *Hox* genes along the anterior-posterior axis correlates with that of their genomic organisation (Duboule and Dolle, 1989; Graham, *et al.*, 1989). Genes at the 5'-end of a *Hox* complex are the most posteriorly expressed, with the further 3' genes being expressed in a progressively more anterior manner. In the hindbrain and cranial neural crest, members of the *HoxA*, *B* and *D* clusters are expressed in overlapping domains along the A-P axis, with the anterior limits of expression coinciding with rhombomere boundaries in a nested manner (Figure 1.3) (reviews see: Wilkinson, *et al.*, 1989b; McGinnis and Krumlauf, 1992; Wilkinson, 1993). Loss (Studer, *et al.*, 1996; Gendron-Maguire, *et al.*, 1993; Rijli, *et al.*, 1993), or gain (Kessel, *et al.*, 1990; Zhang, *et al.*, 1994) of *Hox* gene function in the mouse, leads to changes in hindbrain development, characterised by the transformation of rhombomere identity, suggesting that a combinational *Hox* code specifies the A-P identity of each rhombomere. It has been proposed that the rostral-caudal identity of the developing hindbrain is also conferred to the periphery via the *Hox* gene code expressed by migratory neural crest cells (Hunt, *et al.*, 1991a; Hunt, *et al.*, 1991b). For example, *HoxA2* is expressed in migratory neural crest cells that enter the second branchial arch, and the effect of loss of *Hoxa2* function reveals that it is normally essential for A-P patterning of neural crest derivatives (Gendron-Maguire, *et al.*, 1993; Rijli, *et al.*, 1993; Maconochie, *et al.*, 1999).

### 1.3.3 Transcription factors

A number of other genes exhibit rhombome restricted expression patterns and are important in the segmentation of the hindbrain. Two such transcription factors are the zinc finger gene *Krox20*, and the basic domain-leucine zipper gene *kreisler* (Figure 1.3). *Krox20* is expressed in presumptive r3 and r5, and continues to be expressed in these rhombomeres following their overt segmentation, as well as in the neural crest cells migrating caudo-laterally from r5 (Wilkinson, *et al.*, 1989a). Loss of *Krox20* function results in loss of r3 and r5 and disruption of cranial nerve patterning, suggesting that *Krox20* is required for the maintenance of these rhombomeres (Schneider-Maunoury, *et al.*, 1993; Swiatek and Gridley, 1993).

Subsequent analysis of the *Krox20* null mutants has showed that rhombomere boundaries are formed between r2/r4 and r4/r6, suggesting that overall hindbrain segmentation is maintained (Schneider-Maunoury, *et al.*, 1997). Further to this, analysis has revealed that presumptive r3 cells acquire r2 or r4 identities and presumptive r5 cells acquire a r6 identity (Voiculescu, *et al.*, 2001).

The *kreisler* gene encodes a transcription factor of the Maf subfamily, and is expressed in presumptive r5 and r6 (Cordes and Barsh, 1994). Mouse mutants lacking *kreisler* function have an unsegmented neural tube caudal to r3, which can be attributed to the complete loss of r5 (Cordes and Barsh, 1994; Manzanares, *et al.*, 1999). The two transcription factors *Krox20* and *kreisler*, have been found to be direct transcriptional regulators of a number of *Hox* genes (Sham, *et al.*, 1993; Nonchev, *et al.*, 1996; Manzanares, *et al.*, 1997), thus coupling hindbrain segmentation with the specification of A-P identity.

#### 1.3.4 Restriction of cell movements

The Eph-family of receptor tyrosine kinases and their ligands, the ephrins, are important in mediating cell-contact-dependent signalling that regulates repulsion or adhesion events between two adjacent cells or populations of cells (Friedman and O'Leary, 1996; Wilkinson, 2000; Mellitzer, *et al.*, 2000). Ephrins are all membrane bound ligands, either by a GPI-anchor (ephrin-A class) or by a transmembrane domain (ephrin-B class). EphA receptors bind only GPI ephrin linked ligands and EphB receptors only transmembrane bound ligands; the only exception to this is the promiscuous EphA4 that can bind both types of ephrin classes (Wilkinson, 2000). Eph and ephrins are involved in a number of developmental processes such as retino-tectal mapping, axonal pathfinding, angiogenesis, neural crest cell migration, and hindbrain segmentation (reviews see: Wilkinson, 2000; Wilkinson, 2001).

In the hindbrain, certain Eph receptors and their interacting ephrins have complementary expression patterns, with the Eph-receptors expressed in odd-numbered rhombomeres and ephrins in adjacent even numbered rhombomeres (Becker, *et al.*, 1994; Flenniken, *et al.*, 1996) (Figure 1.3). Recent studies have demonstrated that the restriction of cell intermingling between adjacent rhombomeres is due to receptor/ligand interactions at the boundaries between the two cell populations. This has been demonstrated by *in vivo* blocking of Eph-

receptor function (Xu, *et al.*, 1995), and mosaic activation of Eph receptors and ephrins (Xu, *et al.*, 1999). Further evidence suggests that Eph/ephrin dependent bi-directional signalling is required for this process (Mellitzer, *et al.*, 1999). *Krox20* directly controls the segmental expression of EphA4, thus identifying a mechanism by which the identity and movement of cells are coupled, generating sharply restricted segmental domains (Theil, *et al.*, 1998).

### 1.3.5 Other molecules exhibiting restricted expression patterns in the developing hindbrain

A number of other molecules have been identified with roles in the patterning of the hindbrain/cranial neural crest (review see; (Wilkinson, 1993; Lumsden and Krumlauf, 1996; Holland and Holland, 1999). One molecule, Retinoic acid (RA) is the bioactive metabolite of vitamin A (retinol), and one of its effects is to establish or alter the pattern of specific *Hox* gene activity (review see; (Maden, 2000). In the hindbrain, RA has been found to induce members of the Hox gene complex via retinoic acid receptors (RARs) and retinoic acid response elements (RAREs) (Marshall, *et al.*, 1992). For example, ectopic application of RA results in the loss of anterior identity of the hindbrain and consequently disruption in neural crest cell migration (Gale, *et al.*, 1996). Some RAR family members and cellular retinoic acid binding proteins (*CRABPs*) have been found to exhibit restricted expression patterns within the hindbrain; *RAR $\alpha$*  is expressed in r4 to r7, *RAR $\beta$*  expressed caudally to r7, and *CRABP-1* is expressed in r2 and r4 to r6 (Figure 1.3). It has been found that loss of function of both *RAR $\alpha$*  and *RAR $\beta$*  results in disruption of hindbrain segment identity (review see; (Maden, 2000).

A number of other genes have also been identified whose expression is restricted to specific regions along the A-P axis of the hindbrain and midbrain during early embryonic development. For most of these molecules however, their functions remain less well understood. They encode a variety of molecules including transcription factors of the *Irx* (Goriely, *et al.*, 1999), *Sax* (Schubert, *et al.*, 1995), *Hlx* (Fjose, *et al.*, 1994), *Hes/Her* (Lobe, 1997; Pasini, *et al.*, 2001) and *Ash* (Allende and Weinberg, 1994) families; secreted/cell surface proteins such as Wnt-8C (Hume and Dodd, 1993), *Fjx1* (Ashery-Padan, *et al.*, 1999), cadherins (Inoue, *et al.*, 1997) and collapsins (Shepherd, *et al.*, 1996); transmembrane receptors such as FGF

receptors (Thisse, *et al.*, 1995; Walshe and Mason, 2000); nuclear receptors such as seven-up related proteins (Fjose, *et al.*, 1993) and other factors that act as specific antagonists such as Follistatin (Graham and Lumsden, 1996) and sprouty2 (Chambers, *et al.*, 2000).

## 1.4 NEURAL CREST

The neural crest is a migratory population of cells that forms at the border between the neural plate and the future epidermis (Hall, 1999; LaBonne and Bronner-Fraser, 1999; Le Douarin and Kalcheim, 1999; Christiansen, *et al.*, 2000). In chick, shortly after the fusion of the neural folds (before neural tube fusion in the mouse), neural crest precursor cells undergo an epithelial to mesenchymal transition and become detached from the neural tube. Migration of neural crest cells lateral to the neural tube occurs in a rostro-caudal wave along the neural axis of the developing embryo. Neural crest cells migrate along specific routes through the embryo to form a wide range of diverse derivatives including most neurons and support cells of the peripheral nervous system, smooth muscle, melanocytes, craniofacial cartilage and bone (Noden, 1988; Köntges and Lumsden, 1996).

### 1.4.1 Neural crest induction

A number of models have been proposed to explain the process of neural crest induction at the neural plate border (Baker and Bronner-Fraser, 1997; Nieto, 2001). Heterotropic grafting and *in vitro* culture experiments have been the primary assays used to determine the tissue interactions required to induce neural crest. Grafting experiments were originally undertaken in axolotl embryos and demonstrated that neural crest is formed at the border between the neural and non-neural ectoderm (Moury and Jacobson, 1989; Moury and Jacobson, 1990). Later grafting experiments in chick and *Xenopus* embryos confirmed that this tissue/tissue interaction is conserved between vertebrates and is required for neural crest induction (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). These experiments suggest that neural plate cells will become neural crest cells if given the appropriate signals (review see: Baker and Bronner-Fraser, 1997; LaBonne and

Bronner-Fraser, 1999; Nieto, 2001). The early processes that induce the neural crest cells are largely unknown but recent evidence has implicated a number of signalling molecules with roles during neural crest induction including members of the Wnt, FGF and BMP signalling protein families (Ikeya, *et al.*, 1997; Mayor, *et al.*, 1997; LaBonne and Bronner-Fraser, 1998b). The role of BMPs in the induction of neural crest cell fates is best understood. Described below is the evidence supporting the role of BMPs in neural crest cell induction and an overview of other molecules with roles during neural crest cell induction.

(i) Multiple roles for BMPs during neural crest induction

Evidence suggests that BMP signalling controls a series of neural crest patterning events, initially at the interface between neural and non-neural ectoderm and later in the dorsal neural tube. Two members of the BMP family, *BMP-4* and *BMP-7* are initially expressed in the non-neural ectoderm adjacent to the neural plate and as the neural tube closes become restricted to the dorsal neural epithelium (Watanabe and Le Douarin, 1996; Liem, *et al.*, 1995). The expression pattern of *BMP-4* and *BMP-7* is consistent in both time and space to implicate them with a role in the induction and maintenance of the neural crest cell fate. Application of recombinant BMP proteins to neural plate explants results in the rapid induction of expression of neural crest cell markers (Dickinson, *et al.*, 1995; Liem, *et al.*, 1995; Mancilla and Mayor, 1996). Conversely, inhibition of BMP signalling at the neural plate border by Noggin protein, results in the loss of expression of neural crest markers and therefore the BMP antagonist prevents the induction of neural crest cell fate (Liem, *et al.*, 1997). These data suggest that BMP molecules secreted by the non-neural ectoderm induce the formation of neural crest in the lateral edges of the neural plate.

After the initial induction of neural crest, during the period of trunk neural tube closure, BMPs are reported to have a second role. During the period of trunk neural tube closure, inhibition of BMP signalling by ectopic *Noggin* expressing cells downregulates the zinc finger gene *Slug* and subsequently prevents migration of trunk neural crest cells (Selleck, *et al.*, 1998). These data suggest that BMPs have a number of separate and distinct roles during the induction of neural crest cells.

(ii) *Slug* is a neural crest inducing molecule

One of the earliest known markers of specified neural crest cells is the zinc finger transcription factor *Slug* that is expressed at the neural plate border in presumptive neural crest cells (Nieto, *et al.*, 1994; Mayor, *et al.*, 1995). Application of *Slug* antisense oligonucleotides to chick (Nieto, *et al.*, 1994) or *Xenopus* (Carl, *et al.*, 1999) embryos, results in an inhibition of cranial neural crest cell migration. Recent data suggests that *Slug* acts as a transcriptional repressor since inhibition of *Slug* activity in *Xenopus* embryos by the construction and introduction of a dominant negative *Slug* blocker (during early stages of neural crest induction), prevents the formation of neural crest precursors (LaBonne and Bronner-Fraser, 2000). In non-neural ectodermal regions, *BMP-4* expression precedes *Slug* expression in the adjacent lateral neural plate, suggesting that *Slug* is a candidate target for BMP-4. In a number of experiments, addition of BMP-4 protein has been shown to rapidly induce *Slug* expression (Liem, *et al.*, 1995; Dickinson, *et al.*, 1995; Mancilla and Mayor, 1996), suggesting *Slug* is downstream of BMP-4. Cell lineage analysis within the dorsal neural tube indicates that *Slug* expressing neural crest cells are not committed to become neural crest and can contribute to all ectodermal derivatives such as epidermis, neural tube and neural crest (Selleck and Bronner-Fraser, 1995). Moreover, commitment to a neural crest fate is a relatively late event occurring around the time of neural crest emigration (Selleck and Bronner-Fraser, 1995).

#### 1.4.2 Initiation of neural crest migration

Neural crest cells first become recognisable as a distinct population once detached from the dorsal neural tube. The molecular cues that trigger neural crest cell emigration are largely unknown, but during the delamination of neural crest cells a number of changes occur within the dorsal neural tube. These changes are thought to relate to the generation of motile neural crest cells from the dorsal neural tube and include the breakdown of the basal lamina, re-organisation of the cytoskeleton, changes in the plane of cell division and changes in cell adhesion (reviewed in: Le Douarin and Kalcheim, 1999; Perris and Perissinotto, 2000). The following section focuses on the molecular mechanisms that underlie the generation of a migratory neural crest cell.

In the trunk of the chick embryo, *BMP-4* is expressed uniformly along the dorsal neural tube and one of its antagonists *Noggin*, is expressed in the dorsal neural tube in a high caudal to low rostral gradient (Sela-Donenfeld and Kalcheim, 1999). Ectopic application of *BMP-4* protein causes premature migration of neural crest cells, whereas ectopic application of *Noggin* results in a delay in migration (Sela-Donenfeld and Kalcheim, 1999). Since neural crest emigration occurs in a wave from rostral to caudal, these findings suggest that *BMP-4* and *Noggin* are acting antagonistically to regulate delamination and migration of trunk neural crest cells. The graded expression of *Noggin*, and therefore neural crest cell emigration, is controlled by cues emanating from newly formed somites (Sela-Donenfeld and Kalcheim, 2000), but the nature of these signal(s) has of yet to be identified.

As *BMP-4* has a pivotal role during emigration, a key issue is to identify targets of *BMP* signalling that underlie the epithelial to mesenchymal transition of neural crest cells and migratory behaviour. In chick, the transcription factors *Slug* and *Snail*, members of the *Snail* family, are expressed in pre-migratory and post-migratory neural crest cells respectively. The expression of *Slug* is required for neural crest emigration (Nieto, *et al.*, 1994; LaBonne and Bronner-Fraser, 2000) and has been implicated with a role in epithelial-mesenchymal transitions, for example in the dorsal neural tube and in the limb bud (Ros, *et al.*, 1997). Recent work has shown that in epithelial cell lines, mouse *Snail* protein binds to the promoter and downregulates the expression of the cell adhesion protein *E-cadherin* (Cano, *et al.*, 2000). The ectopic expression of *Snail in vitro* in epidermal keratinocytes, shows that the repression of *E-cadherin* by *Snail* causes the conversion of a non-invasive epithelial cell type to a mesenchymal cell type with invasive like properties (Cano, *et al.*, 2000). These data suggest that *Snail* is important in the epithelial to mesenchymal transition of neural crest cells to allow emigration, but since *E-cadherin* is not expressed in the neural plate, it is likely other members of the cadherin family may also be targets of *Snail*.

At the time of neural tube closure and prior to delamination of neural crest cells from the dorsal neural tube, a number of members of the cadherin family are differentially expressed between the pre-migratory and post-migratory neural crest. *N-cadherin* and *cadherin-6B* transcripts are initially expressed in the closing dorsal neural tube and are downregulated at the initiation of neural crest cell migration

(Nakagawa and Takeichi, 1995). Two other members, *cadherin 7* and *cadherin 11*, are however found to be upregulated during neural crest migration (Nakagawa and Takeichi, 1995; Kimura, *et al.*, 1995; Hadeball, *et al.*, 1998). In the chick embryo, overexpression of *N-cadherin* or *cadherin 7* in the neural tube prevents migration of many neural crest cells (Nakagawa and Takeichi, 1998). Cadherin-mediated interactions between neural crest cells suggest that different adhesion properties are mediated by different cadherin interactions, both during the onset of migration and subsequently during migration through the mesoderm.

The epithelial to mesenchymal transition of neural crest cells also requires *rhoB*, a member of the rho GTP-binding protein binding family. The rho protein family has been implicated with roles in cytoskeleton rearrangements that modulate changes in cell morphology and behaviour (reviews see: Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). The *rhoB* protein is expressed within the dorsal neural tube and by migratory neural crest cells, and is a downstream target of BMP signalling since it can be induced by BMPs in the dorsal neural tube (Liu and Jessell, 1998). Inhibition of *rhoB* function prevents the epithelial to mesenchymal transition, but not the formation of neural crest cells themselves (Liu and Jessell, 1998); this suggests a role for *rhoB* in delamination of the neural crest.

#### 1.4.3 Migratory pathways of neural crest cells

To identify the pathways followed by migrating neural crest cells, several types of cell marking techniques have been used including quail/chick chimera, DiI labeling and some molecular markers. The migratory pathways of neural crest cells differ according to their axial level of origin (Noden, 1975; Ayer-Le Lievre and Le Douarin, 1982). The populations of neural crest cells arising along the neural axis have been designated as cranial, vagal and trunk (Figure 1.2). In some cases, specified neural crest cells migrate along specific routes and differentiate into distinct cell types according to their axial level of origin; thus linking guidance with differentiation of some neural crest cell populations (LaBonne and Bronner-Fraser, 1998a). At other positions along the neural axis, neural crest cells migrate to sites where they encounter inductive signals (Anderson, 1997; White, *et al.*, 2001).

Neural crest cells that migrate from cranial levels contribute to the cranial sensory ganglia and the parasympathetic ciliary ganglion of the eye, whereas others

migrate ventrally to form many of the cartilaginous elements of the facial skeleton (Noden, 1978; Couly, *et al.*, 1992; Couly, *et al.*, 1993). Cranial neural crest cells can be subdivided into five regions: caudal forebrain, midbrain, rostral hindbrain and caudal hindbrain (which overlaps with rostral vagal). Each population of neural crest cells derived from a particular region has a region specific pattern of migration and derivatives. For example, neural crest cells originating in the midbrain migrate as a broad unsegmented sheet under the ectoderm, and at their final destination they contribute to derivatives such as bones of the face, ciliary ganglion and cranial ganglia (Ayer-Le Lievre and Le Douarin, 1982); neural crest cells arising in the hindbrain migrate ventrally and enter the branchial arches to form the bones of the jaw (Köntges and Lumsden, 1996).

Within the gut, the earliest-generated vagal crest cells move as a wave from anterior to posterior to populate the bowel, forming ganglia of the enteric nervous system (Burns and Douarin, 1998; Burns and Le Douarin, 2001). Trunk neural crest cells follow two primary migratory pathways (Weston, 1963): a dorsolateral pathway between the ectoderm and somite, and a ventral pathway through the rostral half of the somitic sclerotome (Rickmann, *et al.*, 1985). Cells following the dorsolateral stream give rise to melanocytes and cells that migrate along the ventral pathway give rise to the peripheral nervous system (PNS) of the trunk. Neural crest cells that form the PNS differentiate into many derivatives including the chain of sympathetic ganglia along the trunk, dorsal root ganglia and the chromaffin cells of the adrenal medulla.

#### 1.4.4 Segmented migration of neural crest cells from the hindbrain

In chick and other vertebrate embryos, neural crest cells migrate from the hindbrain in three distinct streams: neural crest cells from r1 and r2 migrate into the first branchial arch; neural crest cells from r4 into the second branchial arch, and crest cells from r6/7 migrate into the third and fourth branchial arches (Lumsden, *et al.*, 1991), leaving neural crest free regions adjacent to r3 and r5. The molecular cues that divide the migrating cranial neural crest cells in the hindbrain has been the subject of much research, but it is still not entirely clear how this is achieved. Distinct mechanisms may be used in different species to segregate hindbrain derived migratory neural crest cells.

In the chick, it has been proposed that pre-migratory neural crest cells in r3 and r5 are eliminated prior to emigration by a programmed cell death mechanism (reviews see: Graham, *et al.*, 1996; Lumsden and Graham, 1996). Apoptosis occurs throughout the dorsal neural tube; raised levels of cell death occur in dorsal r2/3 and r5 at a time that coincides with neural crest production, in a region that correlates with the expression of a homeobox containing gene *msx2*. *Msx2* is expressed in dorsal r3 and r5, and ectopic expression of the *msx2* gene throughout the A-P axis results in the elimination of migratory neural crest from all axial levels, suggesting that this gene mediates the apoptotic elimination of cranial neural crest from r3 and r5 (Takahashi, *et al.*, 1998). *BMP-4* is also expressed in r3 and r5 and can induce *msx2* in rhombomere explant cultures, suggesting that BMP-4 protein induces *msx2* and ultimately apoptosis in neural crest cells from rhombomeres 3 and 5 (Graham, *et al.*, 1994). Recent evidence supporting these data has implicated the BMP-4 antagonist Noggin, which is expressed in dorsal r4 and migratory r4 neural crest cells as an apoptotic protector of neural crest cells in r4. The direct binding of Noggin to BMP-4 has been shown to inhibit *msx2* induction, and thus prevent neural crest cell depletion by programmed cell death in rhombomere 4 and in early migratory neural crest cells (Smith and Graham, 2001).

Localised injections of the lipophilic dye DiI into the dorsal neural tube of chick or mouse embryos, demonstrates that all rhombomeres produce neural crest cells (Serbedzija, *et al.*, 1992; Birgbauer, *et al.*, 1995). The relatively few neural crest cells that are produced by r3 and r5 migrate either rostrally or cordally to join neighbouring r2, r4 or r6 migratory neural crest streams (Sechrist, *et al.*, 1993; Kulesa and Fraser, 1998; Trainor, *et al.*, 2002b). Studies in the mouse suggest that the division of hindbrain neural crest cells into migratory streams is due to paraxial exclusion zones in the mesoderm adjacent to r3 and r5 (Trainor, *et al.*, 2002b). In the mouse, the *ErbB4* neuregulin receptor is expressed in r3 and r5, and in *ErbB4* null mutation migratory neural crest cells are present adjacent to r3 (Golding, *et al.*, 2000). Transplantation of neural crest cells from the *ErbB4* mutant into a wild type embryo result in correctly patterned migration whereas abnormal migration occurs when wild type neural crest cells are transplanted into an *ErbB4*<sup>-/-</sup> host (Golding, *et al.*, 2000). It is therefore likely that ErbB4 has a role in the guidance of cranial neural

crest cells by creating paraxial exclusion zones lateral to the hindbrain, thus preventing aberrant migration of neural crest cells into the mesenchyme.

In the developing *Xenopus* embryo, neural crest originating from the hindbrain migrates in four adjacent streams, but unlike chick and mouse, there are no gaps between the migrating streams of neural crest cells, and only become segregated on entering the branchial arches (Sadaghiani and Thiébaud, 1987; Smith, *et al.*, 1997). Eph receptors and ephrins have been implicated in restricting migratory streams of cranial neural crest cells from the hindbrain in cranial mesoderm (review see; (Robinson, *et al.*, 1997). *EphA4* and *EphB1* are expressed by migratory r5 neural crest cells and in the mesoderm of the third branchial arch, whereas their interacting ligand *ephrin-B2*, is expressed in r4 migratory neural crest and second branchial arch mesoderm (Smith, *et al.*, 1997). These complementary expression patterns suggest that Eph receptors and ephrin signalling may restrict intermingling between the two adjacent streams of neural crest cells. Disruption of Eph receptor or ephrin signalling by overexpression or inhibition of function leads to the aberrant migration of third arch neural crest cells (Smith, *et al.*, 1997), suggesting that Eph receptor/ephrin signalling is required to maintain the distinct streams of neural crest.

The guidance of neural crest cells into their appropriate branchial arches has been assumed to be crucial for cranial patterning based on evidence that pre-migratory branchial neural crest is committed to a specific anteroposterior identity controlled by Hox gene expression (Hunt, *et al.*, 1991a; Gendron-Maguire, *et al.*, 1993; Rijli, *et al.*, 1993; Krumlauf, 1994). Previous investigations have suggested that branchial neural crest cells maintain their *Hox* expression, and therefore axial identity, following transplantation to a more anterior or posterior position such that they migrate into a more posterior or anterior branchial arch (Noden, 1983; Prince and Lumsden, 1994; Couly, *et al.*, 1998). Other studies suggest however, that neural crest cells can be more plastic in their axial identity (Nieto, *et al.*, 1995; Saldivar, *et al.*, 1996; Hunt, *et al.*, 1998). One possible explanation for this is that if a large enough group of cells are moved then they might maintain their molecular identity due to mutual interactions. A recent investigation in the mouse embryo has demonstrated that when a small population of neural crest cells is transplanted to an alien axial position they lose their original axial identity (marked by expression of a transgenic *Hox* gene reporter) when they migrate into a different branchial arch from

their normal target (Trainor and Krumlauf, 2000). Therefore, branchial arch mesoderm provides permissive factors required to maintain *Hox* gene expression that had been previously set in pre-migratory neural crest, but cannot reprogram and induce a new A-P identity. These findings suggest that in mouse the A-P positional identity established in pre-migratory neural crest cells is maintained by signals in the branchial arches. In the chick, transposition experiments by Noden (1983) have been interpreted to suggest that neural crest cells are prepatterned, but a recent investigation has challenged this interpretation: *In ovo* grafting of the isthmus further caudally in place of r4 inhibits the expression of *Hoxa2* in second branchial arch neural crest cells and results in the duplication of first branchial arch structures. Moreover, ectopic application of FGF8 adjacent to r4 also inhibits *Hoxa2* expression (Trainor, *et al.*, 2002a). These data demonstrate that FGF8 signalling from the isthmus controls branchial arch patterning, suggesting neural crest cells are not pre-patterned and respond to local environmental signals.

#### 1.4.5 Segmental migration of trunk neural crest cells

In the trunk of avian embryos, neural crest cell migration through the somites is segmental, with neural crest cells entering the rostral half of each somitic sclerotome but avoiding the caudal half (Figure 1.2). This segmental migration is required to pattern segmentally arranged sensory and sympathetic ganglia (Krull, 2001). The relationship between neural crest cells and their surrounding tissues has been explored by manipulating the neural tube and/or somites in a number of investigations undertaken primarily in the chick embryo.

Grafting experiments where somites are removed result in the disruption of the repeated pattern of sensory and sympathetic ganglion at sites of somite ablation, suggesting that somites are required to divide migratory neural crest cells (Bronner-Fraser and Stern, 1991). Further grafting investigations have shown that the information necessary to guide neural crest cells is intrinsic to the sclerotome (Stern and Keynes, 1987). Despite a great deal of research only a few molecules have been identified that are predominantly expressed in regions where neural crest cells do not enter and are implicated with inhibitory or repulsive cues on migratory neural crest cells (review see: Le Douarin and Kalcheim, 1999; Perris and Perissinotto, 2000). These molecules include Peanut agglutinin (PNA)-binding glycoprotein (Oakley, *et*

*al.*, 1994; Krull, *et al.*, 1995), Collagen type IX (Ring, *et al.*, 1996), F-spondin (Debby-Brafman, *et al.*, 1999), T-cadherin (Ranscht and Bronner-Fraser, 1991), Versican (Landolt, *et al.*, 1995) and Eph receptors and ephrins (Krull, *et al.*, 1997; Wang and Anderson, 1997). These data suggest that metameric pattern of neural crest cell migration in the trunk requires interactions between many different molecules.

## **1.5 AIMS OF THE PROJECT**

Despite the progress made in dissecting the genetic pathways involved in the segmentation of the vertebrate hindbrain and neural crest, large gaps in current knowledge still exist. To identify further components of the molecular pathways that govern hindbrain and cranial neural crest development a screen of a subtracted chick hindbrain cDNA library was undertaken with the aim to identify genes with special restricted expression patterns. One particular gene that was identified during this screen was selected for further investigation to elucidate its role during cranial neural development.

## CHAPTER TWO

### MATERIALS & METHODS

All molecular biology protocols were carried out as previously described (Sambrook, *et al.*, 1989) and any deviation from this source is detailed below. A complete list of reagents and plasmid vectors used during this project is located in Appendix A.

#### 2.1 BACTERIAL STRAINS

Escherichia coli genotypes:

DH5 $\alpha$  *supE44*  $\Delta$ *lac* U169 ( $\phi$ 80 *lacZ* $\Delta$ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*.

SOLR<sup>m</sup> e14'(McrA')  $\Delta$  (*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ uvrC umuC::Tn5(Kan<sup>r</sup>) lac gyrA96 relA1 thi-1 endA1  $\lambda^R$  [F' *proAB lacI<sup>q</sup>Z* $\Delta$ M15]<sup>c</sup> Su<sup>-</sup> (nonsuppressing).*

XL1-Blue  $\Delta$ (*mcrA*)183  $\Delta$ (*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI<sup>q</sup>Z* $\Delta$ M15 Tn10 (Tet<sup>r</sup>)]<sup>c</sup>.

Bacteriophage type:

$\lambda$ gt10  $\lambda$ stI $\lambda^o$  b527 srI $\lambda$ 3<sup>o</sup> imm434 (srI434)

#### 2.2 MAINTENANCE & MANIPULATION OF BACTERIAL STRAINS

##### 2.2.1 Bacterial growth media and antibiotics

DH5 $\alpha$  *E. coli* were grown in Luria-Bertani (LB) broth (Appendix A). Stock solutions of antibiotics were prepared with double distilled water (DDW) (Ampicillin

stock; 100 mg/ml) and stored at  $-20\text{ }^{\circ}\text{C}$ . Antibiotic stock solutions were filter sterilised through a  $22\text{ }\mu\text{m}$  filter, and added to cooled autoclaved LB broth (Ampicillin working concentration;  $100\text{ }\mu\text{g/ml}$ ).

### 2.2.2 Growth of bacterial cultures

Cultures were prepared by inoculating LB broth containing the appropriate antibiotic with a single bacterial colony, and incubated overnight at  $37\text{ }^{\circ}\text{C}$  at 250 rpm in an orbital shaker.

### 2.2.3 Maintenance and storage of bacterial cultures

For short-term storage (1-2 month), bacterial cells were maintained on LB agar plates (Appendix A) sealed with Parafilm and stored upside-down at  $4\text{ }^{\circ}\text{C}$ . For long-term storage, bacterial cells were stored as glycerol stocks;  $0.7\text{ ml}$  of an overnight culture mixed with  $0.3\text{ ml}$  of  $50\text{ }\%$  (v/v) glycerol in screw-top tubes, and stored at  $-80\text{ }^{\circ}\text{C}$ .

### 2.2.4 Heat-shock transformation of competent cells

Frozen DH5 $\alpha$  competent bacteria (stored as glycerol stocks at  $-80\text{ }^{\circ}\text{C}$ ) were thawed on ice. Cells were dispensed ( $50\text{ }\mu\text{l}$ ) into a pre-chilled  $1.5\text{ ml}$  microfuge tube and between  $1$  and  $5\text{ }\mu\text{l}$  of DNA solution was added and mixed gently. The mixture was incubated on ice for 20 minutes before a  $37\text{ }^{\circ}\text{C}$  heat shock for 5 minutes and cooled on ice for 2 minutes. After this period,  $200\text{ }\mu\text{l}$  of LB was added and the mixture incubated for a further 20 - 30 minutes at  $37\text{ }^{\circ}\text{C}$  before being plated onto a LB-agar containing an appropriate selective antibiotic. The bacterial plates were allowed to dry before being incubated upside-down at  $37\text{ }^{\circ}\text{C}$  for between 12 and 16 hours.

## 2.3 ISOLATION, MANIPULATION & MODIFICATION OF DNA

### 2.3.1 Isolation of plasmid DNA

Small-scale preparation of plasmid DNA was performed using the Qiagen™ Plasmid Miniprep purification kit. Large-scale purifications utilised the Qiagen™ Maxi-kit, according to manufacturers' instructions.

### 2.3.2 Excision of phagemid DNA from Lambda ZAP II

Phagemid DNA was 'rescued' from Lambda ZAP II vectors, according to the manufacturers' instructions (Stratagene; ExAssist™ Interference-Resistant Helper Phage).

### 2.3.3 Restriction enzyme digestion of DNA

All restriction enzymes were used according to the manufacturers' instructions (Promega, New England Biolabs) using the appropriate buffer supplied. The volume of the enzyme used in the reaction was never more than 10 % of the total reaction volume.

Clones that were isolated from the directionally cloned subtracted chick hindbrain cDNA library (pSPORT1) were linearised with *SalI* for subsequent DIG labeled antisense RNA transcription with SP6 RNA polymerase (section 2.7.1) for use in whole mount in situ hybridisation (section 2.7.2).

### 2.3.4 Phenol/chloroform extraction

Equal volumes of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) and DNA were vortexed thoroughly and centrifuged at 13,000 rpm for 2 minutes at room temperature. The aqueous phase was removed to a fresh tube and ethanol precipitated (section 2.3.5).

### 2.3.5 Ethanol precipitation of DNA

DNA was precipitated for a minimum of 30 minutes at -20 °C by adding 0.1 volumes of 3 M sodium acetate (NaOAc) pH 5.8 and 2.5 volumes of 100 % ethanol

(-20 °C). The DNA was pelleted by centrifugation at 13,000 rpm for 20 minutes at 4 °C. The supernatant was removed and the DNA was washed with 70 % (v/v) ethanol and centrifuged as described above for 10 minutes. The pellet was air-dried and resuspended in an appropriate volume of TE (Appendix A) or sterile distilled water.

### 2.3.6 Dephosphorylation of DNA

To prevent self-ligation of compatible ends of vector DNA during ligation, the 5' ends were dephosphorylated. Following digestion of approximately 5 µg of DNA with the appropriate restriction enzyme (section 2.3.3), the DNA was extracted with phenol:chloroform:IAA (section 2.3.4) and ethanol precipitated (section 2.3.5). The dried DNA pellet was resuspended in 50 µl of DDW and quantified. The reaction was buffered with 1x dephosphorylation buffer to which calf intestine alkaline phosphatase (Roche) was added at 1 unit per 1 pmol of 5' terminal phosphorylated DNA fragments (3' recessed, 5' recessed or blunt-ended), in a final volume of 100 µl. Reactions were incubated at 37 °C for 1 hour then extracted with phenol:chloroform:IAA (section 2.3.4) and ethanol precipitated (section 2.3.5).

### 2.3.7 Ligation of DNA fragments

The ligation of DNA fragments was carried out using the 'Rapid DNA Ligation Kit' (Roche/Boehringer Mannheim) according to manufacturers' instructions (Roche). In a typical reaction a 1:3 molar ratio of vector:insert DNA was used, mixed with 1x ligation buffer and 0.1-1 Weiss units of T4 DNA ligase in a total volume of 10 µl. Insert DNA was omitted from control reactions.

Fragments that had been digested with different restriction enzymes producing either 5' or 3' single-stranded overhangs were end-filled prior to ligation using either Klenow polymerase or T4 DNA polymerase respectively. 1 unit of large Klenow DNA Fragment per µg of DNA in a solution of 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT and 20 µM of each of the four dNTPs, were incubated at 15-37 °C for 15-30 minutes. Alternatively, 1 unit of T4 DNA polymerase per µg of DNA in a solution of 50 mM Tris-HCl (pH 8.8), 5 mM MgCl<sub>2</sub>, 5 mM DTT and 20 µM of each dNTPs, were incubated at 15 °C for 15 to 30 minutes.

## 2.4 ANALYSIS OF DNA

### 2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA samples was carried out as follows: 1x TBE (Appendix A) agarose gels containing 1 µg/µl ethidium bromide were run in 1x TBE buffer. The percentage of agarose was dependent on the expected size of the DNA. DNA samples were mixed with 0.1 volumes of gel loading buffer and separated by electrophoresis.

### 2.4.2 Determination of nucleic acid concentration

DNA and RNA were quantified by spectrophotometry at 260 nm. An optical density (OD) of 1 is the equivalent of 50 µl/ml double stranded DNA, 35 µg/ml single stranded DNA and 40 µg/ml RNA.

### 2.4.3 DNA sequencing & analysis

Clones were sequenced from both the 5' and 3' ends using the M13 reverse and M13 universal primers (Pharmacia) in conjunction with a Big Dye terminator kit and a 377 automated DNA Sequencer (Applied Biosystems) at the Advanced Biotechnology Centre, Imperial College, London. Sequencing was analysed for putative open reading frames using the MacVector 5.1 package (Oxford Molecular). Nucleic acid and protein sequence similarity searches were performed using the gapped BLAST algorithms (Karlin and Altschul, 1990) accessed via the internet at <http://www.ncbi.nlm.nih.gov>. Protein family searches (Pfam) were performed at <http://pfam.wustl.edu> and (TIGR) <http://www.tigr.org/tdb/>.

## 2.5 SYNTHESIS OF mRNA

Synthesis of capped mRNA for *in vivo* injection was carried out as previously described (Moon and Christian, 1989). Briefly, sense transcripts were synthesised *in vitro* using 5 µg of linearised plasmid DNA and 50 units of RNA Polymerase (SP6,

T3 or T7; Promega) in the presence of: 10 mM DTT; 1 mM of ATP, CTP and UTP; 0.1 mM GTP; 0.5 mM RNA cap analog (m<sup>7</sup>G(5')ppp(5')G); 10 µl 5x Transcription Optimised buffer (Promega) and 40 units of RNasin Ribonuclease Inhibitor (Promega) in a final volume of 50 µl. The reaction was incubated at 37 °C for at least 1 hour. To remove the DNA template after the RNA synthesis, 5 units of RQ1 DNase (Promega) were added to the reaction and incubated at 37 °C for 30 minutes. The synthetic RNA was purified from unincorporated nucleotides using Croma spin columns (Clontech), ethanol precipitated (section 2.3.5), resuspended in Sigma pure water and analysed by agarose gel electrophoresis (section 2.4.1). Concentration was determined as previously described (section 2.4.2).

To ensure synthesised ΔCS2cE13 mRNA was capable of being translated to produce the appropriate sized polypeptide, *in vitro* translations were performed using the Wheat Germ Extract (Promega) according to the manufacturers' instructions. Chick E13 mRNA was translated *in vitro* using the same conditions but with the following changes; L-<sup>35</sup>S labeled Cysteine (Amersham) and 1 µM/µl of potassium acetate was added to each reaction to optimise translation.

## **2.6 COLONY SCREENING USING RADIOACTIVE LABELED PROBES**

### **2.6.1 Colony blot**

Bacterial clones were plated or gridded onto LB agar and incubated overnight at 37 °C. Colony lifts were performed by placing Colony/Plaque Screen™ hybridisation transfer membranes (DuPont NEN® Research Products) onto the surface of the agar; membrane orientation was marked by piercing the membrane and agar with a needle. Membranes were processed according to the manufacturers' instructions.

### **2.6.2 Hybridisation**

Membranes were pre-hybridised according to the manufacturers' instructions (Clony/Plaque Screen™, DuPont) in aqueous hybridisation buffer (Appendix A). Membranes were incubated in either 'Techne' hybridisation bottles or glass petri

dishes at 65 °C. Hybridisation probes were generated and labeled using the Megaprime™ DNA Labeling System (Amersham) according to the manufacturers' instructions. The labeling reaction was purified by gel exclusion chromatography through Sephadex G-50 column, equilibrated with 3x SSC, and denatured at 100 °C for 10 minutes before being added to the pre-hybridising membranes.

Following hybridisation and washing the membranes were wrapped in Clingfilm and exposed to X-ray film (Kodak). Intensifying screens were used at -80 °C when required. Exposed film was developed in an automated developing machine (Kodak).

## **2.7 DETECTION OF TRANSCRIPTS & EMBRYO MANIPULATION**

### **2.7.1 Generation of DIG labeled probes; *in vitro* transcription**

Linearised cDNAs were used as templates in the synthesis of digoxigenin (DIG; Roche/Boehringer Mannheim) labeled anti-sense RNA probes according to the manufacturers' instructions. Probe integrity and approximate concentration was assessed by agarose gel electrophoresis (section 2.4.1).

### **2.7.2 Whole mount in situ hybridisation**

In situ hybridisations were performed either manually using 'Protocol Four' as previously described (Xu and Wilkinson, 1998), or automatically using an In situPro in situ machine (AbiMed) using a modified version of 'Protocol Four' (Appendix B). Approximately 1 µg of DIG labeled antisense RNA probe was used per in situ hybridisation.

### **2.7.3 Flat-mounting and photography**

Embryos were placed in 75 % (v/v) glycerol and left to equilibrate. Chemically sharpened tungsten needles were used to dissect out the hindbrain; a ventral cut was made in the neural tube and excess ventral mesoderm tissue was removed. The hindbrain was then placed on a microscope slide containing a drop of

75 % (v/v) glycerol, manipulated to attain the correct orientation and a cover slip was lowered over the specimen ensuring it was flattened.

Bright field low power pictures of whole embryos were taken on a stereomicroscope (Leica Wild M10) with a photographic attachment, using an agarose bed to position the embryos. Flat mounted hindbrains were photographed with a Zeiss Axiophot microscope using Nomarski optics. Photographs were taken using Kodak 64T film and processed by N.I.M.R. Photo-Graphics division. Images were digitalised using a Polaroid SprintScan 35 scanner and images were manipulated using AdobePhotoshop3.0.1.

## **2.8 IN OVO ELECTROPORATION**

The electroporation technique was undertaken as previously described (Itasaki, *et al.*, 1999). Electroporated embryos were dissected from the egg, washed in DepcPBS and stained for  $\beta$ -galactosidase as follows: by fixing in 4 % (w/v) PFA for 15 minutes (room temperature), and washing twice in DepcPBS for 10 minutes. The embryos were then incubated in Xgal staining solution (filter sterilised), in the dark at 37 °C until the staining had reached the required intensity. The stain was fixed by incubating in 4 % (w/v) PFA overnight before processing by whole mount in situ hybridisation to assay the distribution of the transcript in question (section 2.7.2).

## **2.9 PRODUCTION & DETECTION OF RECOMBINANT PROTEIN**

The production of recombinant protein was carried out using the *Drosophila* Expression System (DES<sup>®</sup>; Invitrogen<sup>®</sup>). To allow for the correct insertion of cE13 cDNA into the pMT/BIP/V5-HisA expression vector a PCR approach was followed.

### **2.9.1 Construction of pMT/BIP/cE13/V5-HisA expression vector**

Gene specific primers were designed to incorporate pMT/BIP/V5-HisA vector specific restriction sites:

5' end (*KpnI* restriction site)

5'-CGG GGT ACC TTC CTC CCT GCT CAC AGG-3'

3' end (*NotI* restriction site)

5'-ATA AGA ATG CGG CCG CCA TCG CTG AGG ACA TAG-3'

PCR reaction mix: 1 ng cE13 DNA template; 125 ng of each primer; 10x dNTPs; 2.5 units of *Pfu* polymerase (New England Biolabs); 10x *Pfu* buffer; volume was adjusted with DDW. PCR conditions of 30 cycles: 95 °C for 1 minute; 48 °C for 1 minute; 72 °C for 2 minutes and one final cycle of 5 minutes at 72 °C. The entire PCR reaction was loaded on to a 1 % (w/v) agarose/TBE gel to check for size and purity, the band was then excised from the gel with a scalpel blade and purified using the QIAEX II Agarose Gel Extraction kit (Qiagen) according to the manufacturers' instructions. The gel purified DNA was ligated into the *NotI/KpnI* linearised pMT/BiP/V5-HisA plasmid and transformed. Clones with inserts were identified by colony screening (section 2.6), and sequenced (section 2.4.3) to confirm PCR accuracy.

### 2.9.2 Generation & purification of recombinant protein

The pMT/BIP/V5/His-A/E13 construct was introduced to Schneider 2 insect cells (S2) using the calcium chloride technique according to the manufacturers' instructions (Invitrogen®). The recombinant cE13 protein was purified by metal-chelate affinity chromatography using the Xpress™ System ProBond™ Resin (Invitrogen®) to which the purified fusion protein was bound. The resin was prepared according to the manufacturers' instructions. A cleared lysate was prepared under native conditions according to protocol 8 the QIAexpressionist™ handbook (Qiagen). Batch purification of the protein occurred under native conditions and was performed as described in protocol 11, The QIAexpressionist™ handbook (Qiagen).

## 2.10 PROTEIN ANALYSIS

### 2.10.1 Electrophoresis of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis of proteins was carried out using a Bio-Rad PROTEAN™ minigel kit according to manufacturers' instructions. SDS-PAGE gels (10 % (w/v) acrylamide) consisted of a lower resolving gel [1.75 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 1.75 ml 1.5 M Tris-HCl pH 8.8, 10 µl ammonium persulphate (250 mg/ml), 30 µl 10 % (w/v) SDS, 10 µl NNN'N'-tetramethylethylenediamine (TEMED), 3.45 ml distilled water], which was overlaid with water-saturated butanol and allowed to polymerise at room temperature. Once set, the water-saturated butanol was poured off and the gel was rinsed with distilled water and dried with filter paper. The stacking gel [0.5 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 0.9 ml 0.5 M Tris-HCl pH 6.8, 5.1 µl ammonium persulphate (250 mg/ml), 36 µl 10 % (w/v) SDS, 5.1 µl TEMED, 2.17 ml distilled water] was poured on top of the resolving gel, a comb was inserted to allow the formation of wells, and allowed to set at room temperature.

Protein samples were prepared by mixing with an equal volume of 2x SDS sample buffer (Appendix A) and incubating at 100 °C for 3 minutes to denature the samples. Samples were loaded into the wells and electrophoresed in SDS running buffer (Appendix A). Rainbow molecular weight markers (Amersham) were used to estimate protein size. To visualise the bands, gels were stained for 1 hour in Coomassie staining buffer (Appendix A), washed with DDW and then destained in destaining buffer (Appendix A) until bands were visible.

### 2.10.2 Western blotting of proteins

After SDS-PAGE electrophoresis, protein samples were transferred to polyvinylidene difluoride membrane (Sequi-Blot™ PVDF protein sequencing membrane, Bio-Rad) previously permeabilised by immersion in methanol by wet electrophoresis transfer. The membrane was blocked with 5 % (w/v) milk powder (Marvel) in PBTw [Phosphate Buffered Saline (PBS), 0.1 % (v/v) Tween20] for 12 hours at 4 °C, with agitation. The membrane was then incubated with the Anti-V5-HRP antibody (Invitrogen®) at a 1:5000 dilution in 1 % (w/v) milk powder and

PBTw for 2 hours at room temperature, and then washed 6 times in PBTw for 10 minutes with agitation. The bound antibody was detected using SuperSignal® West Pico Chemiluminescent Substrate (PIERCE) according to the manufacturers' instructions, and exposed to X-ray film (Kodak).

## 2.11 CO-IMMUNOPRECIPITATION ANALYSIS

Immunoprecipitation was performed as described (Henkemeyer, *et al.*, 1994; Larrain, *et al.*, 2000). Briefly, conditioned media was removed from either cE13 expressing or Chordin expressing transiently transfected S2 cells and concentrated using a Microcon®-50 spin column (Microconcentrators). Expressing cells were lysed with 2 ml of PLC buffer (Appendix A). Cell debris and unlysed cells were removed by precipitation; centrifugation at 12,000 rpm for 10 minutes at 4 °C. 50 ng of recombinant human BMP-4 protein (R&D Systems) was added to each sample of 200 µl of cell lysate (supernatant) or concentrated media, and allowed to bind with agitation for 6 hours at 4 °C. During this period, 50 µl of protein A-Sepharose beads (Pharmacia Biotech) were washed three times with PLC buffer prior to the addition of 10 µl of rabbit anti-goat antibody (1:10) and incubation for two hours at 4 °C. The beads were pelleted by centrifugation for 2 minutes at 13,000 rpm, washed three times with 1 ml PLC buffer, and resuspended in 50 µl PLC buffer. To this, 10 µl of goat anti-BMP-4 antibody (1:10) was added and incubated for two hours at 4 °C and washed 3 times in PLC buffer. The beads were resuspended in 50 µl PLC buffer, and added to either the BMP-4 (incubated) and cell-lysate or conditioned media samples and incubated overnight at 4 °C. The following day the beads were pelleted for two minutes and the precipitated immune complexes were washed three times with PLC buffer and resuspended in 20 µl loading buffer. Samples were boiled for 2 minutes, electrophoresed through 10 % (w/v) SDS polyacrylamide gels under reducing conditions (section 2.10.1) and electroblotted onto PDVF membranes (section 2.10.2). The membranes were processed as described previously (section 2.10.2), and probed using the anti-V5 HRP conjugated antibody at a dilution of 1:5000.

## 2.12 XENOPUS EMBRYO CULTURE & MANIPULATIONS

### 2.12.1 Isolation & injection of *Xenopus* embryos

*Xenopus laevis* embryos were obtained from *Xenopus* adult females (Sive, *et al.*, 2000) that had been injected 12 hours previously with 700 units of human chorionic gonadotrophin (Sigma). The eggs were expelled by gentle peristalsis of the mother's ventrolateral surface and were fertilised by rubbing them with macerated testis from a sacrificed male. After 5 minutes the eggs were flooded with 10 % (v/v) normal amphibian medium (NAM: Appendix A) and left for 20 minutes to allow for cortical rotation. Prior to injection, embryos were dejellied using 2 % (w/v) cysteine hydrochloride (pH 7.8), washed at least 3 times in 10 % (v/v) NAM and transferred to 75 % (v/v) NAM containing 4 % (w/v) Ficoll. A volume of approximately 5 nl was injected into dejellied embryos at the relevant stage of development using a capillary glass needle driven by an air injector. The injection volume was calculated by injecting liquid into oil and measuring the diameter of the drop using a graticule. By treating the drop as a sphere, the injected volume was determined ( $\text{volume} = \frac{4}{3}\pi r^3$ ). Dorsal and ventral halves of the 4-cell stage embryo can be distinguished due to the lighter pigmentation and smaller size of the dorsal blastomeres compared to the ventral blastomeres. Six hours after injection, embryos were transferred to 10 % (v/v) NAM and allowed to develop to the relevant stage before fixation in MEMFA (Appendix A). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

### 2.12.2 Isolation of *Xenopus* ectodermal explants

For isolation of ectodermal explants (animal caps) *Xenopus* embryos previously injected with the appropriate RNA were allowed to develop as previously described (section 2.12.1). At stage 8-9 ectodermal explants were placed in 75 % (v/v) NAM and the vitelline membrane surrounding the embryo was removed manually using sharpened watchmakers forceps. A circle of tissue from the animal-most region, comprising less than 50 % of the animal hemisphere of the embryo was cut. The ectodermal explants were then allowed to develop to the desired stage in 75 % (v/v) NAM before being flash-frozen on dry ice and stored at  $-80\text{ }^{\circ}\text{C}$ .

### 2.12.3 RNA isolation and RT-PCR of animal caps

RNA was isolated from ectodermal explants (10 - 20) or 2 whole embryos using Trizol™ reagent (Gibco BRL) according to the manufacturers' instructions. The samples were treated with RQ1 DNase (Promega) to remove genomic DNA contaminants according to manufacturers'.

RNA equivalent to 5 ectodermal explants were reverse transcribed using MMLV RTase (Gibco BRL) and random primers (Promega). One-tenth of the reverse transcription reaction was used for the PCR reaction (25 µl). The PCR reaction mix contained 100 µM of each dNTP, 1x AmpliTaq buffer (Perkin Elmer), 1.5 mM MgCl<sub>2</sub>, 0.1 µg of each primer, 1 µC <sup>32</sup>P dCTP and 1.25 units of AmpliTaq DNA Polymerase (Perkin Elmer). PCR conditions of 25 cycles: 93 °C for 30 seconds; 55 °C for 1 minute; 72 °C for 30 seconds. The products of PCR amplification were run in a 5 % (v/v) polyacrylamide gel, the gel was dried (80 °C for 50 minutes under vacuum) and exposed to X-ray sensitive film (Kodak) for between 2 and 12 hours. The markers and primer sequences used in this thesis are listed below (Ruiz i Altaba and Melton, 1989; Hemmati-Brivanlou and Melton, 1994):

NCAM	5'- CACAGTTCCACCAAATGC -3'
	5'- GGAATCAAGCGGTACAGA -3'
BF-1	5'- CCTCAACAAGTGCTTCGTCA -3'
	5'- TAAAGGTGAGTCCGGTGGAG -3'
Xhox3	5'- GTACCTCAACCAACGGCCTA -3'
	5'- GGACTCGGGAGAAGGGTAAC -3'
En-2	5'- CGGAATTCATCAGGTCCGAGATC -3'
	5'- GCGGATCCTTTGAAGTGGTCGCG -3'
Muscle actin	5'- GCTGACAGAATGCAGAAG -3'
	5'- TTGCTTGGAGGAGTGTGT -3'
EF-1α	5'- CAGATTGGTGCTGGATATGC -3'
	5'- ACTGCCTTGATGACTCCTAG -3'

### 2.13 CLONING OF MURINE E13 BY RT-PCR

Total RNA was isolated from 8.5-9.5 days postcoitum (dpc) mouse embryos with Trizol™ (Gibco-BRL), and reverse transcribed using Superscript™ reverse transcriptase kit (Gibco BRL) using oligo (dt)<sub>12-18</sub> primers. PCR was performed with primers designed using the mouse EST (genebank accession number; BE289531) and incorporated *NotI* and *SalI* restriction sites:

Forward primer (*SalI* restriction site),

5'-CTCATCTCAAGGGCAAGCTCTG-3'

Reverse primer (*NotI* restriction site),

5'-CTTCACACATCCTGGGCCAC-3'

PCR consisted of 35 cycles: 95 °C for 1 minute; 55 °C for 1 minute; 72 °C for 2 minutes. The 473bp fragment was then digested with *NotI* and *SalI*, gel purified and sub-cloned into the plasmid pBluescript II KS (+/-) for sequencing and in situ probe generation. For antisense probe generation the plasmid was linearised with *SalI* and transcribed with T7 RNA polymerase.

### 2.14 ANIMALS

Fertilised brown chicken (*Gallus gallus*) eggs were acquired from Winters Egg Farm (Hertfordshire). Frogs (*Xenopus laevis*) and mice (*Mus musculus*) were all supplied by N.I.M.R. animal services.

## CHAPTER THREE

# IDENTIFICATION OF GENES EXPRESSED DURING HINDBRAIN, MIDBRAIN & CRANIAL NEURAL CREST DEVELOPMENT

### 3.1 INTRODUCTION

During the development of the vertebrate central nervous system, the neural epithelium undergoes a series of constrictions to form the presumptive forebrain, midbrain, hindbrain and spinal cord. Within the hindbrain, further subdivisions take place to form 7/8 transient segments termed rhombomeres (r) that form the blueprint for subsequent head development (Chapter 1). For example, branchial and visceral motor neurons exhibit a rhombomere-based segmental organisation (Lumsden and Keynes, 1989). Furthermore, neural crest cells originating from r1/2, r4 and r6 of the hindbrain migrate in a highly patterned manner into branchial arches 1, 2, and 3/4 respectively, with neural crest free regions adjacent to r3 and r5 (Graham, *et al.*, 1993; Bronner-Fraser, 1995; Köntges and Lumsden, 1996).

A number of molecules have been characterised with roles during hindbrain development and these include: genes that establish axial identity (*Hox* clusters: McGinnis and Krumlauf, 1992; Wilkinson, 1993), segmentation genes that form and maintain rhombomere compartments (*Krox20* and *kreisler*: Wilkinson, *et al.*, 1989a; Cordes and Barsh, 1994), and adhesion and repulsion molecules that restrict cell intermingling between rhombomeres (Eph receptors and ephrins: Friedman and O'Leary, 1996; Wilkinson, 2001). Despite the considerable progress made in dissecting the genetic pathways involved in the early patterning of the vertebrate hindbrain development large gaps remain in current knowledge. Therefore, the aim of this project was to identify previously unknown genes with potential roles in neural tissue development, especially within the hindbrain region.

Numerous techniques have been employed to identify genes involved in the development of a particular tissue and these can be divided into 3 groups: structure/interaction screens; phenotype screens; and expression pattern screens. Identifying homologues or relatives of known genes with roles in development (either between species or within families) is one screening method that utilises techniques such as cross-species hybridisation, degenerate PCR or sequence database searching. The basis of this approach is the conserved function of genes in different species, and the use of specific gene families (such as homeobox genes) in developmental processes. Another more focussed approach is to identify molecule(s) that interact with a known molecule via a two-hybrid screen.

Genetic screening assesses the various phenotypes following random mutagenesis of a model organism's genome. Genetic screens in *Caenorhabditis elegans* (Young and Hope, 1993; Hope, 1991) and *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980) have been the primary source of understanding for the mechanisms that underlie patterning in invertebrates. In vertebrates, large-scale mutagenesis screens have been performed in *Danio rerio* (zebrafish: Driever, *et al.*, 1994; Haffter, *et al.*, 1996) and *Mus musculus* (mouse: Friedrich and Soriano, 1991; Wurst, *et al.*, 1995). Genetic screens have limitations as some genes may be missed in the screening procedure due to redundancy or as the result of a subtle phenotype. Another approach has been to identify genes that when overexpressed lead to specific phenotypes; for example a functional screen to identify dorsalising factors in *Xenopus laevis* originally identified Xwnt-8 that has subsequently shown to be an integral part of this process (Smith and Harland, 1991). The limitation of gain of function screens is that they can only be employed to identify genes for certain processes, as the methodology requires an amenable bioassay. Both genetic and functional screening procedures analyse a resulting phenotype, and as such have an advantage compared to expression pattern screening (see below) as the gene function(s) can be readily identified.

There are also screens that do not address gene function but assess gene expression patterns. Identification and analysis of a gene's transcript distribution may provide an insight as to the gene's function; expression pattern screening utilizes techniques such as gene traps, microarray and in situ hybridisation. The advantage of gene traps is that the inserted cassette (typically  $\beta$ -galactosidase) allows a clear

visualisation of a genes expression pattern and potentially allows rapid identification a genes function, but this process is technically difficult and slow (Friedrich and Soriano, 1991; Hope, 1991; Wurst, *et al.*, 1995). Microarray screening is a relatively fast technique capable of profiling gene expression patterns in many thousands of genes within a single experiment, but results can vary due to spatial resolution of the hybridisation signal when compared to the in situ hybridisation technique (DeRisi, *et al.*, 1996; Iyer, *et al.*, 1999). In situ hybridisation screens allows for the direct expression analysis of a gene, but is relatively slow compared with microarray screening (Gawantka, *et al.*, 1998; Neidhardt, *et al.*, 2000; Wertz and Herrmann, 2000; Thut, *et al.*, 2001). Using expression pattern screening techniques to identify genes with spatially restricted expression pattern(s) does not necessarily indicate that the gene has an important function within that tissue. For example, the distribution of gene transcripts does not imply that the gene's functional product will have a similar profile due to possible post-translational control. In addition, screens focused on identifying genes with restricted transcript expression domains may overlook genes with more widespread (or diffuse) expression patterns as these molecules may have key functions in a particular sub-domain. The greatest limitation when identifying genes by expression profile screening is that the subsequent functional analysis of an isolated gene is likely to be relatively slow and time consuming, but recent techniques such as RNAi and antisense morpholino technology make these types of screens more feasible.

This chapter describes the results of a combined subtracted hybridisation and whole mount in situ hybridisation screen, to identify molecules with potential roles in the developing cranial neural tissue of the chick (*Gallus gallus*) hindbrain.

### 3.1.1 Rationale of screen

Prior to my arrival, a project was initiated in this laboratory to combine the construction of a non-PCR based subtracted hindbrain cDNA library with a large scale whole mount in situ hybridisation screen. Chick embryos were chosen due to their relatively large embryonic hindbrain that enables the extraction of sufficient material to construct a conventionally cloned cDNA library, thereby circumventing the need for a PCR based amplification step that can introduce bias and generate artifacts (Smith and Gridley, 1992). Two directionally cloned cDNA plasmid

libraries were constructed, one from chicken embryonic hindbrains during the period of hindbrain segmentation (HH stage 9- to 12) and the other from blastula stage whole chicken embryos (prior to the formation of the primitive streak, Stage X: Eyal-Giladi and Kochav, 1975). Subtractive hybridisation (Harrison, *et al.*, 1995) was employed to remove clones common to both the 'pre-streak' stage and hindbrain cDNA libraries to generate a subtracted cDNA chick hindbrain library. The subtraction procedure was envisaged to remove ubiquitously expressed genes and enrich for hindbrain expressed genes and this was confirmed by subsequent analysis prior to my project.

Previously, 106 randomly chosen cDNA clones from the subtracted cDNA library were screened by whole mount in situ hybridisation (J. H. Christiansen and V. Robinson) and 16 clones were found that exhibit restricted expression patterns in the hindbrain or neural crest. In order to present the study in completeness, this data has been included in this chapter. These data have been published (Christiansen, *et al.*, 2001), and in this thesis they have been updated.

### 3.2 COMPLEXITY OF THE SUBTRACTED cDNA LIBRARY

In order to calculate the number of clones required to screen the library to completeness, the number of independent clones within the library was determined. To calculate the complexity of the subtracted cDNA hindbrain library,  $1.2 \times 10^3$  clones were randomly picked and manually gridded onto agar plates. These clones were then lifted onto nylon membranes and screened using radioactively labeled probes made from 9 other randomly chosen clones. The number of positively hybridising colonies per probe ranged from 0 to 2 (Table 3.1), with an average number of 0.6 per  $1.2 \times 10^3$  colonies. This indicates that a particular gene is likely to be represented in the library once in every  $2 \times 10^3$  clones.

Probe	A	B	C	D	E	F	G	H	I
Frequency	1	2	0	2	1	1	0	0	0

Table 3.1: Summary of the frequency of the 9 randomly picked clones used to screen  $1.2 \times 10^3$  clones from the subtracted cDNA library by southern hybridisation

### 3.3 IDENTIFICATION OF CLONES WITH RESTRICTED EXPRESSION PATTERNS IN DEVELOPING CRANIAL NEURAL TISSUE

In order to identify genes with potential roles in neural tissue development, expression patterns of randomly selected clones from the subtracted cDNA hindbrain library were assessed by whole mount in situ hybridisation of chicken embryos. The expression of previously identified hindbrain segmentation genes, such as *Krox20*, first reach detectable levels (by in situ hybridisation) at HH stage 9- and continue to be expressed throughout the period of hindbrain segmentation (Irving, *et al.*, 1996). To analyse expression during both early and late embryonic hindbrain development, at least 3 chick embryos ranging in age from HH 9- to 12 were analysed for each probe. In situ hybridisation screening was performed using an in situ hybridisation robot (Abimed), following optimisation of in situ hybridisation conditions (Appendix B).

In total, 445 digoxigenin (DIG) labeled anti-sense RNA probes were generated from randomly selected clones and used as probes for whole mount in situ hybridisation during this study. Thirty-nine of the 445 clones (9 %) were found to have restricted expression patterns in the midbrain, hindbrain or cranial neural crest (Figure 3.1), with no gene with spatially restricted expression pattern found more than once. Three hundred and thirty seven (76 %) of the clones screened were expressed either at uniform levels throughout the neural tube suggestive of neural epithelial specific genes or exhibited non-regionalised expression at varying levels of intensities in the embryo, suggestive of ubiquitously expressed genes (data not shown). Signal was undetectable in 69 (15 %) of the clones screened (data not shown), which may reflect either low levels of transcript expression or insensitivity of the probe under the hybridisation conditions utilised.

A range of different expression patterns were identified within the developing cranial neural tube with some clones expressed in broad domains, for example within the whole hindbrain (clone 1F9) or midbrain (clone 0B3), others in specific rhombomeres (clones 0A7 and 0A11) and others in specific populations of cells

within particular rhombomeres (clones 0A6, 4E4 and 4G2). Some clones are expressed at the isthmic constriction between the midbrain and hindbrain (clones 0A6, 0B8, 1C6, 3F2 and 3G1), in migratory neural crest cells (2F10), or at sites adjacent to the developing hindbrain e.g. surface ectoderm lateral to r3 (clone 2H10) or head mesoderm (clone 0B2; Figure 3.1).

### **3.4 SEQUENCE ANALYSIS OF CLONES EXHIBITING RESTRICTED DOMAINS OF EXPRESSION**

Clones exhibiting restricted expression patterns were sequenced from both ends using vector specific primers and the resulting sequences were deposited in the Genbank expressed sequence tag database (dbEST; corresponding accession numbers appear in Figure 3.1). In order to determine whether any of the clones exhibiting restricted expression patterns encode proteins with known function or were related to known proteins, conceptual translations of the sequences in all 6 reading frames were compared against the NCBI (tBLASTx; <http://www.ncbi.nlm.nih.gov>) and TIGR (<http://www.tigr.org>) protein databases. Expected (e) values lower than  $10^{-6}$  were taken to represent highly significant sequence similarity suggestive of a homologue or closely related genes, these values are listed in Figure 3.1. Expected values of between  $10^{-2}$  and  $10^{-6}$  are suggestive of more distantly related genes or conserved motifs, and are not included in this thesis.

#### **3.4.1 Genes with previously characterised roles in neural development**

Sequence analysis of three clones that exhibit regionalised neural expression (0B5, 3H10 and 4B3) were shown to represent molecules with previously characterised expression or function during early cranial neural development. Clone 0B5 is expressed caudally along the neural tube from r5 and corresponds to the homeobox containing transcription factor HoxD3. Analysis of murine HoxD3<sup>-/-</sup> embryos has shown that this gene has a role in governing A-P identity of numerous structures including the neural tube and derivatives such as the vertebra and skull (Condie and Capecchi, 1993).

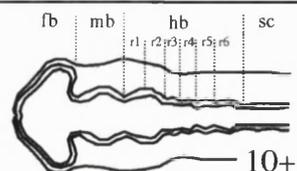


Figure 3.1: Patterns and sequence similarity of screened clones exhibiting restricted expression domains within cranial neural tissue of the developing chick embryo

The right-hand panels show images of whole mount *in situ* hybridisations of each positive clone. The HH stage of each embryo is indicated in the lower right of each photograph. The left-hand side of the figure shows the results of sequence similarity searches of the 39 cDNA clones, those hits greater than  $10^{-6}$  (indicating distant family members or motifs) are not indicated. The clone name allocated during this screen is shown in bold, and the corresponding EST accession numbers (5' and 3' sequence) allocated by Genbank are shown in italics. Sequence similarity values and identities are shown after the appropriate EST accession numbers and similarities refer to the best-hit following protein database searching using the tBLASTx algorithm. The 'e value' is calculated using the Karlin-Altschul statistic (Karlin and Altschul, 1990) and denotes the degree of similarity; smaller numbers denote better matches and a value of 0 indicates a direct match. Where known, the type of protein encoded is designated below accession numbers. The line drawing at the top of the figure shows gross cranial neural tube architecture at HH stage 10+. The inserted image in 0A7 is a cross-section through r5 of a HH stage 11- embryo. Abbreviations: d, dorsal; fb, forebrain; mb, midbrain; hb, hindbrain; r, rhombomere; sc, spinal cord; v, ventral.

**Clone name***Genbank accession number* Sequence similarity; e value

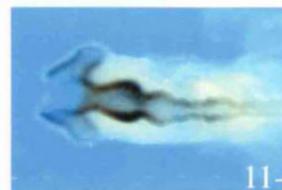
Protein type

**0A2**

*BF724032(5')* Chick brain type creatine kinase (B-CK); e= 0.0  
Enzyme

**0A3**

*BF724033(5')* No significant sequence similarity  
*BF724034(3')* No significant sequence similarity

**0A4**

*BF724035(5')* No significant sequence similarity  
*BF724036(3')* No significant sequence similarity

**0A5**

*BF724037(5')* Human MGC11034; e-32  
*BF724038(3')* No significant sequence similarity  
Hypothetical protein of unknown function

**0A6**

*BF724039* Human FLJ10813; e-40  
Hypothetical protein of unknown function

**0A7**

*BF724040(5')* ] Chick cellular inhibitor of  
*BF724041(3')* ] apoptosis-1 (chIAP1); e= 0.0  
Component of intracellular signalling cascade

**0A8**

*BF724042(5')* Human doublecortin (Dbct); e= 0.0  
*BF724043(3')* Human doublecortin (Dbct); e= 0.0  
Cytoskeleton associated protein

**0A9**

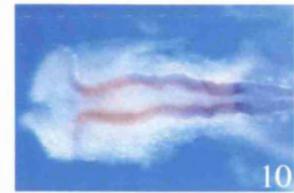
*BF724044* No significant sequence similarity



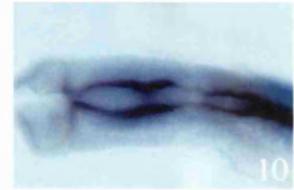
Figure 3.1

**0A10***BF724045(5')* Chick Sin1; e= 0.0*BF724046(3')* Chick Sin1; e= 0.0

Component of intracellular signal cascade

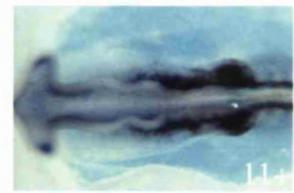
**0A11***BF724047(5')* Mouse haem-regulated inhibitor of translation (HRI); e-159*BF724048(3')* Mouse haem-regulated inhibitor of translation (HRI); e-159

Serine/threonine kinase

**0A12***BF724049* No significant sequence similarity*BF724050(3')* No significant sequence similarity**0B2**

5' sequence No significant sequence similarity

3' sequence No significant sequence similarity

**0B3***BF724051(5')* Human L15344; e-119*BF724052(3')* No significant sequence similarity

Hypothetical protein of unknown function

**0B5***BF724053(5')* Chick HoxD3; e= 0.0

Transcription factor

**0B6***BF724054(5')* No significant sequence similarity*BF724055(3')* No significant sequence similarity**0B7**

5' Sequence No significant sequence similarity

3' Sequence No significant sequence similarity

**0B8***BF724056(5')* No significant sequence similarity*BF724057(3')* No significant sequence similarity

Figure 3.1 (continued)

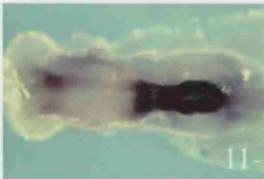
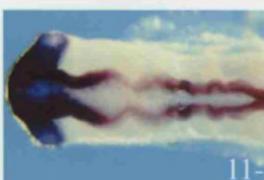
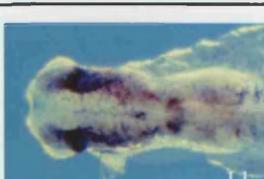
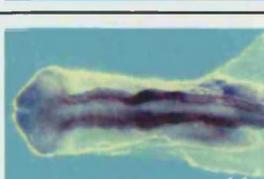
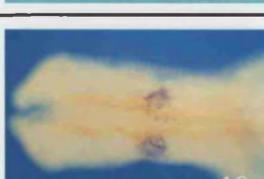
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<p><b>1F9</b>  <i>BF724062(5')</i> Human KIAA0575; e-29  <i>BF724063(3')</i> No significant sequence similarity            Hypothetical protein of unknown function</p>	
<p><b>2B10</b>  <i>BF724064(5')</i> No significant sequence similarity  <i>BF724065(3')</i> No significant sequence similarity</p>	
<p><b>2F6</b>  <i>BF724066(5')</i> No significant sequence similarity  <i>BF724067(3')</i> No significant sequence similarity</p>	
<p><b>2F10</b>  <i>BF724068(5')</i> No significant sequence similarity  <i>BF724069(3')</i> No significant sequence similarity</p>	
<p><b>2H4</b>  <i>BF724070(5')</i> No significant sequence similarity  <i>BF724071(3')</i> No significant sequence similarity</p>	
<p><b>2H10</b>            5' Sequence Human CAB66706; e-19            3' Sequence No significant sequence similarity            Hypothetical protein of unknown function</p>	
<p><b>3D9</b>  <i>BF724072(5')</i> No significant sequence similarity  <i>BF724073(3')</i> No significant sequence similarity</p>	

Figure 3.1 (continued)

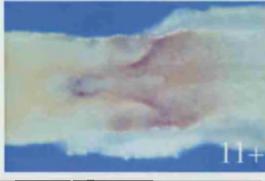
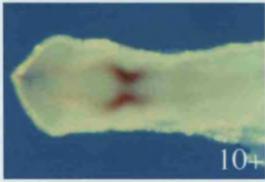
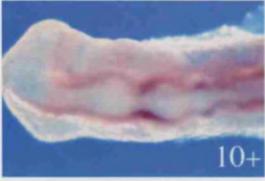
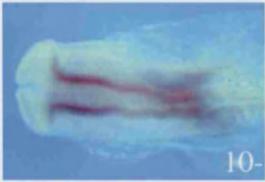
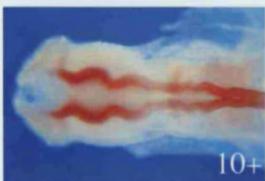
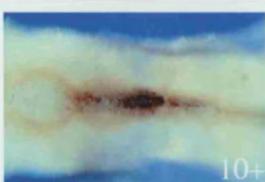
<p><b>3E2</b>  <i>BF724074</i>(5') Human G2; e-72  <i>BF724075</i>(3') No significant sequence similarity  Hypothetical protein of unknown function</p>	
<p><b>3E11</b>  <i>BF773561</i>(5') No significant sequence similarity  <i>BF773562</i>(3') No significant sequence similarity</p>	
<p><b>3F2</b>  <i>BF724076</i> No significant sequence similarity</p>	
<p><b>3F10</b>  <i>BF724077</i>(5') Mouse spermidine synthase (SPDSY); e-33  <i>BF724078</i>(3') No significant sequence similarity  Enzyme</p>	
<p><b>3G1</b>  <i>BF724079</i>(5') No significant sequence similarity  <i>BF724080</i>(3') No significant sequence similarity</p>	
<p><b>3H10</b>  <i>BF724081</i>(5') Chick EphB3; e= 0.0  Receptor tyrosine kinase</p>	
<p><b>4B1</b>  <i>BF724082</i>(5') Mouse ULIP4; e-84  <i>BF724083</i>(3') Mouse ULIP4; e-84  Enzyme</p>	
<p><b>4B3</b>  <i>BF724084</i>(5') Chick Msx1; e= 0.0  Transcription factor</p>	
<p><b>4E4</b>  <i>BF724085</i>(5') No significant sequence similarity  <i>BF724086</i>(3') No significant sequence similarity</p>	

Figure 3.1 (continued)

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**4F10**

*BF724087(5')* No significant sequence similarity  
*BF724088(3')* No significant sequence similarity



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**4F11**

*BF724089(5')* Zebra-finch cannabinoid receptor-1 (CB1);  $e=0.0$   
*BF724090(3')* No significant sequence similarity  
Transmembrane protein



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**4G1**

*BF724091(5')* No significant sequence similarity  
*BF724092(3')* No significant sequence similarity



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**4G2**

*BF724093(5')* No significant sequence similarity



Clone 0B5 encodes the receptor tyrosine kinase EphB3 (Sajjadi and Pasquale, 1993), that is expressed in r3 and r5 of the developing hindbrain. Eph receptors (expressed predominantly in odd numbered rhombomeres) in conjunction with their ligands, the ephrins (expressed predominantly in even numbered rhombomeres), mediate bi-directional signalling that restricts cell intermingling between rhombomeres (Wilkinson, 1995; Wilkinson, 2001).

Clone 4B3 encodes Msx1, a homeobox containing transcription factor that is expressed in the developing embryo at sites of epithelial-mesenchymal interactions including pre-migratory neural crest in the hindbrain (Yokouchi, *et al.*, 1991; Feledy, *et al.*, 1999). *Msx1*<sup>-/-</sup> mice display midline and other craniofacial abnormalities such as cleft palate, due to neural crest developmental defects (Satokata and Maas, 1994).

The identification of these three genes with known roles in neural development suggests that the screening method employed can identify genes of functional importance.

### 3.4.2 Identification of known molecules with previously uncharacterised expression in cranial neural tissues

Nine of the 39 clones identified with restricted expression patterns encode molecules that are identical or closely related to proteins with previously characterised expression, or functions in tissues other than early embryonic neural tissue. These cover a range of molecules including: (i) enzymes, (ii) intracellular components of signalling cascades, (iii) membrane bound receptors, (iv) cytoskeleton associated proteins, and (v) specific inhibitors of other proteins (Figure 3.1 and Figure 3.2).

#### (i) Segmentally expressed enzymes

Four clones encode different types of enzymes (0A2, 0A11, 3F10 and 4B1). Clone 0A2 is expressed throughout the hindbrain except for r5 at varying levels in different rhombomeres and sequence analysis shows it is identical to the chicken *brain-type creatine kinase (B-CK)* gene (Prichard, *et al.*, 1983). The B-CK protein is the brain isoenzyme of creatine kinase and is localised to the nucleoplasm; being highly expressed in the nucleus of proliferating astrocytes suggestive of a role in cell division/nuclear division (Manos and Bryan, 1993). The B-CK enzyme is involved in

energy metabolism transferring high-energy phosphoryl groups from creatine phosphate to the nucleoside diphosphate adenosine diphosphate (ADP), generating adenosine triphosphate (ATP) (Hossle, *et al.*, 1986). B-CK is found in a number of mature tissues and has a documented role during late rat brain development, being expressed during astrocyte cell divisions and myelination (Manos and Bryan, 1993).

Clone 0A11 is expressed strongly in r5, and nucleotide sequence analysis suggests that it is a serine/threonine kinase highly related to the haem-regulated eukaryotic initiation factor subunit-2 alpha (eIF-2 $\alpha$ ) kinase (haem-regulated inhibitor, HRI: Chen, *et al.*, 1991; Berlanga, *et al.*, 1998). Within erythroid tissues, the HRI enzyme modulates the synthesis of globin with hemin and iron availability (Chen, *et al.*, 1991). Subsequent sequencing of a full length clone corresponding to 0A11 (Genbank accession number AF330008) has confirmed that it is highly related to, yet divergent from, HRI (Appendix C) as well as two other eIF2 $\alpha$  kinases: interferon-inducible double-stranded RNA dependent protein kinase (PKR: Meurs, *et al.*, 1990) and pancreatic eIF-2 $\alpha$  kinase (PEK: Shi, *et al.*, 1998). Interestingly, the catalytic domain (a four amino acid motif: Chen, *et al.*, 1991) is conserved between 0A11 and HRI (Appendix C). All three of these kinases modulate mRNA translation in eukaryotes by phosphorylating the eIF-2 $\alpha$  component of the translational initiation complex. This prevents binding of the initiator methionine transfer RNA (Met-tRNA<sub>i</sub>) to the 40S ribosomal subunit and thus inhibits the initiation of protein translation (Kimball, *et al.*, 1999).

Clone 3F10 is expressed throughout the midbrain and hindbrain with highest expression in r1 and exhibits moderate amino acid similarity to the carboxyl terminus of spermidine synthase (SPDSY) (Appendix C: Myöhänen, *et al.*, 1994). The SPDSY enzyme is part of the polyamine biosynthetic pathway and is required for the production of spermidine from its precursor putrescine (Korhonen, *et al.*, 1995). It is known that polyamines have a role in the regulation of cell growth and differentiation, but despite the research undertaken and its relative simple structure, little is known about spermidine's mode of action (Janne, *et al.*, 1991).

Clone 4B1 is expressed in the midbrain, hindbrain (r4 to r6) and in neural crest cells emigrating from r4, albeit at lower levels. In addition, 4B1 exhibits high sequence similarity with the dihydropyrimidinase-related protein ULIP-4 (unc-33 Like Phosphoproteins), an amidohydrolase related to Urease (Holm and Sander,

1997). The *C. elegans unc-33* gene product is an intracellular protein involved in the control of neuritic outgrowth and axonal guidance (Hedgecock, *et al.*, 1985). Determination of the entire sequence of clone 4B1 (Genbank accession number AF330010) shows that it is very highly related to the amino terminal half of ULIP-4 (Appendix C).

(ii) Components of intracellular signal transduction cascades

Two clones represent intracellular components of signal transduction pathways (0A7 and 0A10). Clone 0A7 is expressed at high levels in the midbrain and r5 at HH stage 11-, and within r5 expression is restricted to the ventral two-thirds of the segment (Figure 3.1). Sequence analysis shows that clone 0A7 encodes for a peptide identical to the chick cellular inhibitor of apoptosis-1 protein (ch-IAP1; (You, *et al.*, 1997). Ch-IAP1 is recruited and binds to type 1 or type 2 tumour necrosis factor alpha (TNF $\alpha$ ) receptor after TNF $\alpha$  ligand binding (Shu, *et al.*, 1996). This interaction is required to suppress the transcriptional activation of initiator caspases (Wang, *et al.*, 1998) and ch-IAP1 can also bind directly to certain effector caspases (Roy, *et al.*, 1997), both resulting in an inhibition of apoptosis (Deveraux and Reed, 1999).

Clone 0A10 is expressed at low levels in r3 and r5; initial sequence analysis showed that 0A10 is most similar to JC310 (Appendix C). The human JC310 gene (human Sin1), when overexpressed in *Saccharomyces cerevisiae* (budding yeast), is capable of rescuing a temperature sensitive RAS<sup>val19</sup> mutation, suggesting that Sin1 interacts directly with RAS proteins (Colicelli, *et al.*, 1991). Complete sequencing of 0A10 identified it as the chicken homologue of Sin1 (Sty1 interacting protein), a *Schizosaccharomyces pombe* (fission yeast) protein that interacts with Sty1 (also known as Spc1 or pHH1; (Degols, *et al.*, 1996). Sty1 is a mitogen-activated protein kinase (MAPK), an intermediate in the stress-activated MAP signalling pathway that is required for the induction of mitosis and cell survival in response to changes in extracellular conditions (review see; (Marshall, 1994; Treisman, 1996). The Sin1 protein is phosphorylated following stress in a Sty1 independent manner and is required for stress-dependent transcription via Atf1, the substrate of Sty1/Spc1 (Wilkinson, *et al.*, 1999). Clone 0A10 is functionally equivalent to yeast Sin1 as chimaeric fusions of the two proteins can rescue loss of Sin1 function in yeast

(Wilkinson, *et al.*, 1999). This suggests that chick 0A10 may be required for the propagation of MAPK signalling.

(iii) Transmembrane proteins

Clone 4F11 is expressed in specific dorsal cells posterior to r6 at HH stage 11, and is identical to zebra-finch transmembrane G-protein coupled central cannabinoid receptor-1 (CB1; (Chakrabarti, *et al.*, 1995). Cannabinoid anandamide, the psychoactive ingredient of *Cannabis sativa*, binds to and activates the CB1 receptor (Abood, *et al.*, 1997). Activated cannabinoid receptors transduce signals across the membrane stimulating signal transduction pathways that regulate adenylate cyclase activity, Ca<sup>2+</sup> and K<sup>+</sup> channels and the mitogen-activated protein kinase (MAPK) pathway (review see; (Howlett, 1998). Mice with targeted disruptions in the *CB1* gene are viable but display reduced locomotor activity and hypoalgesia (Ledent, *et al.*, 1999; Zimmer, *et al.*, 1999).

(iv) Cytoskeleton associated proteins

At HH stage 10, clone 0A8 is expressed strongly in r2, r4 and also in migratory r4 neural crest cells and its sequence is highly similar to doublecortin. Mutations in human *doublecortin* result in X-Linked Laminar Heterotopia and Lissencephaly syndrome, a disease that is characterised by abnormal cortical lamination in the cerebral cortex. The disruption of the normal six-layered neocortex is due to the abnormal migration of cortical neurons (des Portes, *et al.*, 1998; Gleeson, *et al.*, 1998). The *doublecortin* protein has been shown to directly bind and stabilise microtubules via interactions with tubulin, and is expressed widely in migrating neurons (Francis, *et al.*, 1999; Horesh, *et al.*, 1999; Gleeson, *et al.*, 1999; Taylor, *et al.*, 2000). Complete sequencing of the 0A8 clone (Genbank accession number AF330009) confirmed that it is the chick homologue of *doublecortin* (Appendix C).

(v) Specific antagonists of other proteins

Clone 1D10, expressed in the fore-, mid- and hindbrain at HH stage 11-, shares moderate amino acid similarity to the amino terminus of human ribonuclease/angiogenin inhibitor protein (RI/RAI: Appendix C and Schneider, *et al.*,

1988). RAI has previously been isolated from adult cerebrum and inhibits both secretory and non-secretory type ribonucleases (Nadano, *et al.*, 1994). RNase activity within a cell is regulated by the formation of RNase/inhibitor complexes and the ratio of RNase to RAI is high in proliferating tissue yet low during catabolic metabolism; the ration of RNase to RAI regulates mRNA turnover and thus protein synthesis (Quirin-Stricker, *et al.*, 1968).

### 3.4.3 Clones encoding for proteins of unknown function

The majority of clones identified with restricted expression domains within developing neural tissue code for proteins of unknown function. Six of these clones: 0A5 (expressed in the forebrain and r3 at HH stage 10-), 0A6 (expressed at the isthmus and midbrain at HH stage 10+), 0B3 (expressed throughout the midbrain at HH stage 11+), 1F9 (expressed throughout the hindbrain at HH stage 11-), 2H10 (expressed in ectoderm lateral to r2/3 at HH stage 10+) and 3E2 (expressed in r3 and r5 at HH stage 11-), exhibit sequence similarities to human hypothetical proteins. These are respectively; MGC11034 (Genbank: direct submission), FLJ10813 (Genbank: direct submission), L15344 (Ambrus, *et al.*, 1993; Ambrus, *et al.*, 1996), KIAA0575 (Genbank: direct submission), CAB66706 (Koehrer, *et al.*, 2000) and, probable mucin protein G2 (U10991; (Foord and Rose, Unpublished). These ‘virtual’ proteins have all been identified by *in silico* analysis of human genomic sequencing data and are of unknown function.

The remaining 21 clones exhibiting restricted expression in cranial neural tissues identified in this screen have no significant sequence similarity to any known protein sequence in public domain databases. These data are summarised in Figures 3.1 and 3.2.

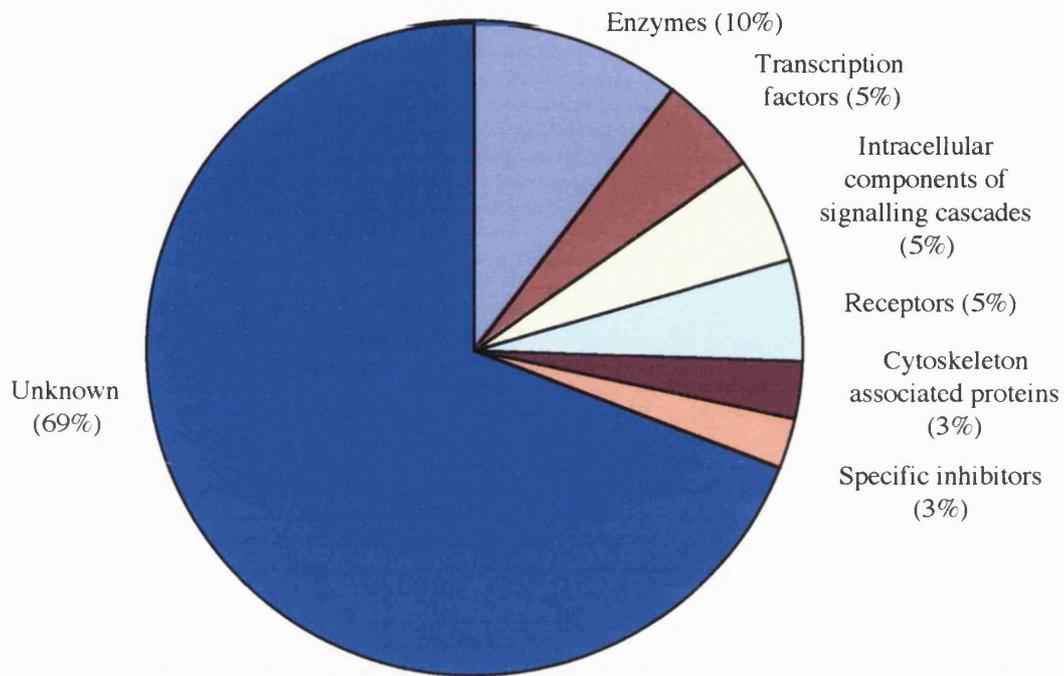


Figure 3.2: Functional classification of clones identified during the screen with expression restricted to cranial neural tissues

ESTs corresponding to 69 % of clones are similar to hypothetical proteins or have no significant sequence similarity and therefore encode proteins of unknown function. Clones coding for molecules with known or inferred functions include: enzymes (10 %); transcription factors (5 %); intracellular components of signalling cascades (5 %); receptors (5 %); cytoskeleton associated proteins (3 %); and specific protein inhibitors (3 %).

### 3.5 DISCUSSION

This chapter describes a whole mount in situ hybridisation screen of a subtracted chick hindbrain cDNA library designed to identify genes with spatially restricted expression during chick neural tissue development.

#### 3.5.1 Use of an in situ hybridisation screen to identify transcripts with spatially restricted expression patterns within developing cranial neural tissue

Until recently, the in situ hybridisation technique has largely been ignored in larger scale screening projects to assess gene expression patterns, largely due to the high amount of 'hands-on' time required for this method. The introduction of automated robots (Plickert, *et al.*, 1997) that have the capacity to carry out multiple in situ hybridisations reactions (as used during this project), greatly reduces the amount of manual processing and increases the viability of undertaking a large scale in situ hybridisation screen. The in situ hybridisation technique allows the spatial and temporal expression pattern of a gene to be assessed and this expression profile may provide insights as to the genes function. Moreover, if an expression pattern of a gene correlates with another gene it may provide evidence of a functional relationship. The relevance of this approach has been demonstrated recently when a novel gene was identified during a large scale in situ hybridisation screen aimed at investigating gene expression during early *Xenopus* development (Gawantka, *et al.*, 1998). The unknown gene shared an almost identical expression profile to bone morphogenetic protein 4 (*BMP-4*) and characterisation of this novel gene, termed BAMBI (BMP and Activin Membrane-Bound Inhibitor), demonstrated that it is a pseudoreceptor and inhibitor of TGF- $\beta$ /BMP signalling (Onichtchouk, *et al.*, 1999). Recently the term 'synexpression groups' has been coined to describe genes that function in the same process and share spatial expression pattern in a number of different tissues (Niehrs and Pollet, 1999). No synexpression groups were identified during this in situ hybridisation screen, but one clone (OB7) is expressed in a domain that overlaps with *BMP-4*. Clone OB7 (cE13) has been investigated and these data are presented in Chapters 4, 5 and 6.

Individual clones sourced from the subtracted chick hindbrain cDNA library were used to generate probes for use in a whole mount in situ hybridisation screen. Almost 9 % of the screened clones have been found to have expression profiles characterised by restricted expression within chick embryo cranial neural tissue. Comparing the percentage of genes with restricted expression patterns with other in situ hybridisation screens suggests that this screen has a similar efficacy. A whole mount in situ screening strategy employed to identify novel genes in the developing murine gonads identified 8.6 % of the total number of differential expressed clones (identified using a subtractive procedure) with restricted expression patterns (Wertz and Herrmann, 2000). However, these findings differ to two other large-scale whole mount in situ hybridisation screens; one of a blastula stage *Xenopus* cDNA library where 26 % of the randomly selected clones from the unbiased library exhibited restricted expression domains (Gawantka, *et al.*, 1998); and in another screen in the developing murine embryo, 18 % of clones identified (using a subtractive procedure) exhibited restricted expression patterns (Neidhardt, *et al.*, 2000). Although the basis of these differences is unclear it may reflect the type of enrichment strategy that was utilised. Sequence analysis of clones identified in these screens with spatially restricted expression patterns suggests the proportion of clones identical to or highly related to sequences in databases (76 % (Gawantka, *et al.*, 1998); 66 % (Neidhardt, *et al.*, 2000); and 52 % (Wertz and Herrmann, 2000)) are much higher than in this screen (31 %). This finding may reflect that there are far more mouse and *Xenopus* sequences in public domain databases than chick.

The subtracted chick hindbrain cDNA library was found to have a complexity of one clone in every  $2 \times 10^3$  clones screened. This project has screened less than a quarter of the library and has identified 39 clones exhibiting regionalised expression, and therefore theoretically between 100 and 200 clones with restricted expression patterns within developing neural tissues remain to be identified within the cDNA library.

### 3.5.2 Identification and sequence analysis of genes exhibiting restricted domains of expression within embryonic cranial neural tissue

Clones that exhibited regionalised domains of expression were sequenced from both the 5' and 3' ends and this routinely produced at least 700 nucleotides of

high-quality sequence data. ESTs identified that exhibit restricted domains of expression but do not share any similarity to known molecules will at least serve as useful markers.

Sequence analysis of the 39 clones with regionalised expression shows that three clones correspond to genes (*HoxD3*, *EphB3* and *Msx1*) with previously known expression and functions within the developing hindbrain. The identification of these clones confirms the validity of the subtractive hybridisation and whole mount in situ hybridisation screening approach to identify genes with essential roles during chick embryonic cranial neural development.

Based on sequence similarities of the clones identified with known or predicted functions, four encode enzymes, two encode transcription factors, two intracellular components of signalling cascades, two transmembrane receptors, one a cytoskeleton associated protein and one a specific antagonist. From this, one may expect there to be a similar diversity of functions in the 27 clones that have no assigned identities, but molecular identification of these clones will not be possible until more data has been deposited in public domain databases such as the new chick EST database (<http://www.chick.umist.ac.uk>), and/or by obtaining further sequence data of these clones using gene specific primers. The number of clones identified with similarity to hypothetical proteins of unknown function has increased since the original analysis of the sequences (Christiansen, *et al.*, 2001), due to the speed and amount of data generated by the human genome sequencing project.

### 3.5.3 Enzymes exhibiting segmental expression patterns in the developing chick hindbrain

Recently the zebrafish homologue of B-CK has been cloned and its expression pattern is reported to be highly dynamic. Zebrafish embryos with 10 pairs of somites express B-CK in a dorsal band of r3 and the trigeminal ganglia (Dickmeis, *et al.*, 2001). B-CK in chick embryos with 14 pairs of somites (HH stage 11-) is expressed throughout the hindbrain at varying levels, but intriguingly not in r5. It would be interesting to extend the chick B-CK expression profile to determine if B-CK expression is conserved between the species. This raises the question why is an enzyme associated with energy metabolism expressed at different levels in different rhombomeres. One reason may be due to different energy requirements within a

particular rhombomere or subpopulation of cells within a rhombomere; for example the requirement for B-CK could be due to an increase in mitotic rate.

#### 3.5.4 Central cannabinoid receptor-1 maybe expressed in early reticular neurones

Clone 4F11, is highly related to zebra-finch transmembrane G-protein coupled central cannabinoid receptor-1 (CB1; (Chakrabarti, *et al.*, 1995), and is likely to be the chick homologue. The cannabinoid ligand binds and activates the CB1 receptor, initiating a number of intracellular signal transduction pathways (Abood, *et al.*, 1997; Howlett, 1998). In the adult brain, the CB1 receptor is expressed in neurons in the hippocampus, substantia nigra and nerve fibers; it is expressed throughout the forebrain but is distributed only sparsely in the hindbrain (Moldrich and Wenger, 2000). Within the developing hindbrain the first neuronal cell type to be detected by neurofilament staining are the reticular neurones at HH stage 13 (Lumsden and Keynes, 1989; Glover and Petursdottir, 1991). Clone 4F11 is expressed caudally from r6 at HH stage 11, and these data suggest that the chick CB1 gene is expressed in early reticular neurons and is an early marker for differentiating neuronal cell-bodies in the developing chick hindbrain. At this time however, it is unclear if CB1 is expressed in early differentiating reticular neurons as co-labeling/mapping has not been performed. Another possibility could be that the chick CB1 receptor is expressed in a different (neuronal) cell type.

#### 3.5.5 Co-expression of apoptotic regulatory genes in rhombomere 5

In the chick hindbrain, neural crest cells migrate away from the dorsal neural tube in a highly patterned manner forming three distinct streams. Neural crest cells from r1 and r2 migrate towards the first branchial arch (BA), r4 crest cells enter BA2 and crest from r6 migrates into BA's 3, 4 and 5, resulting in neural crest free regions adjacent to rhombomeres 3 and 5 (Lumsden, *et al.*, 1991). All rhombomeres produce neural crest cells, but r3 and r5 produce significantly less neural crest cells than their even-numbered neighbouring rhombomeres (Graham, *et al.*, 1993). One mechanism proposed to explain this is the apoptotic elimination of neural crest cells from r3 and r5, *BMP-4* is expressed in dorsal r3 and r5 and can induce the homeobox gene *Msx2* (Graham, *et al.*, 1993; Graham, *et al.*, 1994). *Msx2* has been shown to be sufficient

to induce the apoptotic elimination of neural crest from all rhombomeres (Takahashi, *et al.*, 1998). Two clones identified during this screen (0A7 and 0A11) that are expressed in r5 encode for peptides that are identical to or highly related to proteins with roles in regulating cell death (ch-IAP1 and eIF2 $\alpha$  kinase-related, respectively).

Clone 0A7 encodes for chick cellular inhibitor of apoptosis-1 protein (ch-IAP1) which has previously been shown to suppress apoptosis (You, *et al.*, 1997). Apoptosis is mediated in part by caspases, which are proteases that lead to the disassembly of structural proteins within the cell (Thornberry and Lazebnik, 1998). Caspase activation occurs in a cascade dependent manner with pro-apoptotic signals from cytotoxic agents activating death receptor signalling, which leads to the activation of 'initiator' caspases (inert precursors), which then hydrolyse and activate 'effector' caspases that ultimately disassemble the cell. Chick IAP1 can attenuate the apoptotic process in two ways (Figure 3.3). Intracellular ch-IAP1 is recruited to the Tumour Necrosis Factor alpha receptor-1 (TNF $\alpha$ R1) following TNF binding and is part of a signal transduction cascade (Ashkenazi and Dixit, 1998) that leads to the phosphorylation and degradation of the Inhibitor of  $\kappa$ B (I- $\kappa$ B). Following this, active NF- $\kappa$ B complex is released from the I- $\kappa$ B/NF- $\kappa$ B complex and is translocated to the nucleus allowing the transcriptional activation of numerous survival genes, including ch-IAP1 itself (Wang, *et al.*, 1998). Alternatively, ch-IAP1 can directly bind to and inhibit certain effector caspases (Figure 3.3: Roy, *et al.*, 1997).

Clone 0A11 encodes a novel serine threonine kinase that is highly similar (and has a conserved catalytic domain) to a family of kinases that phosphorylate the 2 $\alpha$  subunit of the eukaryotic initiation factor (eIF2 $\alpha$ ). These include the haem-regulated inhibitor of translation (HRI: Chen, *et al.*, 1991), the interferon-inducible double-stranded RNA dependent protein kinase (PKR: Meurs, *et al.*, 1990) and pancreatic eIF2 $\alpha$  kinase (PEK: Shi, *et al.*, 1998). The 2 $\alpha$  subunit of eukaryotic initiation factor normally binds both guanosine 5'-triphosphate (GTP) and the initiator methionine-transfer RNA (Met-tRNA<sub>i</sub>) to present and transfer Met-tRNA<sub>i</sub> to the 40S subunit of the ribosome. During this process, GTP is hydrolysed to guanosine 5'-diphosphate (GDP) and in order to allow a second round of translational initiation to occur, GDP must be exchanged for GTP on the eIF2 $\alpha$  subunit by a second initiation factor, eIF2B. Phosphorylation of eIF2 $\alpha$  results in its

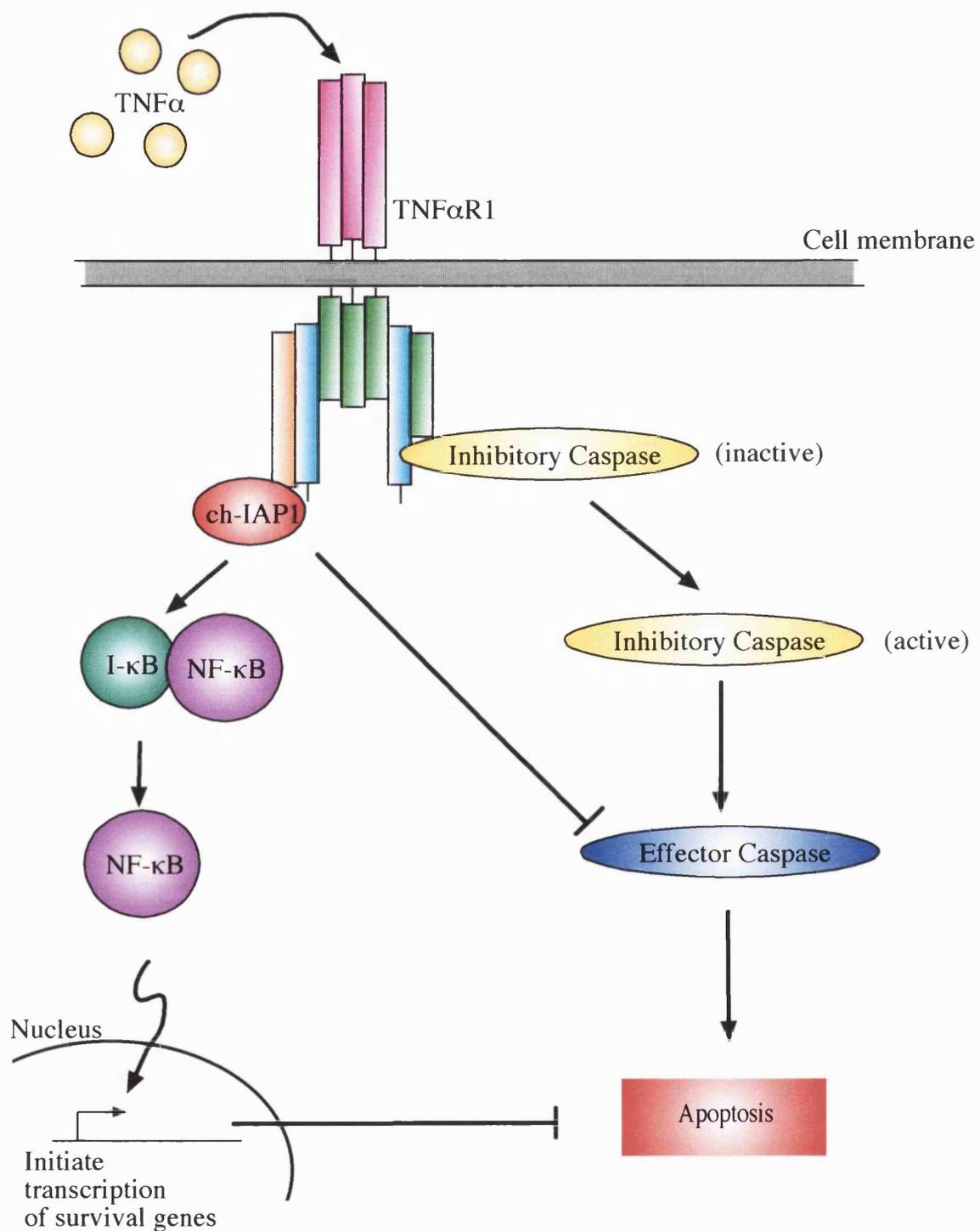


Figure 3.3: Anti-apoptotic role of ch-IAP1 in the TNF $\alpha$  signalling pathway

Cartoon illustrating the inhibitory apoptotic function of ch-IAP1 in the TNF $\alpha$  signalling pathway; see text for details.

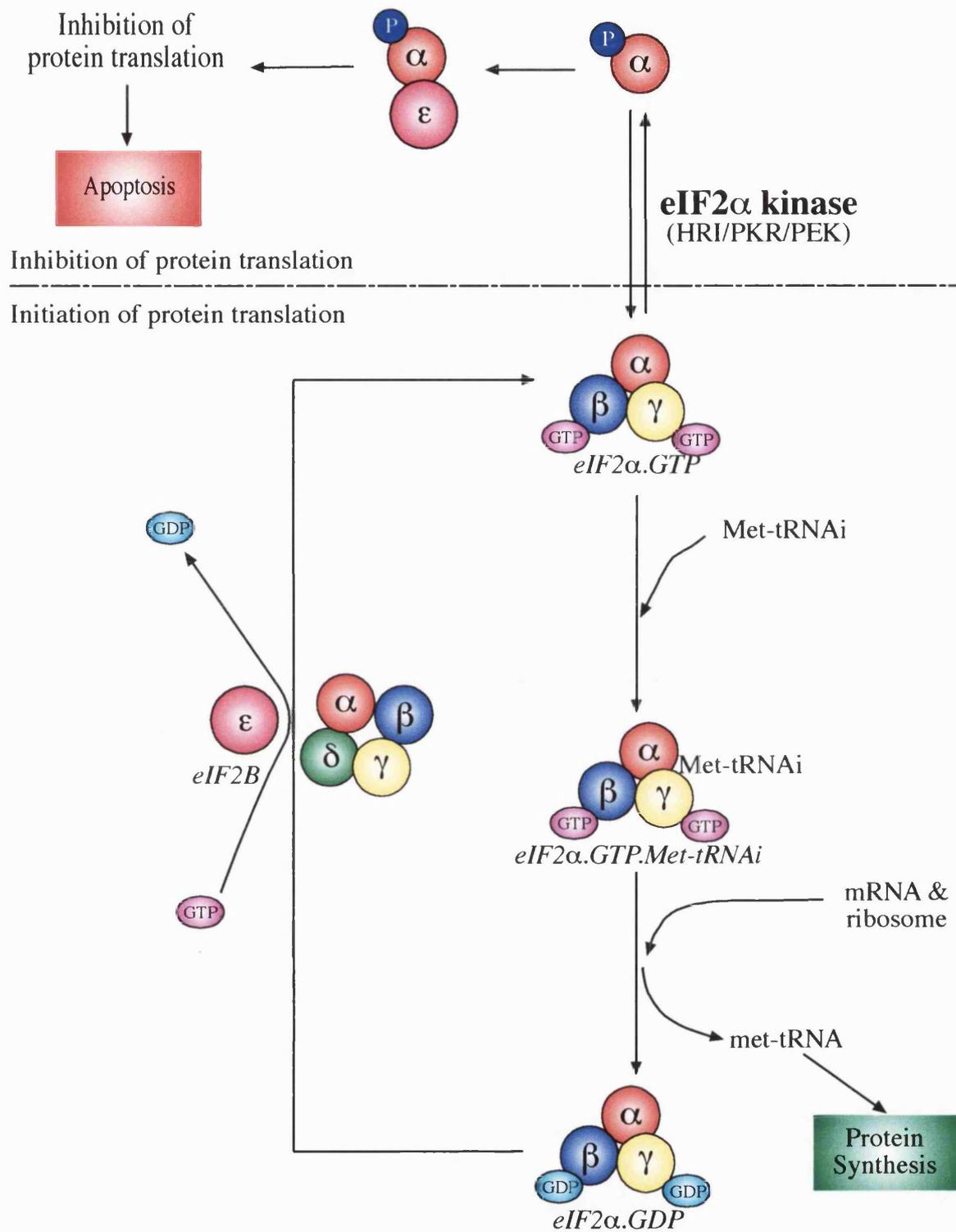


Figure 3.4: Inhibition of protein translation and cell death by eIF2 $\alpha$  kinase

Cartoon illustrating the position of eIF2 $\alpha$  kinase relative to the initiation of protein synthesis and its role in impeding translation that ultimately results in apoptosis; see text for details.

binding and sequestering eIF2B which subsequently leads to an inhibition of protein synthesis (Kimball, *et al.*, 1999). One direct effect of inhibiting protein translation via the phosphorylation of eIF2 $\alpha$  by eIF2 $\alpha$  kinase can be the induction of apoptosis (Figure 3.4: Lee and Esteban, 1994; Srivastava, *et al.*, 1998).

In rhombomere 5, eIF2 $\alpha$  is expressed at similar levels along the entire dorso-ventral axis of the neural epithelium, whereas the expression of chIAP1 is found to be limited to the ventral two-thirds of the neural tube (Figure 3.1; 0A7). Given that eIF2 $\alpha$  kinase can potentially phosphorylate eIF2 $\alpha$ , a process that can subsequently promote apoptosis, the expression of eIF2 $\alpha$  kinase in r5 suggests that this rhombomere is inherently programmed to undergo apoptosis. The overlapping expression of ch-IAP1 in the ventral two-thirds of r5 could protect these cells from apoptotic elimination, leaving the presumptive dorsal neural crest cells vulnerable to programmed cell death (Figure 3.5). It would therefore be interesting to investigate the putative roles eIF2 $\alpha$  kinase and ch-IAP1 may play in this process, and to determine if this process is related to the BMP/msx cell death pathway. One experiment to investigate if apoptosis is inhibited in r5 would be the overexpression of ch-IAP1 by *in ovo* electroporation and subsequent analysis to determine the relative levels of cell death.

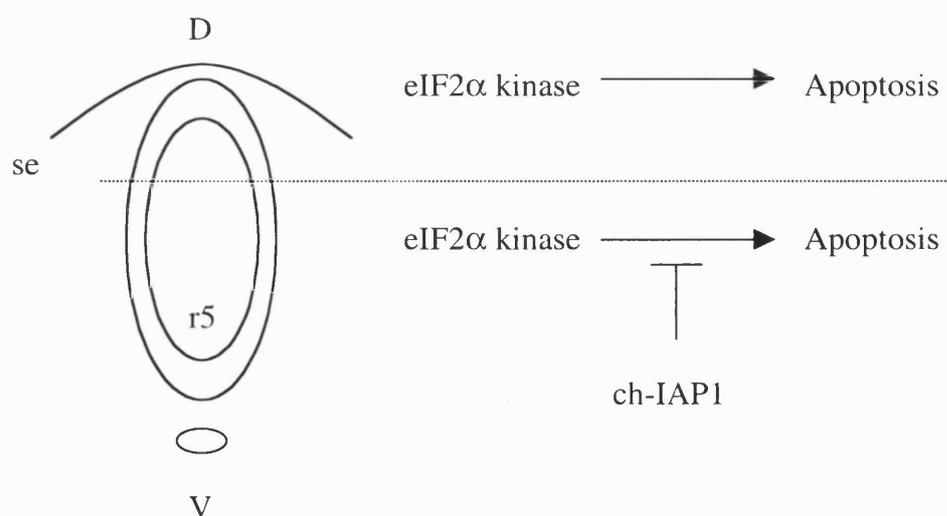


Figure 3.5: Proposed relationship between eIF2 $\alpha$  kinase and ch-IAP1 in r5

Abbreviations: D, dorsal; r5, rhombomere 5 lumen; se, surface ectoderm; V, ventral. Dotted line denotes dorsal limit of ch-IAP1 expression domain.

### 3.5.6 Genes expressed in neural crest migrating from rhombomere 4

Clone 0A8 is initially expressed in r2, r3 and r4, and then later is expressed at high levels in r2, r4 and in neural crest cells migrating from r4. Sequence analysis suggests that 0A8 is likely to represent the chick homologue of *doublecortin* (Appendix C and (Christiansen, *et al.*, 2001). *Doublecortin* was first identified by virtue of its mutation in the human disorder X-linked lissencephaly (des Portes, *et al.*, 1998; Gleeson, *et al.*, 1998). This disease is classified as a Type I lissencephaly and is characterised by the presence of four cortical layers in contrast to unaffected individuals who have six (Barkovitch, *et al.*, 1996). During the third and fourth month of human development, normal cortical layering is established by a series of migratory waves of neuronal cell bodies. The phenotype displayed in X-linked lissencephaly can be attributed to the failure of almost all migratory cortical neurons to reach their usual destination (Barkovitch, *et al.*, 1996). The *doublecortin* gene encodes an extracellular protein that contains several potential phosphorylation sites (Gleeson, *et al.*, 1998) and one tubulin binding domain (Taylor, *et al.*, 2000). The doublecortin protein associates with microtubules, and increases their bundling and stability (Horesh, *et al.*, 1999). *Doublecortin* is widely expressed in migrating cortical neurons, and loss of *doublecortin* function results in disruption of neuronal migration (Barkovitch, *et al.*, 1996). This suggests that the doublecortin protein influences neuronal migration by exerting an effect on the cytoskeleton which leads to changes in cell shape. In situ hybridisation experiments described in this chapter show that chick *doublecortin* is expressed in a set of migratory neural crest cells that emerge from r4. Motility of neural crest cells, like migrating neurons, is governed by changes in cell shape (Le Douarin and Kalcheim, 1999). Since *doublecortin* is expressed in migratory neural crest cells, this protein may also play a role in mediating motility of migratory neural crest cells through changes in cell shape. However, since *doublecortin* is only expressed in a subset of migratory neural crest cells, it is unlikely that the gene product has a universal role in mediating neural crest cell migration. Similarly, because individuals with X-linked lissencephaly are not missing any r4-derived craniofacial structures (Barkovitch, *et al.*, 1996), it is also unlikely that *doublecortin* is a necessary component required in r4 derived neural crest cell migration. Further analysis is still required to ascertain what role, if any,

the doublecortin protein plays in neural crest cell migration, but as *doublecortin* is also expressed in many non-migratory cells (for example r2 and r4), the gene product may also regulate other microtubule-dependent and/or independent processes.

Growth cones at the tips of developing axons are guided by both attraction and repulsion molecules, and include Nectrin (Serafini, *et al.*, 1994; Colamarino and Tessier-Lavigne, 1995) and Semaphorin protein families (Kolodkin and Ginty, 1997; Luo, *et al.*, 1993). These extracellular guidance molecules are well studied, but less is known about the intracellular signalling molecules that potentiate these signals. Some intracellular components have however, been identified such as the ULIP protein family (Unc-33 Like Phosphoproteins; (Hedgecock, *et al.*, 1985). Clone 4B1 is expressed in migratory neural crest cells derived from r4, and sequence analysis found it to be highly similar to the neural specific intracellular ULIP proteins. The ULIP proteins contain several highly similar motifs and subdomains that have been highly conserved throughout evolution (Hedgecock, *et al.*, 1985; Byk, *et al.*, 1996). Sequence comparison of the complete 4B1 clone shows that it is most related to ULIP4 (Dihydropyrimidase related protein-4; Drp4), but amino acid alignment of clone 4B1 identified a region of approximately 250 residues missing at the carboxyl terminus (Appendix C and (Christiansen, *et al.*, 2001). This region is conserved in all other ULIP family members and therefore clone 4B1 may represent an alternatively spliced product.

ULIPs are strongly expressed in neuronal growth cones of the developing brain and in regions of the neuromuscular junction of the adult (Byk, *et al.*, 1998). A number of ULIP proteins have been shown to regulate neuronal outgrowth including *unc-33*, which when mutated in *C. elegans* results in motor un-coordination and defective patterns of axonal outgrowth due to microtubule anomalies (Hedgecock, *et al.*, 1985; Hedgecock, *et al.*, 1987). Other family members include the early marker of differentiating rat cortex neurons ULIP2 (Toad-64, Turned On After Division; (Minturn, *et al.*, 1995), and chick ULIP4 which when phosphorylated is essential for collapsin-mediated neuronal growth cone collapse (Goshima, *et al.*, 1995). Overexpression of a related ULIP family member, human ULIP2 (CRMP-2) in a mouse neuroblastoma cell line demonstrates that ULIP2 associates with microtubules and mediates cell shape changes via interactions with the cytoskeleton (Gu and Ihara, 2000).

As well as regulating repulsive guidance cues for axonal outgrowth, Collapsins have been implicated in controlling neural crest cell migration (Eickholt, *et al.*, 1999). Collapsin-1 is localised to territories adjacent to cranial and trunk neural crest cell migratory pathways, its receptor neuropilin-1, is expressed in migratory crest cells (Shepherd, *et al.*, 1996; Eickholt, *et al.*, 1999). *In vitro* assays using recombinant collapsin-1 protein demonstrate that collapsin-1 acts in an inhibitory manner on the migration of neural crest cells by inducing morphological changes in the migratory crest cells, reminiscent to those that occur during growth cone collapse (Eickholt, *et al.*, 1999). Clone 4B1 is expressed in migratory r4 neural crest cells that co-express neuropilin-1, raising the possibility that ULIPs may mediate collapsin-induced repulsion of migrating neural crest cells.

Chapters 4, 5 and 6 describes the sequence analysis, transcript distribution, and functional analysis of clone 0B7 (cE13) that was identified during this screen, and the experiments aimed at elucidating its function within the developing embryo.

## CHAPTER FOUR

### ISOLATION & SEQUENCE ANALYSIS OF E13

#### 4.1 INTRODUCTION

Cranial neural development involves the coordinated functions of many molecules (Chapter 1), but due to the complexity of the nervous system and the relatively few genes that have been identified more remain to be identified. To address this, an approach was undertaken to identify new genes that are involved in neural patterning by screening a subtracted chick hindbrain cDNA library by whole mount in situ hybridisation (as described in Chapter 3). A number of genes were identified that exhibit restricted domains of expression within developing neural tissues. Clone 0B7 was identified during this screen by virtue of its restricted expression pattern in the dorsal neural tube at sites that correspond to a population of developing neural crest cells.

This chapter describes the isolation and sequence analysis of an 0B7 cDNA with an entire open reading frame (cE13), and the identification and sequence analysis of mouse and zebrafish homologues.

## 4.2 MOLECULAR CHARACTERISATION OF CHICK E13

### 4.2.1 Isolation of chick E13

Nucleotide sequencing of clone 0B7 showed that this clone comprises 562 bp of sequence and conceptually translating this sequence suggested that a complete open reading frame (ORF) was not present. In order to isolate a cDNA clone with a longer ORF, a radiolabeled probe was generated from clone 0B7 and used to screen a whole chick 12 - 15 somite stage cDNA library constructed in  $\lambda$ ZAP (M. A. Nieto). The library was screened by filter hybridisation and six positively hybridising clones were identified, the largest clone of which was found to encompass an insert of approximately 2.6 kb (called cE13). Nucleotide sequencing of the entire insert by sequence walking using gene specific primers was obtained in both the sense and anti-sense strands along their entire length.

### 4.2.2 Structure of the chick E13 cDNA

The cDNA clone isolated from the  $\lambda$ ZAP library (cE13) contained 2604 nucleotides of sequence. Sequence analysis of cE13 identified several potential open reading frames, the longest of which spans 2036 bases between nucleotide positions 127 and 2163 (Figure 4.1). Conceptual translation of this open reading frame predicts a protein comprised of 678 amino acid residues with a calculated molecular weight of 75026 Daltons (Da). The cE13 cDNA contains a putative ATG start codon with a consensus Kozak translational initiator sequence (GCCACCatgG) (Kozak, 1996) and an in-frame stop codon 75 bp upstream of the ATG initiation codon, which suggests that the start codon at nucleotide position 127 is the true site of translational initiation. This prediction has been substantiated by experiments in which synthetic cE13 mRNA was translated *in vitro*; a protein product with a molecular weight of approximately 75 kDa was generated and this correlates with the predicted size of the protein (Chapter 6, Figure 6.3; A). The termination codon, amber, is followed by a 420 nucleotide untranslated region (UTR) ending with a poly (A) tail that is preceded 24 bp upstream by the AATAAA consensus polyadenylation signal.



Figure 4.1: Nucleotide sequence and predicted amino acid sequence of chick E13

The chick E13 cDNA clone contains 2604 nucleotides of sequence, the longest predicted open reading frame is preceded by the favourable Kozak translational initiation sequence (in bold). Numbers on the right of the sequence indicate the positions of nucleotides and amino acids. The open reading frame is indicated in triplet codons and the predicted amino acids are shown as single-letter abbreviations. The 678 amino acid protein encoded by cE13 has a predicted molecular weight of 75026 Da, the putative translational initiation codon (ATG) is at nucleotide position 127 and stop codon (amber; TGA) is at position 2163 and is indicated by an asterisk. The putative polyadenylation signal is boxed in grey and the poly(A) tail is shown in italics. The hydrophobic secretory signal amino acid sequence is in turquoise and the signalase cleavage site is indicated by an arrow-head. Six potential sites of N-linked glycosylation are underlined in black and the potential multimerisation motif (CGLCG) is underlined in red. The predicted cE13 protein also contains five cysteine-rich repeat (CR) motifs that are identified in green and a von Willebrand Factor type D (VWFD) domain in red.

TGGGACTCCC CGACGGGCGG GCATCTCCCT TCCTCCCCC GGGCCCCCC GTAACCCAGG GCCGGGGTTC GGCGCGGGCT CCGGCGTCCC GCAGGCAGCG 100  
 GCACCCGCGG CGGGCAGCCC **GGGACC ATG CCC** CGG CCC GGC GGG AGC CCG GCG GGG ACA CCC GGC CTC GTC CCG GCC GCT GCC GTC CTC CTG GGC 195  
 M P R P G G S P A G T P G L V P A A A V L L G 23  
 GCC CTC TGC CTC CCC GGG GCG CCG GCC TCC TCC CTG CTC ACA GGA TCC ATT GCA AAA TGT GAA AAC GAA GGG GAA ATT CTA CAG ATA CCA 285  
 A L C L P G A P **▲** S S L L T G S I A K C E N E G E I L Q I P 53  
 TTT ATC ACA GAC AAC CCT TGT ATA ATG TGT GTT TGC CTG AAT AAG GAA GTG ACC TGC AGA AGA GAG AAG TGT CCC CTG CTT TCT AAG GAA 375  
 F I T D N P C I M C V C L N K E V T C R R E K C P L L S K E 83  
 TGT GCT CTT GTC ATT AAG CAG AGA GGA GCT TGC TGT GAA CGT TGC AAA GAC TGC ACT TTT GGA GGA AAG ACA TAC AAC AGC TCT ATG AGA 465  
 C A L V I K Q R G A C C E R C K D C T F G G K T Y N S S M R 113  
 TGG CAC CTC CCT AGC AAT CCC TGT ATT ACA TAC CAG TGC CAG GAG GGA GTG ATA ATA GAG TCG GAG GTA CAG TGT GTT GTT CAT TGC AAA 555  
 W H L P S N P C I T Y Q C Q E G V I I E S E V Q C V V H C K 143  
AAC CCA TCC AAA GTT GTG GGG ATG TGT TGC CCT GTG TGT CCA GGT TGT ATA TTT GAA GGG AGA CAT TAC AAT GAA GGA GAA GAA TTT AGG 645  
 N P S K V V G M C C P V C P G C I F E G R H Y N E G E E F R 173  
 CCT GAA GGA AAT AAA TGC ACC AAG TGC TCT TGT GTT GGT GGC AGG ACC CAG TGC ATC CAA GAA GTC TGC CCC ATT CTC TCG TGT CCC CAG 735  
 P E G N K C T K C S C V G G R T Q C I Q E V C P I L S C P Q 203  
 CAC CTC AGT CAC ATT CCT GCA GGA CAG TGT TGC CCC AAA TGC TTA GGA CAG AGG AAA GTG TTT GAC CTC CCA TTT GGA AGC TGT CTC TTT 825  
 H L S H I P A G Q C C P K C L G Q R K V F D L P F G S C L F 233  
 CAT AGC AAT GTG TAT GAT AAT GGA TCC TCA TTT ATT TAT GAC AAC TGT ACA GTG TGT ACA TGC AAG GAT TCA ACA GTG ATA TGT AGG AAG 915  
 H S N V Y D N G S S F I Y D N C T V C T C K D S T V I C R K 263  
 CGG TGC TTG CTT CCT GGA GAG TGC AAT AAA AAC CAA GAC CAC TGC TGC AAG GAA TGT GTC TCC TAC ATC ACT CCT GAA GAA ATG AAA GTG 1005  
 R C L L P G E C N K N Q D H C C K E C V S Y I T P E E M K V 293  
 TGC AAG TTT GGC AAT AAG ATA TTC CAG GAT GGA GAG ATG TGG TCC TCT GTG AAC TGC ACC ATC TGT GCT TGT GTG AAA GGC AAG ACA GAG 1095  
 C K F G N K I F Q D G E M W S S V N C T I C A C V K G K T E 323  
 TGT CGC AAG AAG CAA TGC ATT CCA GTC AAC AGC TGT CCT CAT GGT AAA ATC CTG AAC AGA AAA GGA TGC TGT CCT ATT TGC ACT GAA AAG 1185  
 C R K K Q C I P V N S C P H G K I L N R K G C C P I C T E K 353  
 CCT GGA GTT TGC ACT GTG TTT GGA GAC CCC CAC TAC AAC ACT TTT GAT GGA CGG ACA TTT AAC TTT CAG GGA ACA TGT CAG TAC GTT TTG 1275  
 P G V C T V F G D P H Y N T F D G R T F N F Q G T C Q Y V L 383  
 ACG AAA GAC TGC TCC TCC TCT GCC TCG CCC TTC CAG GTG CTG GTG AAA AAC GAT GCC CGT CGG ACC CGT TCT TTC TCC TGG ACA AAG TCA 1365  
 T K D C S S S A S P F Q V L V K N D A R R T R S F S W T K S 413  
 GTG GAC CTT GTG TTG GGC AGA AGC ACC ATC AGC CTC CAG CAG CAC CTC ACA GTG AAG TGG AAT GGG ACC CGT ATC TCA CTA CCC TGC GAG 1455  
 V D L V L G R S T I S L Q Q H L T V K W N G T R I S L P C E 443  
 ACA CCA CAA TTT CAG ATC GAT TTG GAT GGT TAT TTG CTG AAA GTG ACC ACC AAA GCA GGC CTG GAA ATC TCA TGG GAC GGA GAC AGT TTT 1545  
 T P Q F Q I D L D G Y L L K V T T K A G L E I S W D G D S F 473  
 GTG GAA GTC ATG GCT GCA CCA CAT CTG AAG GGC AAA CTC TGT GGC CTG TGT GGT AAC TAC AAT GGG CAC AAG CGA GAT GAC CTG ATT GGG 1635  
 V E V M A A P H L K G K L C G L C G N Y N G H K R D D L I G 503  
 GGG GAT GGG AAT TTT AAG TTT GAC GTG GAC GAC TTT GCT GAA TCC TGG CGT GTG GAG TCT AAT GAG TTC TGC AGT CGC CCG CAG AGG AAG 1725  
 G D G N F K F D V D D F A E S W R V E S N E F C S R P Q R K 533  
 CCT GTG CCT GAG CTC TGC CAT GGG ACT GTC AGG GTG AAA CTC CGG GCT CAC CGG GAA TGC CAG AAG CTC AAA GCC TGG GAT TTT CAG AGC 1815  
 F V P E L C H G T V R V K L R A H R E C Q K L K A W D F Q S 563  
 TGT CAT TCA ACG GTG GAT TAC ACA ACT TTT TAC CGG TCA TGT GTG ACA GAC ATG TGT GAG TGC CCA GTC CAT AAG AAC TGC TAC TGC GAG 1905  
 C H S T V D Y T T F Y R S C V T D M C E C P V H K N C Y C E 593  
 TCA TTC CTG GCC TAT GCT CGA GCC TGC CAG CGG GAA GGG CTG AGA GTG CAG TGG ATA CCT GAG CAG CAC TGT GCA GCA ACC CAG TGT AAA 1995  
S F L A Y A R A C Q R E G L R V Q W I P E Q H C A A T Q C K 623  
 CAC GGT GCA GTT TAT GAC ACC TGT GGT CCG GGA TGC GTC AAA ACA TGC GAT AAC TGG AAT GAG ATT GGC CCA TGC AAC AAG CCC TGC GTT 2085  
 H G A V Y D T C G P G C V K T C D N W N E I G P C N K P C V 653  
 GCA GGA TGC CAC TGT CCA GCA AAT TTA GTT CTT CAC AAA GGA AGA TGC ATC AAG CCA GTC CTA TGT CCT CAG CGA TGA CATTGTGCC 2173  
 A G C H C P A N L V L H K G R C I K P V L C P Q R \* 678  
 CTCTCCATGG ACACAAATGC GGGTGTCACT GACCCATCTG CAGCTCACAG ACAGCAAAGG ACTGCCGCTG TTCATGTGCA GGTCTGCGA AGTACAGCTC 2273  
 TACTTACAGA GTGTAATAT GTCCTGTGT GTATTCATGT GTGTTTATTT ATACACACAT GGCCCCAACA AAGATATATA AATGTATACT GTGTGTATAT 2373  
 AGATACACGT ATCTATACAC AAGTGCCCTA AACCTAAGTA CAACCCATAT ATTTATATAC ATGTACACAC ACACAGATAT ACATCGTTTC TATATACCAT 2473  
 AGAAGGATGA ATTATTATAT GTATATTTTT TGCTATAAAG TTTATGTATT ATTTCTTCTA AAACCTTAT CTGTAAGTCA TTGTATAAAT **AAACCAGGTG** 2573  
 TTTTTCATAT AAAAAAAAAA AAAAAAAAAA A 2604

### 4.2.3 Predicted structure of the chick E13 protein

The predicted amino acid sequence of the cE13 protein was analysed for features of secondary structure using the MacVector5.1 protein toolbox. The hydrophobicity profile of the predicted amino acid sequence was determined using the Kyte/Doolittle hydrophobicity scale (Kyte and Doolittle, 1982), and indicated a prominent number of hydrophobic residues within the first 30 N-terminal amino acids, characteristic of a classical hydrophobic leader peptide. Further analysis revealed the presence of a candidate leader sequence cleavage site present between amino acids alanine (A) and serine (S) (residues 32 and 33 respectively), that conform to the '(-3,-1)-rule' of von Heijne (von Heijne, 1983), and also to weight-matrix data compiled from 450 different eukaryotic signalase cleavage sites (von Heijne, 1986) (Figure 4.1). Analysis of the amino acid residues encoding cE13 using the Argos and von Heijne transmembrane prediction program does not identify any obvious hydrophobic membrane spanning motifs (von Heijne, 1992). Six potential N-linked glycosylation sites are present in the cE13 protein (Kornfeld and Kornfeld, 1985) (Figure 4.1). The presence of a leader sequence and no transmembrane domains suggests that the cE13 protein is exported from the cell.

Initial searches of both nucleotide and amino acid sequences public databases (NCBI; <http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Search Tool (BLAST) program algorithm (Karlin and Altschul, 1990) with the entire E13 nucleotide sequence or predicted amino acid sequence initially confirmed that it was novel. However, cE13 is similar to a number of proteins, the most similar being *Xenopus* Kielin ( $e = 3 \times 10^{-88}$ ; Genbank accession number AB026192; Matsui, *et al.*, 2000). Searches to identify protein motifs (NCBI and PFAM; <http://www.pfam.wustl.edu>) identified two different types of amino acid repeat motifs that are characterised primarily by their spacing of cysteine residues (Figure 4.2). At the amino terminus, five sequential von Willebrand Factor type C domains (VWF) (Bork, 1993) are present between amino acids: 43 and 98; 101 and 156; 159 and 217; 231 and 282; and 294 and 350 (Figures; 4.1, 4.2 (A) and 4.4). Amino acids numbered 357 to 768 share similarity to a 'von Willebrand Factor Type D' (VWFD) (Bork, 1993) domain (Figures; 4.1, 4.2, (B) and 4.4).

Figure 4.2: Amino acid alignments of cE13 with VWFC & VWFD domains

Three different protein motifs are identified by database searching with the predicted cE13 amino acid sequence. A: Alignment of cE13 with VWFC motif; (i), cE13 amino acids number 43 to 98 have 23 identical residues; (ii), cE13 amino acids number 101 to 156 have 21 identical residues; (iii), cE13 amino acids number 159 to 217 have 27 identical residues; (iv), cE13 amino acids number 231 to 282 have 26 identical residues; and (v), cE13 amino acids number 294 to 350 have 28 identical residues. B: Alignment of cE13 amino acid number 357 to 678 with VWFD domain of Bovine VWF (bVWF: accession number CAA70525) amino acid numbers 378 to 709 have 102 identical residues. Domains were identified using BLAST (<http://www.ncbi.nlm.nih.gov>) and aligned using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994). Conserved residues are highlighted with an asterisk and conservative amino acid substitutions with a decimal point.

**A**

<i>(i)</i>			
cE13	43	CENEQEILQIP--FITDN-P-----C-IMCVC-----LNKEVTCRREKCP---LLSK	82
VWFC	1	CVQNGVVYENGETWKPDSQPNGVDKCTYICTCDDIEDAVRLGGKVLCDKITCPPELLPSL	60
		* . * . * * * * * * * * * * * * * * * * * *	
cE13	83	EC-----ALVIKQRGACER--C	98
VWFC	61	DCPNRRVDALVIPPGGCCPEWVC	84
		. * * * * * * * * * *	
<i>(ii)</i>			
cE13	101	CTFGGKTYNSSMRWHLPSNP---CITYQCQE-----GVIIESEVQC-----VV	140
VWFC	1	CVQNGVVYENGETWKPDSQPNGVDKCTYICTCDDIEDAVRLGGKVLCDKITCPPELLPSL	60
		* . *	
cE13	141	HCKNP---SKVV--GMCCP--VC	156
VWFC	61	DCPNRRVDALVIPPGGCCPEWVC	84
		* * * * * * * * * * * * * * * * * *	
<i>(iii)</i>			
cE13	159	CIFEGRHYNEGEEFRPEG--N---KCT-KCSC-----VGGRTQCIQEVCP-----IL	199
VWFC	1	CVQNGVVYENGETWKPDSQPNGVDKCTYICTCDDIEDAVRLGGKVLCDKITCPPELLPSL	60
		* . *	
cE13	201	SCPQ---HLSHIPAGQCCPK--C	217
VWFC	61	DCPNRRVDALVIPPGGCCPEWVC	84
		* * * * * * * * * * * * * * * * * *	
<i>(iv)</i>			
cE13	231	CLFHSNVYDNG-----SS--FIYDNT-VCTCKDST-----VICRKR-C---LLPGE	270
VWFC	1	CVQNGVVYENGETWKPDSQPNGVDKCTYICTCDDIEDAVRLGGKVLCDKITCPPELLPSL	60
		* . . *	
cE13	271	CNKNQ---D-----HCCKE--C	282
VWFC	61	DCPNRRVDALVIPPGGCCPEWVC	84
		* * * * * * * * * * * * * * * * * *	
<i>(v)</i>			
cE13	294	CKFGNKIFQDGEMW---SSVN---CT-ICAC-----VKGKTECRKKQC-----IPVN	233
VWFC	1	CVQNGVVYENGETWKPDSQPNGVDKCTYICTCDDIEDAVRLGGKVLCDKITCPPELLPSL	60
		* . . . . * * * * * * * * * * * * * * * * * *	
cE13	41	SCPHG---KILNRKG-CCP--IC	350
VWFC	61	DCPNRRVDALVIPPGGCCPEWVC	84
		* * . . . * * * * * * *	

**B**

cE13	357	CTVFGDPHYNTFDGRTFNFGQTCQYVLTKDCSSASPFQVLVKNDAARRTRSFVSWTKSVDL	416
bVWF	387	CLITGQSHFKSFDHRHFTFSGVCQYLLAQDCQDHSFSVVIETVQCADDP-DAVCTRSVTV	445
		* . *	
cE13	417	VLG---RSTISLQOHLTVKWNTRISLPCETPQFQIDLGDG-YLLKVTTKAGLEISWDGDS	472
bVWF	446	RLPSPHHGLLKLKHGGVALDGDQVQIPLLQGLRIQHTVTASLQLNFGEDLQIDWDGRG	505
		* . . . * * * * * * * * * * * * * * * * * *	
cE13	473	FVEVMAAPHLKGLCLCGNYNGHKRDDLIGDGNFKFDVDDFAESWRVESNEFCSRQR	532
bVWF	506	RLLKLSVPYAGRTCGLCGNYNGNRDDFLTPAGLVEPLVEHFGNSWKLRAD--CEDLQE	563
		. . . * * * * * * * * * * * * * * * * * *	
cE13	533	KVPPELCHGTVRVKLAHRECQKLKAWDFQSCHSTVDYTTFYRSCVTDMECEPVHKNCYC	592
bVWF	564	QPS-DPCSLNPRLTKFADQACAILTSPKFEACHSAVSPLPYLRNCRYDVCACSDGRDCLC	622
		* * . * * * * * * * * * * * * * * * * * *	
cE13	593	ESFLAYARACQREGLRVQWIPEQHCAATQCKHGAVYDTCGPGCVKTCDNWNEIG-PCNKP	651
bVWF	623	DAVANYAACARRGVHVGWREPSFCALS-CPHGQVYQCGTPCNLTCRSLSHPDEECTEV	681
		. . *	
cE13	652	CVAGCHCPANLVLHK-GRCIKPVLCPQR	678
bVWF	682	CLEGCFPPGLFLEDTGSCVPKACPCY	709
		* . * * * * * * * * * * * * * * * *	



cE13



Chordin



Procollagen IIA



Tsg



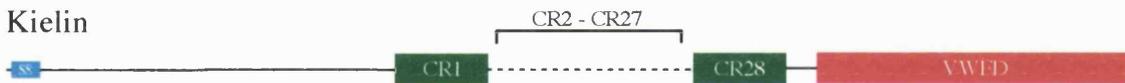
Crim-1



Neuralin-1



Kielin



Cv-2



Figure 4.4: Schematic comparison of CR motif containing proteins

Schematic comparison of cE13 with Chordin and CR motif containing molecules. Domains are highlighted as follows: ss, secretory signals in blue; CR, cysteine-rich repeat motifs in green; and VWFD, von Willebrand Factor type D domains in red.

Further analysis of the five VWFC domains coded by cE13, identified that they are more similar to cysteine-rich repeat (CR) motifs than VWFC domains because of the size and spacing of the ten cysteine residues in the motifs (Figure 4.3). CR motifs are found in a number of secreted proteins such as Chordin (Sasai, *et al.*, 1994), Type IIA procollagen (Zhu, *et al.*, 1999), Twisted gastrulation (Tsg) (Mason, *et al.*, 1994), Crim-1 (Kolle, *et al.*, 2000), Neuralin-1 (Coffinier, *et al.*, 2001) and *Xenopus* Kielin (Matsui, *et al.*, 2000) (Figure 4.4).

Analysis of the VWFD domain identified a five amino acid sequence (CGLCG) found in a number of proteins that form multimers; such as von Willebrand Factor (VWF) and Mucin family members (Figure 4.1: Mayadas and Wagner, 1992; Perez-Vilar and Hill, 1998). Searches of the PFAM databases with the cE13 VWFD domain identified a Trypsin Inhibitor Like cysteine-rich (TIL: <http://www.pfam.wustl.edu>) domain between residues 622 and 675, but on further investigation this amino acid motif was found to be a sub-domain of the larger VWFD domain.

### **4.3 CHICK E13 IS SIMILAR TO DROSOPHILA CROSSVEINLESS 2**

Routine sequence database searching undertaken during this project identified a *Drosophila melanogaster* gene similar to cE13 called Crossveinless 2 (Cv-2) (Genbank accession number AF288223: Conley, *et al.*, 2000). Direct comparison of the entire predicted chick E13 protein with the 751 amino acid Cv-2 protein using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994), shows that they are 30 % identical and are 44 % similar (Figure 4.5). Amino acid alignments show the number and order of CR motifs and VWFD domains are conserved between the two proteins (Figures 4.4 and 4.5). The region of highest identity between cE13 and Cv-2 is within the amino two-thirds of the VWFD domain, and the lowest corresponds to the hydrophobic leader sequences and the carboxyl portion of the VWFD domain that is not conserved in *Drosophila* Cv-2 (Figure 4.5). Amino acid sequence comparisons also identified that the potential multimerisation motif (CGLCG) is conserved between the two proteins (Figure 4.5).



Figure 4.5: Alignment of *Drosophila* Cv-2 with chick E13 protein

Amino acid sequence alignment of *Drosophila* Cv-2 (Cv-2) with cE13 protein using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994). Asterisks denote amino acids common to all four proteins and dots mark similar amino acid residues. Protein motifs are coloured accordingly: leader sequence in blue; CR motifs in green and VWFD domain in red. The putative multimerisation motif is boxed.

Cv-2	1	MCCQSSGQWKFPAQQPRKSLASRRRHTGFRPSTQILLI LIAVLLALIQGRITVDAGA	GDSL	60
cE13	1	MPK-----PGGSPAGTPGLVPAAVLLGALCLPAPAS	SSLLT	37
		* * * * *	* * * * *	
Cv-2	61	GVRQSENEGEEVQLKNQPFITCFKCECQNGFVNCR-LTCTPPVN-DCYILDKSNGTCCR		118
cE13	38	GSIAKENEGEILQIP-FITDNPCIMCVCLNKEVTCRREKCFLLSKECALVIKQRGACCE		96
		* * * * *	* * * * *	
Cv-2	119	FKKGESEFRMSYESCSEWNTDFDPCKTYKCVATVVFETFOKCYSCDNNOLQPPKPGEC		178
cE13	97	FKCKDTEFGKTYNSSMRWHLPSNPCITYQCQEGVIESEFVQCVVHCKNPSK---VVGMC		153
		* * * * *	* * * * *	
Cv-2	179	PTCQGRKINGDTVAFGHEVDASIDDRCLVCCRGTCITCSKRTCPVLPCEMSKQIKRPDE		238
cE13	154	PVCPGELTEGRHYKSEEFKPEFGNKTKKSCZGGKPKLIQEVLPILSLCPQLSHIPAGC		212
		* * * * *	* * * * *	
Cv-2	239	CCPRCPQNHSFLPVP-GRCLFNKSVYPEKTQFMPDRCTNCTCLNGTSVCQRPTCPILECA		297
cE13	213	CCPKCLGQRKVFDFLPGSCLFHSNVYDNGSSFIYDNTVCTCKDSTVICRKRCLLPGECM		272
		* * * * *	* * * * *	
Cv-2	298	PEFQEPDGCCTPCA---VAEVRSECSLDGIVYQNNETWDMGPCRSCRCNGGTIRCAQMR		354
cE13	273	-KNC--DHCKKEGVSYITPEEMKVCFKGNKIQDGENWSSVNCTICACVKGKTECRKKQC		329
		* * * * *	* * * * *	
Cv-2	355	PAVKCRANEELKQPPGECQRCVETAGTCTVFGDPHFERTFDGKFFSFQGSCKYLLASDCM		414
cE13	330	IPVNSCPHGKILNRKG-CCPICTEKPGVCTVFGDPHYNTFDGRTFNFQGTQYVLTKDCS		388
		* * * * *	* * * * *	
Cv-2	415	GKT--FHRIITNEGRGTRRSSTAKTVTLSLRNIKVM LGORMRVKVNGRVTLIPYFVVAGG		472
cE13	389	SSASPFQVLVKNDAARRTRSESWTKSVDIVLGRSTISLQCHITVKWNGTRISLPCETPQ--		446
		* * * * *	* * * * *	
Cv-2	473	QNVTIERLADGGAVMLRSEMGLTLEWNGAGFLQVSVPAKFKKRLGGLCSNFGSSRDLLT		532
cE13	447	----EQIDLDDGYLLKVTTKAGLEISWDGDSFVEVMAAPHLKGLCGLCSNYNGHKRDLLI		502
		* * * * *	* * * * *	
Cv-2	533	GKDGESHGJDFVWHFANSWKVCGPKSCSE-KRF---FLAATPTRDRKSNFYCHPLSVPA		588
cE13	503	GGDC--NPKFTVDDFAESWRVFSNFCSRPQRKPVPELCHGTVVKYI RAHRECUKIKAW		559
		* * * * *	* * * * *	
Cv-2	589	LFGECNERLNFENYKAACRMDVCECPSGD-CHCDSFAAYAHECRRLGVLDPDRSATNCP		647
cE13	560	DFQSCHSTVDYTTFYRSCVTDMCCEPVHKNCYCESFLAYARACQREGLRV-QWIPEQHCA		618
		* * * * *	* * * * *	
Cv-2	648	AGWRRNATLSSFKGNQFYGDPSFSRMKGRROKNHQLRLQLQEQQRSKQGQKGRHKPGG		707
cE13	619	ATQCKHGAVYDTCGFGCVKTCDNWNEIGPCNKFCVAGCHCFANLVLHKGRCIKPVLCP--		676
		* * * * *	* * * * *	
Cv-2	708	HNQLDRQGHNGLDKDLQKEFILKHPSSFLYPRAPDRTPPPLH	751	
cE13	660	-----ANLVLHKGRCIKPVLCPQ	678	
		* * * * *	* * * * *	

#### 4.4 IDENTIFICATION AND SEQUENCE ANALYSIS OF MURINE E13

BLAST searching the translated EST database dbEST (NCBI) with cE13 identified a mouse EST clone (Genbank accession number BE289531) that shared identity with cE13 over a portion of the VWFD domain. This 497 bp mouse EST sequence was then used to search the TIGR mouse gene index database (<http://www.tigr.org>) and a 2998 bp cDNA was identified (Genbank accession number AK014221) that is contiguous with BE289531. Subsequent to this, the AK014221 sequence was used to re-search the dbEST mouse database, and a 485 bp EST sequence (Genbank accession number BB860694) was identified that overlaps with AK014221 and extends the mouse E13 sequence a further 100 bp upstream. These three nucleotide sequences were assembled to produce a 1.9 kb contiguous nucleotide sequence (mE13).

Conceptual translation of the mE13 sequence produce a predicted protein composed of 646 amino acid residues. Analysis of the predicted mE13 amino acid sequence using the programs described previously identified that there is no leader sequence, and using the Argos and von Heijne transmembrane prediction program, no obvious hydrophobic membrane spanning motifs are present (von Heijne, 1992). Four potential N-linked glycosylation sites are present in the mE13 protein (Kornfeld and Kornfeld, 1985), and a potential multimerisation motif (CGLCG: Mayadas and Wagner, 1992; Perez-Vilar and Hill, 1998) (Figure 4.6). At the amino terminus, five sequential CR motifs are present between amino acids: 11 and 66 (CR1); 69 and 124 (CR2); 127 and 185 (CR3); 198 and 249 (CR4); and 261 and 317 (CR5). Amino acid number 324 to 646 are similar to a VWFD domain (Bork, 1993) (Figure 4.6). The predicted 645 amino acid mouse E13 protein when aligned with the 678 amino acids of chick E13 using the BLOSUM30 ClustalW alignment program (Thompson, *et al.*, 1994) show they are 84 % identical and 93 % similar (Figure 4.6).

## 4.5 IDENTIFICATION AND SEQUENCE ANALYSIS OF ZEBRAFISH E13

Searches of the TIGR zebrafish EST database with the full-length chick E13 cDNA identified a highly related 677 bp EST (Genbank accession number BG985626). Conceptual translation of the 677 bp nucleotide sequence produces a predicted zebrafish E13 protein composed of 225 amino acid residues (zE13<sup>3'</sup>). Analysis of the predicted zE13 amino acid sequence identified one potential N-linked glycosylation site (Kornfeld and Kornfeld, 1985) and a potential multimerisation motif (CGLCG: Mayadas and Wagner, 1992; Perez-Vilar and Hill, 1998) (Figure 4.6). Amino acids 1 to 7 correspond to the carboxyl portion of the fifth CR motif coded by cE13 and amino acids 14 to 225 are similar to the VWFD domain of cE13 (Figure 4.6). Alignment of the 225 amino acid sequence that comprises zE13<sup>3'</sup>, with the 678 amino acids of chick E13 using the BLOSUM30 ClustalW alignment program (Thompson, *et al.*, 1994), show that they are 73 % identical and 84 % similar (Figure 4.6). To identify further sequence the full-length chick E13 cDNA sequence was used to search a translated version of the zebrafish Ensembl Trace Server EST database (<http://www.ensembl.org>). One read is 52 % identical to the first 50 amino acids (hydrophobic leader sequence) of cE13 (zE13<sup>5'</sup>); Ensembl reference number Z35724: Figure 4.6).

The most notable feature of the multiple amino acid sequence alignment of chick E13 with the partial mouse E13, zebrafish E13 (5' end) and *Drosophila* Cv-2 amino acid sequences is the conservation of the majority of the cysteine residues (Figure 4.7). The highest degree of similarity between the four amino acid sequences is localised to the amino portion of the VWFD domain, 34 % (78 out of 225 amino acids) of residues are conserved between chick, mouse, zebrafish and *Drosophila* (Figure 4.7). Interestingly, within the VWFD domain the potential multimerisation motif (CGLCG: Mayadas and Wagner, 1992; Perez-Vilar and Hill, 1998) is conserved between all four species (Figure 4.7).



Figure 4.6: Partial amino acid sequence alignment of chick, mouse and zebrafish E13

Amino acid sequence alignment of chick E13 (cE13), mouse E13 (mE13) and zebrafish E13 (zE13) protein sequences. The alignment was performed using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994). Asterisks denote amino acids common to all four proteins and decimal points mark similar amino acid residues. Protein motifs are coloured accordingly: Leader sequence in blue; CR motifs in green; and VWFD domain in red. Potential N-linked glycosylation sites are underlined and putative multimerisation motifs are boxed.

zE13 <sup>(5')</sup>	1	MFPGNRSCRSQCDFLFFLPHS-----GTEAS	CENEGEVLHHPNITDNP	44
mE13	1		SSFLTGSVAKCENEGEVLQISFITDNP	28
cE13	1	MPRPGGSPAGTPGLVPAAAVLLGALCLPGAP	SSLLTGSIAKCENEGEILQIPFITDNP	60
		**      *      **      *      *****		
zE13 <sup>(5')</sup>	45	I	SCVCI	50
mE13	29	IMCVCLNKEVTCKREKCPVLSRDCALAIKQRGACCERC	KGCTHEGRTYNSSFKWQTPAEP	88
cE13	61	IMCVCLNKEVTCRREKCPPLSKECALVIKQRGACCERC	KDCTFGGKTYNSSMRWHLPSNP	120
		*          *          *          *          *          *          *          *          *		
zE13				
mE13	89	CVLRQCQEGVVTSEVRCVVHCKNPAEHQGACCP	CPGCVFEGVQYREGEFQPEGNKCI	148
cE13	121	CITYQCQEGVVIIESEVQCVVHCKNPSKVVGMC	CPGCVIFEGRHYNEGEEFRPEGNKCT	180
		*          *          *          *          *          *          *          *		
zE13				
mE13	149	TCSCVGGRTQCVRVCFILSCPQHLSTPFGQCCPK	CLGQRKVFDDL-LGSLFRSDYDN	207
cE13	181	TCSCVGGRTQCIEVCFILSCPQHLSHIPAGQCCPK	CLGQRKVFDDLPGSLFHSNVYDN	240
		*****          *****          *          *****          *          *****          *		
zE13				
mE13	208	GASFVYDNCVCTCKDSTMVCKKKCSHPGVCNS	DEDACEDCLLRVPPEDIKVKFGSKI	267
cE13	241	GSSFLYDNCVCTCKDSTVICRKRCLLPGECNKNQ	DHCCKEQVSYITPEEMKVKFGNKI	300
		*          *          *          *          *          *          *          *		
zE13 <sup>(3')</sup>	1		FGCCPVC	17
mE13	268	FRDGMWSSVNCISACVKGKTECRKKQCVVSSCP	QGGKILNRKGCCPICEKPGVCTVF	327
cE13	301	FQDGMWSSVNCITICACVKGKTECRKKQCVVNS	CFHGKILNRKGCCPICEKPGVCTVF	360
		*          *          *          *          *          *          *          *		
zE13 <sup>(3')</sup>	18	GDPHYNTFDGRTFNFQGTCKYVLTKDCSPAAS-	FTVLVKNDARRTRSFWSKVELHTSG	76
mE13	328	GDPHYNTFDGRTFNFQGTQYVLTKDCSSPAS	PFQVLVKNDARRTRSFWSKVELMLGE	387
cE13	361	GDPHYNTFDGRTFNFQGTQYVLTKDCSSSASP	PFQVLVKNDARRTRSFWSKVDLVLGR	420
		*****          *****          *          *****          *          *****          *		
zE13 <sup>(3')</sup>	77	LSISLHQHLLTVRQNGTRIALPFHSTGVHIDL	DGYLLKLTTIAGLEITWDGDSLVEVVAAP	136
mE13	388	STVSLQQHLLTVRWNGSRIALPCHTPHFHIDL	DGYLLKVTTRAGLEISWDGDSFVEVMAAP	447
cE13	421	STISLQQHLLTVKWNTRISLPCETPQFQIDL	DGYLLKVTTKAGLEISWDGDSFVEVMAAP	480
		..*          *          *          *          *          *          *          *		
zE13 <sup>(3')</sup>	137	HLKGQLCGLCGNYNGQRDDSLGGDQFKFDV	DELAESWRVEDNELCEVQNRRTSFLCA	196
mE13	448	HLKGKLCGLCGNYNGHKRDDLIGGDGNFKF	DVDDFAESWRVESNEFCNRPQRKVPPELCC	507
cE13	481	HLKGKLCGLCGNYNGHKRDDLIGGDGNFKF	DVDDFAESWRVESNEFCNRPQRKVPPELCH	540
		*****          *****          *          *****          *          *****          *		
zE13 <sup>(3')</sup>	197	GTVKVKLRAHRTCQKLSWGVSEVPLSVD		225
mE13	508	GTVKVKLRAHRECQKLSWEPQTCHSTVDYTT	FYRSCVDMCECPVHKNCYCESFLAYTR	567
cE13	541	GTVRVKLAHRECQKLAWDFQSCHSTVDYTT	FYRSCVDMCECPVHKNCYCESFLAYAR	600
		***          *****          *          *****          *          *****          *		
zE13				
mE13	568	ACQREGIKVHWEPQQSCAATQCKHGAVYDTC	GPVCVTKCDNWNEIGPCNKPCIAGCHCPA	627
cE13	601	ACQREGLRVQWIPEQHCAATQCKHGAVYDTC	GPVCVTKCDNWNEIGPCNKPCVAGCHCPA	660
		*****          *          *          *****          *          *****          *		
zE13				
mE13	628	NLVLHKGRCIKFVLC	PQR	646
cE13	661	NLVLHKGRCIKFVLC	PQR	678
		*****		



Figure 4.7: Multiple amino acid sequence alignment of chick E13, mouse E13, zebrafish E13 and *Drosophila Cv-2*

Comparison of chick E13 with the predicted mouse E13, zebrafish E13 and *Drosophila* Crossveinless 2 (*Cv-2*: Genbank accession number AF288223) amino acid sequences. The sequences were aligned using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994). Identical residues are boxed in black and conservative amino acid substitutions are boxed in grey.

cE13 1 MPRPGGSPAGTPGLVPAAAVLLGALCLPGAPASSLIT 37  
mE13 1 SSFILT 5  
zE13 1 0  
Cv-2 1 MCCQSSGQWKFPAPQQPRKSLASRRRHTGFRPSTQLLLILIAVLLALLQGRTVDAGADSL 60

cE13 38 GSIAKCENEGEILQIPFIT-DNPCIIMCVLNLKEVTCRREKCPLLSKECALVIRKRGACCE 96  
mE13 6 GSVAKCENEGEVLOISFIT-DNPCIIMCVLNLKEVTCRREKCPVLSRDCALAIRKRGACCE 64  
zE13 1 0  
Cv-2 61 GVRQSCSNEGEVQLKNQPQIFTCFKCEONGFVNCR-DTCCPPVN-DCYILLKSNGTCCR 118

cE13 97 RCKDCTFGGKTLNNSMRHLPSPMPCITYOQEGVITESEVQCVVHCN---PSKVVGMCC 153  
mE13 65 RCKGCTHECRTYNSSFKWQTPAEPVCLVRCQEGVVTSEVRCVVHCN---PAEQGACC 121  
zE13 1 0  
Cv-2 119 RCKGCSFRGMSYBESGSBWNDEPDPCKTYKCVATVVVTEITIQKCYSCDNNQLQPPRPGEC 178

cE13 154 PFCPCGCFIFGRHYNEGEEFRP-EGNKICTKCSVGGRTQCIEVCPILSCFQHLSHIPAGQ 212  
mE13 122 PFCPCGCVFEGVQYREGEEFQP-EGNKICTKCSVGGRTQCVREVCPIILSCFQHLSHTPSQ 180  
zE13 1 0  
Cv-2 179 PTCQGGCKINGQTVAECHHEVDASIDDRCLVCCRCRQTLTCSKKTCPVLPCCMSKQIKRPDE 238

cE13 213 CCPKCLGQRKVFIDLPPGSCCLFHSNVDNGSSFIYDNCVCTCKDSTVIGRKRCLLPGEN 272  
mE13 181 CCPKCLGQRKVFIDL-LGSCCLFRSDVDNGASFIYDNCVCTCKDSTMVGKKKCSHPGVGN 239  
zE13 1 0  
Cv-2 239 CCRPCP-QNHSFLPVPCKLANKSVYPEKTOEMPRCTNCTCLNGTSVQRPTCPILBGA 297

cE13 273 KNQ---DHCCKECVSYITPEEMKVCKFGNKIFQDGMWSSVNCITGACVKGKTECRKKQC 329  
mE13 240 SDE---DACCEDCLLRVPPEDIKVCKFGSKIFRDGEMWSSVNCISIGACVKGKTECRKKQC 296  
zE13 1 0  
Cv-2 298 PEFQEPDGCPCRCAVA---EVRSECSLDGIVYONNETWDMGPCRSRCRNGETIRCA MRC 354

cE13 330 IPVNSCPHGKILNRKGCCPITCTEKPGMCTVEGDPHYNTFDGRTFNFGTTCQYVLTKDCS 388  
mE13 297 VPVSSCPQGKILNRKGCCPITCTEKPGMCTVEGDPHYNTFDGRTFNFGTTCQYVLTKDCS 355  
zE13 1 45  
Cv-2 355 PAVKCRANEELKQPPFECQRCVETACTCTVEGDPHYNTFDGRTFNFGTTCQYVLTKDCS 414

cE13 389 SSASPFQVILVKNDAARRTRSFWSWTKSVDLNVGRSTISIQHLLTVKXNGTRISLPCETPQ-- 446  
mE13 356 SPASPFQVILVKNDAARRTRSFWSWTKSVEMLGESTVLSQHLLTVRWNGSRIALPCHTPH-- 413  
zE13 46 PAAS-FTVILVKNDAARRTRSFWSWTKSVEHTSGLSISIQHLLTVRWNGTRIALPCHSTG-- 102  
Cv-2 415 GKTF--HIRLTNEGRTTRSSWAKTWTLSLRNLKVNIGQIRMRKXVNGTRVTLFYPVAVAG 472

cE13 447 ----FQIDLDGYLLEVTTRAGLEISWDGDSFVEVMAAPHLKGLDGLGNGNYNGHKRLI 502  
mE13 414 ----FHIDLDGYLLKVTTRAGLEISWDGDSFVEVMAAPHLKGLDGLGNGNYNGHKRDDL 469  
zE13 103 ----VHIDLDGYLLKVTTRAGLEITWDGDSFVEVMAAPHLKGLDGLGNGNYNGQRDDSL 158  
Cv-2 473 QNVTIERLADGGAVMLRSEMGLTLEINGAGFLOVSVPAKFKRRLDGLGNGFNGSSRDDLT 532

cE13 503 GGDGNF---D--VDFAESWRVESHNFNRPOKRVPELCHGTVRVKTKAHEECOKLKA-W 559  
mE13 470 GGDGNF---D--VDFAESWRVESHNFNRPOKRVPELCHGTVRVKTKAHEECOKLKS-W 526  
zE13 159 GGDGQFKFD--VDELAESWRVEDNDELCEVQNFRETSFLCAGTVRVKTKAHEECOKLKS-W 215  
Cv-2 533 GKDGFRSHGDDEVWHFANSWKVGGPKSCSR-KRE---FLAATPTRDKRKSNFYCHPLSVPA 588

cE13 560 DFQSCNSTVLYTTTFYRSCVTDMCCECPVHKNCYCESFLAYARACOREGLRV-CWIPEQHC 618  
mE13 527 EFQTCNSTVLYTTTFYRSCVTDMCCECPVHKNCYCESFLAYARACOREGLRV-CWIPEQSC 585  
zE13 216 GVSEVPLSVD 225  
Cv-2 589 LFGEENRNLNPNENYKAACRMVCECPSG-DCHCDSFAVAHECRRLGVOLPDRSATNCP 647

cE13 619 ATQCKHGAVYDTCG-----PGCVKTCDNWN-----EIGPCNKPCVAGCHCBA- 660  
mE13 586 ATQCKHGAVYDTCG-----PGCVKTCDNWN-----EIGPCNKPCVAGCHCBA- 627  
zE13 226 225  
Cv-2 648 AGWRRNATLSSFKGNQFYGDPSFSRMKGRRQKNHQLRLQLQOEEQQRSKQGQKGRKPGG 707

cE13 661 -NLV-----LHKGRCIKPV-----LCPOR 678  
mE13 628 -NLV-----LHKGRCIKPV-----LCPOR 645  
zE13 226 225  
Cv-2 708 HNQLDRQGHNGLDKDOLOKEFILKHVPSFLYPRAPDRTPPPPLH 751

## 4.6 DISCUSSION

### 4.6.1 Chick E13 is an extracellular protein comprising a number of cysteine-rich repeat motifs

Initial nucleotide sequence analysis and amino acid translation of the predicted full-length chick E13 cDNA (cE13) indicated that the encoded cE13 protein was novel. The presence of a hydrophobic leader sequence, signalase cleavage site and the absence of any obvious membrane spanning domains, suggests that the mature cE13 protein functions extracellularly. BLAST sequence analysis shows that cE13 is most similar to *Xenopus* Kielin, and although Kielin and cE13 comprise CR motifs followed by one VWFD domain, they are not orthologous as Kielin is comprised of 28 cysteine-rich (CR) motifs whereas cE13 encodes 5 CR motifs.

CR motifs are characterised by the spacing of 10 cysteine residues including the carboxyl-terminal motif CCXXC which are required for correct protein folding and function (Mayadas and Wagner, 1992). A number of molecules have been identified that contain one or more CR motifs, these include: Chordin (Sasai, *et al.*, 1994); fibrillar procollagen type IIA (Zhu, *et al.*, 1999); CHordin Like (CHL: Nakayama, *et al.*, 2001); Twisted gastrulation (Tsg: Mason, *et al.*, 1994); Cysteine-rich motoneuron-1 (Crim-1: Kolle, *et al.*, 2000); Neuralin-1 (Coffinier, *et al.*, 2001) and Kielin (Matsui, *et al.*, 2000). The CR motifs are required for protein/protein interactions, Chordin for example, is an extracellular molecule that contains four CR motifs and can bind directly to bone morphogenetic protein-4 (BMP-4) molecules. The resulting BMP-4/Chordin protein complex prevents the ligand from binding to its receptor and initiating its intracellular signalling cascade. *In vitro* studies have shown that isolated CR motifs one and three bind BMP-4, albeit at a 5 to 10 fold less affinity than full-length Chordin (Piccolo, *et al.*, 1996; Larrain, *et al.*, 2000). Other molecules containing CR motifs can bind BMP family members and modulate BMP signalling with activities similar to Chordin. These include CHL (Nakayama, *et al.*, 2001), type IIA procollagen (Zhu, *et al.*, 1999) and Tsg (Oelgeschläger, *et al.*, 2000). Interestingly, Tsg has been shown to both promote and inhibit BMP signalling (Oelgeschläger, *et al.*, 2000; Chang, *et al.*, 2001; Scott, *et al.*, 2001). These data demonstrate that molecules containing CR motifs can interact with and modulate

BMP molecules. Therefore, as the cE13 protein contains five CR motifs, cE13 may interact with members of the BMP family members (Chapter 6).

The VWFD motif is involved in the formation and regulation of both intra- and intermolecular disulphide bonds in the formation of protein multimers (Azuma, *et al.*, 1993; Jorieux, *et al.*, 2000). For example, a number of molecules contain VWFD domains and function as multimers, some of which are extracellular associated proteins and include: von Willebrand Factor,  $\alpha$ -tectorins, Zonadhesins and Mucin family members (Sadler, 1998; Legan, *et al.*, 1997; Gao and Garbers, 1998; Perez-Vilar and Hill, 1999). The VWFD domain contains a five amino acid motif (CGLCG) that is required for the formation of inter-molecular disulphide bonds (Azuma, *et al.*, 1993; Mayadas and Wagner, 1992; Perez-Vilar and Hill, 1998). This five amino acid motif is also similar to one found at the active site of disulphide isomerase, an enzyme found in the lumen of the endoplasmic reticulum required for correct protein folding via the formation of disulphide bonds (Bulleid and Freedman, 1988). The presence of a VWFD domain and the five amino acid multimerisation motif suggests that the mature cE13 protein may function as a multimer.

#### 4.6.2 Chick E13 is the homologue of *Drosophila* Crossveinless-2 (Cv-2)

Sometime after the cloning of cE13 cDNA, routine database analysis identified a gene similar to cE13 called Crossveinless-2 (*Cv-2*) (Conley, *et al.*, 2000). Analysis showed that the leader sequence, number and organisation of CR motifs and VWFD domain are conserved between the two molecules. Like the *Cv-2* protein, the second CR motif coded by cE13 lacks two cysteine residues and is the least conserved of the five. These data suggests that chick E13 is the homologue of *Drosophila Cv-2*. Transcripts of the *Cv-2* gene are expressed in the developing cross-veins of the *Drosophila* wing and disruption of the gene results in a wing phenotype that lacks the majority of cross-veins. Molecular analysis shows that these mutant flies have a decreased in the activity of an intracellular component of the Decapentaplegic (Dpp) signalling pathway termed Mothers Against Dpp (MAD: Conley, *et al.*, 2000). In the developing *Drosophila* wing, the Dpp signalling pathway (Posakony, *et al.*, 1990; Podos and Ferguson, 1999) provides anterior-posterior (A-P) positional information to the developing wing. Dpp is a *Drosophila* structural and functional orthologue of BMP-4 (Padgett, *et al.*, 1993; Graff, *et al.*,

1994) and is expressed in a narrow strip of cells in the centre of the disc at the A-P compartment boundary (Podos and Ferguson, 1999). At later stages of wing development, Dpp expression becomes elevated in presumptive veins and is required for vein fate maintenance (Posakony, *et al.*, 1990; Yu, *et al.*, 1996). Conley and co-workers (2000) propose that since *Cv-2* encodes putative Dpp/BMP interacting motifs and the loss of *Cv-2* function results in a reduction of activity of Dpp signalling cascade components, *Cv-2* protein is therefore required for highest levels of Dpp signalling within the developing cross-veins of the *Drosophila* wing.

#### 4.6.3 Identification of vertebrate E13 homologues

Database searching identified the putative mouse and zebrafish E13 homologues. Comparison of the complete chick E13 amino acid sequence with the partial mouse E13 and zebrafish E13 amino acid sequences identified the complete conservation of cysteine residues between the three species. From these partial amino acid sequences it appears that the number and order of the CR motifs and VWFD domain are conserved between chick, mouse and zebrafish, but the identification of full-length zebrafish nucleotide sequence is required to confirm that all domains are conserved.

Since E13 appears to be evolutionarily conserved between vertebrates and invertebrates, it can be reasoned that the biochemical function of E13 is also conserved. Further analysis is required to test this hypothesis. The following chapters investigate the transcript distribution of chick and mouse E13, and assays designed to determine the functional relationship between cE13 and BMPs.

## CHAPTER FIVE

### EXPRESSION OF E13 DURING DEVELOPMENT

#### 5.1 INTRODUCTION

Analysing the temporal and spatial expression pattern of a gene is a necessary step in developing hypotheses about the function of the gene product during development. Clone 0B7, as described in Chapter 3, was identified during an in situ hybridisation based screen to identify genes with restricted expression patterns in developing chick cranial neural tissues. The preliminary analysis of 0B7 (now termed cE13) expression pattern indicated that one site of expression is in the cells of the dorsal hindbrain and spinal cord between HH stage 9- to 12. This expression pattern suggests that cE13 transcripts are found at sites corresponding to pre-migratory neural crest cells. This chapter describes a detailed comparative study of the expression of cE13 and a number of pre-migratory and migratory neural crest markers in the chick to analyse possible roles of cE13 in neural crest cell development. In addition, to gain further insight into other possible functions of E13, a detailed examination of other sites of E13 expression was undertaken in both the chick and mouse embryo.

## 5.2 SPATIAL ANALYSIS OF E13 EXPRESSION DURING CHICK DEVELOPMENT

Chick E13 digoxigenin labeled anti-sense mRNA was synthesised from the 0B7 clone (described in Chapter 3) and was used as probe in in situ hybridisations on chick embryos ranging from HH stage 3 (intermediate streak; approximately 12hrs of incubation) to HH stage 29 of development.

### 5.2.1 Dorsal neural tube

As described in Chapter 1, neural crest cells form in the chick along the anterior posterior length of the dorso-lateral most part of the neural plate. Shortly after the fusion of the neural folds, neural crest cells migrate laterally away from the neural tube along specific pathways to various destinations in the developing embryo (Hall, 1999; Le Douarin and Kalcheim, 1999; Christiansen, *et al.*, 2000). Neural crest cells originating from the midbrain and hindbrain anterior to r2 migrate away in a broad un-segmented sheet, whereas neural crest cells migrating from the hindbrain (between r2 and r6) are divided into three distinct streams adjacent to the even numbered rhombomeres, leaving neural crest free regions lateral to odd numbered rhombomeres (Kulesa and Fraser, 1998; Krull, 2001). In the trunk, neural crest cells also migrate in a segmented pattern and follow two primary migratory pathways; a dorsolateral pathway between the ectoderm and somite, and a ventral pathway through the rostral half of the somitic sclerotome (Rickmann, *et al.*, 1985).

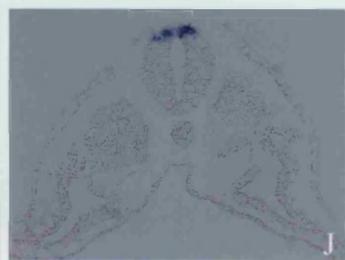
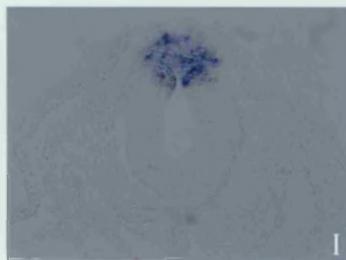
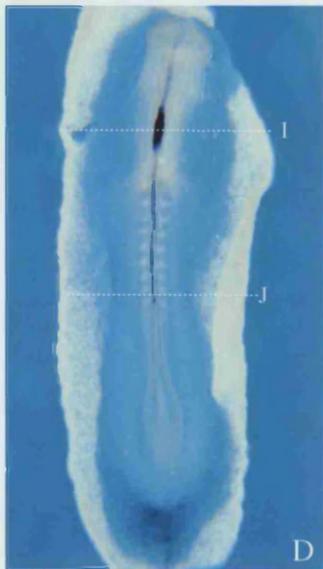
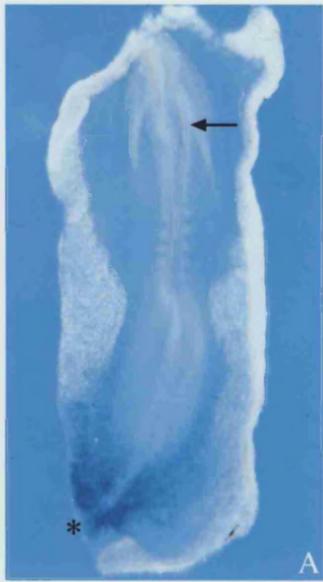
#### (i) Expression pattern of cE13 in the dorsal neural tube

Chick E13 transcripts in the developing chick neural tube and lateral plate mesoderm are first detected at HH stage 8, with expression observed in a few punctate cells localised in the presumptive hindbrain and in the posterior of the embryo surrounding the regressing primitive streak (Figure 5.1; arrow and asterisk in panel A respectively). By HH stage 9- expression is upregulated and becomes less mosaic in the hindbrain (Figure 5.1; panel B). Between HH stages 9- and 9, expression of cE13 is further upregulated in the hindbrain and at HH stage 9, a line of cE13 expressing cells extends along the dorsal midline rostrally from r1/2 to the midbrain and caudally from r5/6 along the trunk (Figure 5.1; panels B and C).



Figure 5.1: Expression pattern of cE13 during cranial neural crest maturation

Whole mount in situ hybridisation of HH stage 8 to 10+ chick embryos with an cE13 riboprobe. Expression of cE13 is first detected at HH stage 8 (A), in the presumptive hindbrain (arrow) and lateral plate mesoderm (asterisk). At HH stage 9-, cE13 expression is upregulated within the hindbrain (B), and by HH stage 9 cE13 expression extends rostrally and caudally from this region into midbrain and spinal cord territories (C). This expression pattern persists during HH stage 9+ (D) and 10- (E). By HH stage 10 (F), cE13 expression in the hindbrain and vagal region of the trunk is downregulated; expression in the hindbrain is just detectable at HH stage 10+ (G). Arrow in panel (G) identifies cE13 expression lateral to the midbrain. Panel (H) shows a lateral view of a HH stage 10+ embryo and the arrow indicates cE13 positive cells adjacent to the midbrain and the arrowhead identifies cE13 positive endoderm overlying the developing heart. Transverse sections through the neural tube show that cE13 transcripts are restricted to the dorsal neural tube, (I) and (J) are cross-sections of a HH stage 9+ embryo as indicated by the dotted lines in panel (D).



Expression of cE13 continues to be upregulated in the dorsal hindbrain, peaking at around HH stage 9+ and 10- (Figure 5.1; panels D, E, I and J). During HH stages 10 and 10+, cE13 expression is progressively downregulated in the hindbrain (Figure 5.1; panels F, G). At HH stage 10+, cE13 transcripts are detected in a region of head mesenchyme adjacent to the midbrain (Figure 5.1; arrows in panels G and H). Expression of cE13 in the trunk continues to progress in an anterior to posterior direction along the embryo as development proceeds; expression is lost in the vagal region of the neural tube at HH stage 10 (Figure 5.1; panel F). In the dorsal neural tube, expression of cE13 is detected for the last time at HH stage 19 (Figure 5.3; panel D).

Initial interpretation suggested expression of cE13 in the dorsal neural tube at these stages corresponds to sites of neural crest development. To examine whether this is the case, a comparative analysis of cE13 expression in the hindbrain region of the neural tube between HH stages 9- to 10+ was undertaken with a number of neural crest associated markers (Figure 5.2). This period starts prior to neural crest cell migration, includes the time when cranial neural crest cell migration is initiated, and continues during the period of migration. Marker genes used in this study include: *Slug*, which is initially expressed in pre-migratory neural crest cells and later is detected in migratory neural crest cells (Nieto, *et al.*, 1994); *AP-2*, which is also detected in both pre-migratory and post-migratory cranial neural crest cells (Shen, *et al.*, 1997); *Sox10*, which is expressed in migratory neural crest cells (Southard-Smith, *et al.*, 1998; Cheng, *et al.*, 2000); *rhoB*, which is expressed in predominantly pre-migratory neural crest cells and transiently in migratory neural crest cells (Liu and Jessell, 1998), and *cad6B*, which is detected in neural crest cell populations prior to migration (Nakagawa and Takeichi, 1995). *BMP-4* has two centres of expression in the hindbrain, focused in dorsal r3 and r5 (Graham, *et al.*, 1993; Graham, *et al.*, 1994).

(ii) Expression profile of known neural crest cell markers

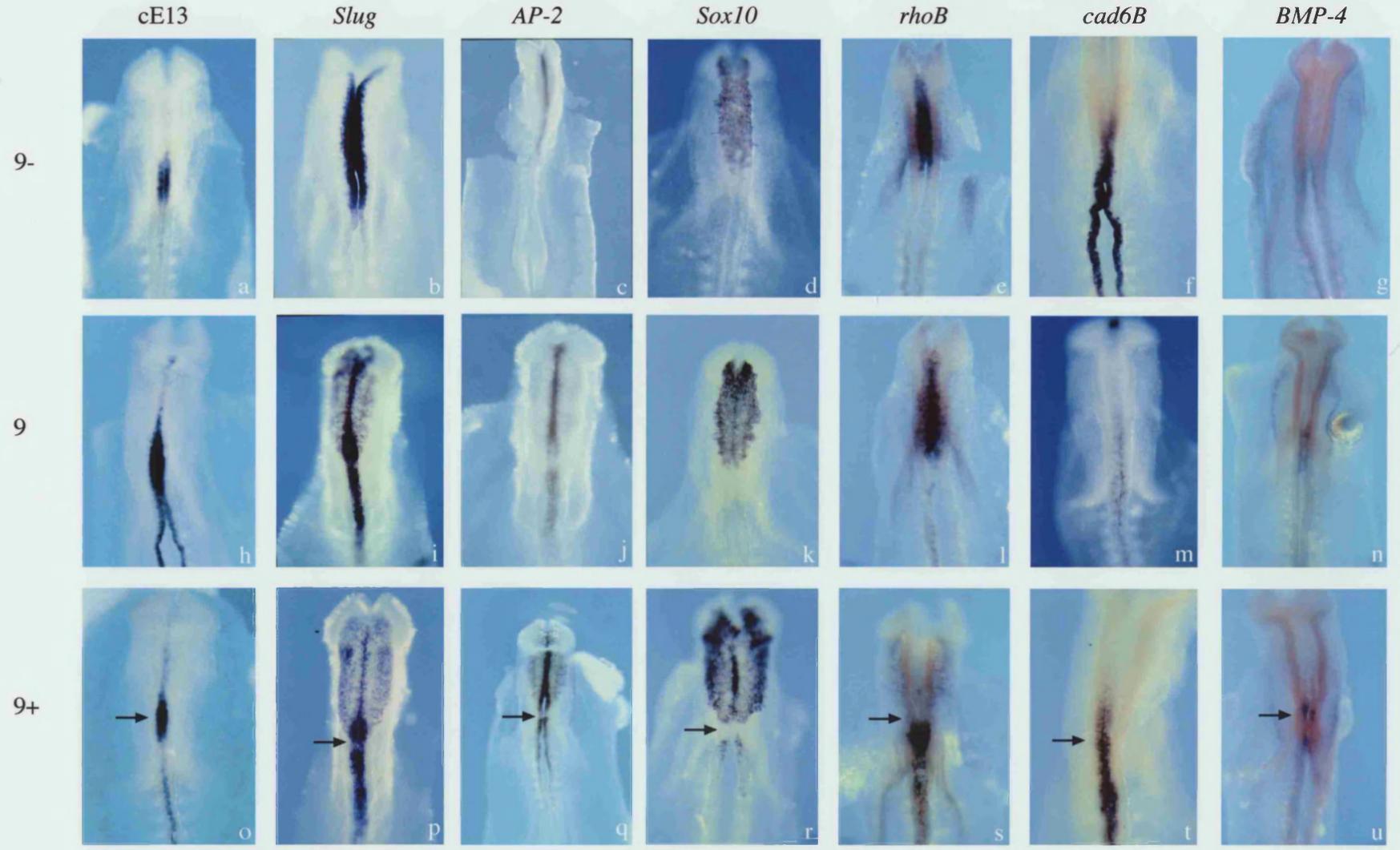
The neural crest markers *Slug* and *AP-2* appear to label different populations of neural crest cells. At HH stage 9-, pre-migratory neural crest cells expressing *Slug* in the dorsal neural tube are detected from presumptive r2 in the hindbrain to the posterior forebrain (Figure 5.2; panel b). *AP-2* expression at HH stage 9- is also detected in pre-migratory neural crest cells from r2 to the posterior forebrain, but unlike *Slug*, *AP-2* is expressed in the vagal region of the neural tube (Figure 5.2; panel c). A comparison of pre-migratory *Slug* expressing neural crest cells with *rhoB* expressing cells shows that *rhoB* labels a different population of pre-migratory neural crest cells. For example at HH stage 9-, *Slug* expressing pre-migratory neural crest cells extend further anterior in the forebrain and posterior in the hindbrain than *rhoB* positive cells (Figure 5.2; panels b and e). *Cad6B* labels a different population of pre-migratory neural crest cells than the *Slug*, *AP-2* and *rhoB* neural crest markers. At HH stage 9-, *cad6B* is expressed caudally from the presumptive hindbrain, whereas pre-migratory *Slug*, *AP-2* and *rhoB* expressing cells are located anterior to the presumptive hindbrain (Figure 5.2; panels b and f). In both early and late stage embryos analysed, no positive pre-migratory *AP-2* or *rhoB* cells are detected in r3 (Figure 5.2; panels: j and l'; l and g', respectively), but pre-migratory *Slug* and *cad6B* positive neural crest cells are detected in r3 (Figure 5.2; panels: I and k'; t and h', respectively).

*Slug*, *AP-2*, *Sox10* and *rhoB* also appear to identify different populations of migrating neural crest cells. For example, neural crest cells expressing *Sox10* have initiated migration from regions anterior to r3 by HH stage 9- (Figure 5.2; panel d), but *Slug* and *rhoB* positive neural crest cells are detected migrating in these regions from HH stage 9+ suggesting they mark different populations of motile neural crest cells (Figure 5.2; panels i and l). *AP-2* positive migratory neural crest cells are detected migrating from the dorsal midline anterior to the hindbrain at HH stage 9+ (Figure 5.2; panel q), two HH stages after migrating *Sox10* neural crest cells and one HH stage after *Slug* and *rhoB* positive motile neural crest cells are detected. These data suggest that *Slug*, *AP-2*, *Sox10*, *rhoB* and *cad6B* all label different populations of pre-migratory and/or migratory neural crest cells.



Figure 5.2: Comparative expression profile of cE13 with known neural crest markers

The detection of cE13, *Slug*, *AP-2*, *Sox10*, *rhoB*, *cad6B* and *BMP-4* by whole mount in situ hybridisation during the period preceding and during cranial neural crest migration (HH stage 9- to 10+). Chick E13 expression pattern is confined to the dorsal neural tube at sites that correspond to a subpopulation of pre-migratory neural crest cells. The transcription factors *Slug* and *AP-2* are expressed in different populations of pre-migratory and post-migratory cranial neural crest cells. *Sox10* is expressed in an early population of migratory neural crest cells. The GTP binding protein *rhoB* is expressed in a subpopulation of both pre-migratory and post-migratory neural crest cells. The adhesion molecule *cad6B* is expressed in a population of pre-migratory neural crest cells that extend caudally from the hindbrain and is rapidly downregulated as migration commences. The *BMP-4* gene, a member of the TGF $\beta$  superfamily, is expressed predominantly in the dorsal neural tube with expression centred in dorsal r3 and r5. Arrows identify (presumptive) rhombomere 3.



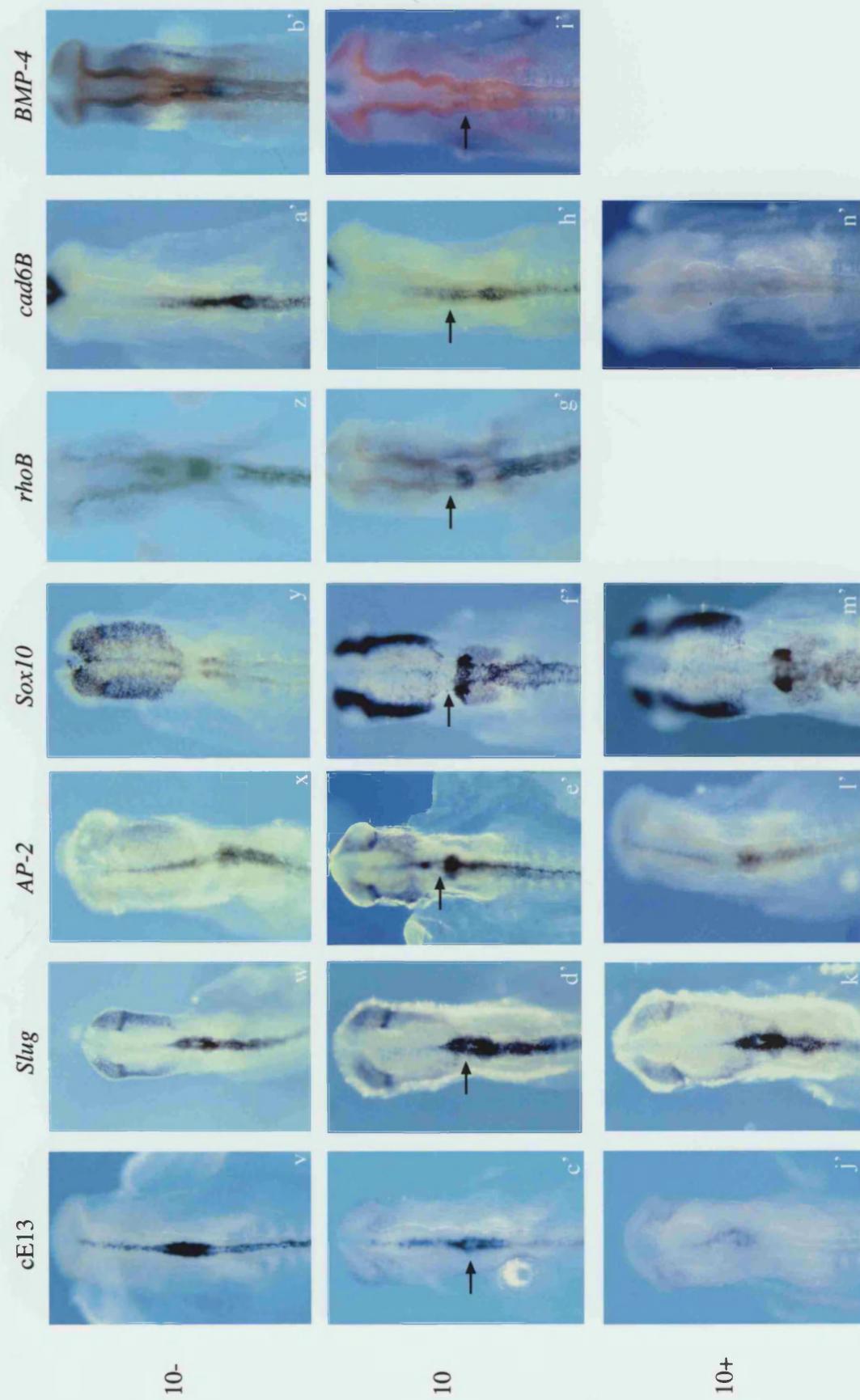


Figure 5.2 Continued

(iii) Comparative expression profile of cE13 with known neural crest markers

At HH stage 9-, cE13 is expressed caudally from presumptive r1/2 to r5 and overlaps with pre-migratory *Slug*, *AP-2*, *rhoB* and *cad6B* expressing neural crest cells. Chick E13 expression at this stage is however restricted to a subdomain of these pre-migratory neural crest cells in presumptive r1/2 to r5 of the hindbrain (Figure 5.2; panels a, b, c, e and f). Slightly later in development, at HH stage 9+, cE13 expression is maintained in the hindbrain and a line of cE13 expressing cells are observed extending rostrally from r5/r6 and a few scattered cells anterior from r1/r2. This expression pattern in the rostral hindbrain, midbrain and posterior forebrain correlates with some *Slug*, *AP-2* and *rhoB* positive cranial neural crest cells that are still found associated with the neural tube at this time (Figure 5.2; panels o to s). At this stage in the hindbrain the main domain of cE13 expression correlates with pre-migratory *Slug* and *AP-2* positive neural crest cells, and some pre-migratory *rhoB* and *cad6B* expressing hindbrain neural crest cells (Figure 5.2; panels o to t). At this stage, expression of cE13 is detected in r3 (Figure 5.2; panel o), no expression of pre-migratory *AP-2* and *rhoB* neural crest cells are detected in r3 (Figure 5.2; panels q and s), but *Slug* and *cad6B* positive pre-migratory neural crest cells are detected in r3 (Figure 5.2; panels p and t). Chick E13 expression is progressively downregulated after HH stage 10- and this reduction in expression occurs at a time when the last pre-migratory positive *Slug*, *AP-2*, *Sox10* and *rhoB* positive cranial neural crest cells are detected in r4 (Figure 5.2; panels j' to m'). Indeed, at this stage expression of the pre-migratory neural crest marker *cad6B* is lost in the dorsal hindbrain (Figure 5.2; panel n').

Another molecule that is expressed in a subset of pre-migratory neural crest cells is BMP-4 (Graham, *et al.*, 1994). At HH stage 9+ and 10-, *BMP-4* expression is localised to dorsal regions of r3 and r5 (Figure 5.2; panels u and b'), these two foci of expression occur prior to *AP-2* and *Sox10* positive r4 neural crest cell migration at HH stage 10 (Figure 5.2; panels e' and f'). At HH stage 9+ and 10-, cE13 expression is centred in the hindbrain, extending from r1/2 to r6 (Figure 5.2; panels o and v) and therefore, portions of cE13 expression appear to coincide with *BMP-4* expression just prior to neural crest cell migration.

## 5.2.2 Other sites of chick E13 expression

### (i) Surrounding regressing primitive streak

Neurulation starts at approximately HH stage 2 (initial streak stage) and divides the ectoderm into neural (neural plate) and non-neural domains. As neurulation proceeds, a secondary site of gastrulation continues in the posterior region of the embryo surrounding the primitive streak (Kessel and Pera, 1998). This site of secondary gastrulation produces cells that ultimately become incorporated into the presomitic mesoderm and somites as somitogenesis continues in a rostral to caudal direction along the embryo (McGrew and Pourquie, 1998).

At HH stage 8, expression of cE13 is detected in ectoderm and lateral plate mesoderm surrounding the regressing primitive streak (Figure 5.3; panels A and B) and is detected until HH stage 10+ (Figure 5.1; panel G). Expression of cE13 is not detected by whole mount in situ hybridisation in any embryo younger than HH stage 8.

### (ii) Developing inner ear

The progenitor of the chick ear, the otic placode, is first observed as a thickening in the surface epithelium lateral to r5 at HH stage 10. The otic placode invaginates at HH stage 11 and continues to deepen, forming the otic cup by HH stage 13. At HH stage 17, the edges of the otic cup fuse to form an enclosed cavity termed the otic vesicle or otocyte and this transient developmental structure later differentiates into the inner ear (Steel and Brown, 1994; Fekete, 1996).

From HH stage 13, cE13 transcripts are detected in the posterior lip of the otic cup (Figure 5.3; panel C). By HH stage 19 expression is lost in the otic vesicle (Figure 5.3; panel D). Apparent signal detected in the otocyte after this developmental period using the whole mount in situ hybridisation technique may be artifactual and due to 'trapping' of reagents in this structure (e.g. Figure 5.3; panel H).

### (iii) Developing heart

The primitive heart is first visible at around HH stage 9, initially as a simple tube that loops to the right-hand side of the embryos at HH stage 10 (Stalsberg and DeHaan, 1969; Redkar, *et al.*, 2001). As development continues the primitive heart

becomes divided into three main regions: the bulbo-ventricle, atrium and sinus venosus (Lyons, 1996). The bulbo-ventricle (future right ventricle) is a continuous structure with the outflow tract that in turn leads to the aortic sac and the aortic arteries.

Chick E13 expression is first detected in the outflow tract of the developing heart at HH stage 13 (Figure 5.3; panel C). Expression of cE13 is maintained in this structure at least until HH stage 22 (Figure 5.3; panels D and H).

#### (iv) Sites of sympathetic ganglia differentiation

The sympathetic ganglia, along with the parasympathetic and enteric ganglia, are formed from neural crest and are the main components of the autonomic nervous system (Le Douarin and Kalcheim, 1999). The chain of sympathetic ganglia that form along the trunk are portions of trunk derived neural crest cells that migrate along the ventral migration pathway and undergo adrenergic differentiation at site adjacent to the dorsal aorta.

At HH stage 19 whole mount in situ hybridisation detects cE13 transcripts at locations where the primary sympathetic strands of the ganglia form (Figure 5.3; panel D). Cross-sections at this stage identify cE13 transcripts in mesenchymal tissue adjacent to the dorsal aorta (Figure 5.3; panel E), and in mesenchymal tissue lateral to the developing lung bud (Figure 5.3; panel E).

#### (v) Developing head

The chick face is formed from four pairs of primordia that are initially composed of undifferentiated mesenchyme surrounded by a layer of epithelial tissue. Three pairs of facial primordia: the frontonasal mass, the lateral nasal process and the maxillary primordia fuse to form the face and upper beak. The lower beak is derived from the paired mandibular primordia (Francis-West, *et al.*, 1994).

At HH stage 13, cE13 expression is detected in a region of the head, caudo-ventral to the developing eye that appears to localise with a lateral region of the maxilla primordia (Figure 5.3; panel C). By HH stage 19, the caudo-ventral site of cE13 expression remains and another is detected dorso-caudal to the developing eye (Figure 5.3; panel D). Cross-sections through the caudo-ventral patch of expression identify cE13 transcripts localised in mesodermal tissue (Figure 5.3; panel F).

Expression of cE13 continues to be expressed in this region until HH stage 22 (Figure 5.3; panel H). Chick E13 is also expressed in sclerotome surrounding the dorsal aorta ventral to the branchial arches at HH stage 13 (Figure 5.3; panel c), and by HH stage 19 expression becomes more widespread (Figure 5.3; panel D). Histological sectioning identifies cE13 expression in the epithelium of Rathke's pouch (Figure 5.3; panel F), the link between the foregut and the anterior neural tube (the infundibulum) that is the primordium of the anterior and intermediate lobes of the pituitary gland.

(vi) Nephrogenous mesenchyme

The intermediate mesoderm, the precursor of the urino-genital system, forms along the trunk in an anterior to posterior direction between the somites and the lateral plate at approximately the same time as somite formation. At around HH stage 18 the intermediate mesoderm thickens to form the nephrogenous mesenchyme. This transient structure forms the non-functional pronephric kidneys that in turn form the active embryonic mesonephric kidneys, and ultimately the fully operational metanephric kidneys by HH stage 41 (reviews see; (Vize, *et al.*, 1997; Kuure, *et al.*, 2000).

Expression of cE13 is observed in the developing nephrogenous mesenchyme at HH stage 19, and cross sections identify cE13 expression adjacent to the early nephric ducts (Figure 5.3; panels D and G).

(vii) Intersomitic region

In the chick, between HH stage 7 and HH stage 22 approximately 50 pairs of somites are formed. These form in an anterior to posterior direction from paraxial mesoderm as immature 'balls' of radially orientated epithelial cells (Christ and Ordahl, 1995; McGrew and Pourquie, 1998; Stockdale, *et al.*, 2000). As development proceeds somites become dorsoventrally compartmentalised and differentiate on their ventral side to form a mesenchymal compartment termed the sclerotome (that will contribute to some bones and the vertebrae). On the dorsal side epithelial identity is retained, becoming the dermomyotome that will contribute to muscle and dermis (Shapiro, 1992; Christ, *et al.*, 2000; Olivera-Martinez, *et al.*, 2000).

Chick E13 expression is detected by in situ hybridisation at HH stage 19 in the intersomitic region (Figure 5.3; panel D). Expression of cE13 is upregulated in the intersomitic region in an anterior to posterior direction along the trunk as development proceeds, until by HH stage 22 cE13 expression is detected along the A-P axis of the trunk (Figure 5.3; panel H).

(viii) Developing limb

The chick limbs are first visible as thickenings within the lateral plate mesoderm; the presumptive wings forming at HH stage 16 lateral to somites 15 to 20, the leg precursors form between somites 26 and 32 at HH stage 17. These limb precursors grow to form buds that are comprised of a mesodermal core surrounded by a sheath of ectoderm. Extensive growth, patterning and morphogenesis occur to eventually form the mature limbs (Summerbell, *et al.*, 1973; Tickle and Eichele, 1994; Capdevila and Izpisua Belmonte, 2001; Tickle and Munsterberg, 2001).

Transcripts of cE13 are first detected in the developing limb buds at HH stage 22, in a discrete domain within the anterior leg bud (Figure 5.3; panel H). Chick E13 transcripts are not detected by whole mount in situ hybridisation in the developing wing at, or prior to this stage. Preliminary data suggests that at later stages cE13 transcripts are expressed in the wing and leg (approximately HH stage 31) localised to sites surrounding the forming digits (data not shown).

(ix) Developing eye

Optic placodes form as ectodermal thickenings at HH stage 9 and are induced by interactions between surface ectoderm at the lateral most aspects of the diencephalon. As development proceeds the optic placode invaginates to form the optic pit, and continuing growth leads to the formation of the optic vesicle by about HH stage 12. The presumptive neural retina is derived from the inner layer of the optic cup facing the lens; its development is closely linked with that of the lens (Prada, *et al.*, 1991). The lens vesicle is initially observed at HH stage 12 as a thickening of the surface ectoderm that contacts the deepening optic vesicle (Prada, *et al.*, 1991; Chow and Lang, 2001). The presumptive lens sinks beneath the surface of the ectoderm (the future cornea) at around HH stage 18, and lens cells continue to

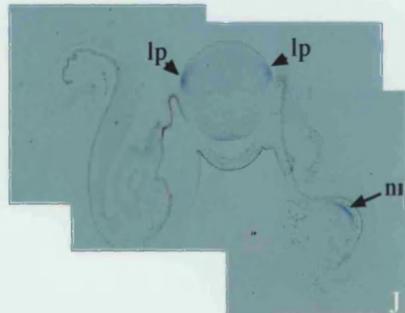
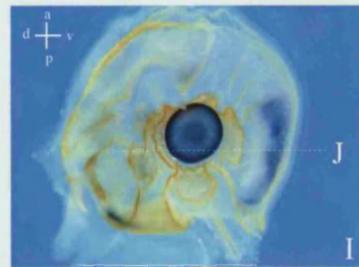
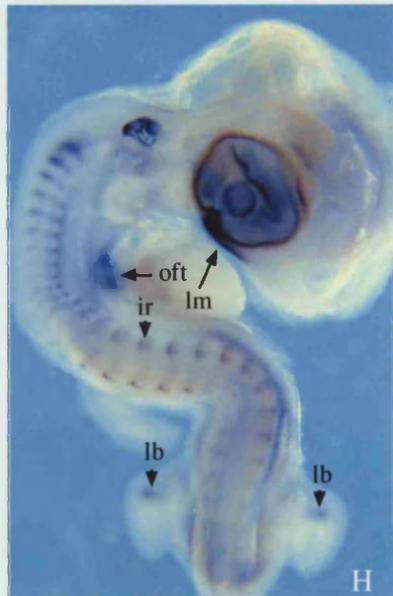
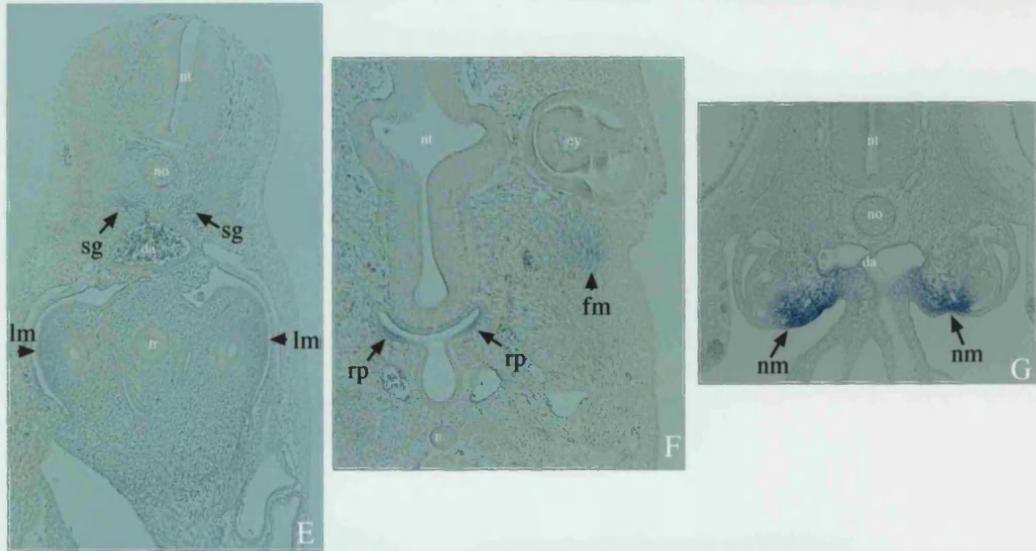
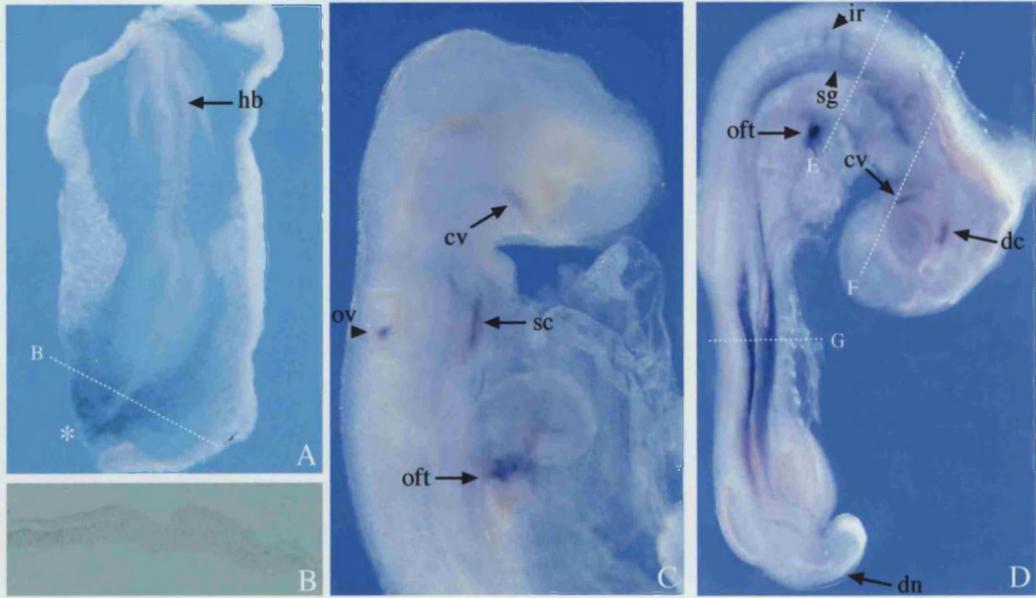
divide and change shape eventually forming its characteristic lentoid shape by HH stage 23/24 (Saha, *et al.*, 1992; Chow and Lang, 2001).

In the developing eye the first detectable level of cE13 expression is at HH stage 22 (Figure 5.3; panel H). To confirm that expression was not due to reagent trapping, eyes were isolated from HH stage 29 embryos, punctured to prevent trapping (a process which makes them flat), and whole mount in situ hybridisations performed. Chick E13 is expressed in a ventro-central and ventro-equatorial (Prada, *et al.*, 1991) portion of the eye and in the developing lens (Figure 5.3; panel I). Sectioning confirms that cE13 transcripts are observed in the pigmented layer of the retina and at the equatorial region of the developing lens (Figure 5.3; panel J).



Figure 5.3: Expression profile of chick E13

Digoxigenin-labeled antisense cE13 RNA was hybridised to embryos ranging from HH stage 8 to HH stage 29. Transcripts encoding cE13 are first detected surrounding the posterior primitive streak at HH stage 8 (asterisk in A), cross sections identified expression in lateral plate mesoderm and overlying ectoderm (B), and in a few scattered cells in the hindbrain (A; hb). At HH stage 13 (C), cE13 transcripts are detected in a region of the head caudo-ventral to the eye (cv), at the posterior lip of the otic cup (oc), the outflow tract of the heart (oft) and sclerotome surrounding the dorsal aorta between the branchial arches (sc). D: Whole mount of a HH stage 19 embryo shows cE13 expression in the intersomitic region (ir), near the dorsal aorta (sg), the outflow tract of the heart (oft), two sites caudal-ventrally (cv) and dorso-caudally (dc) to the eye and dorsal neural tube in the tail region (dn). Dotted lines through the embryo illustrated in (D), represent sections shown in; (E) cE13 expression in at site of sympathetic ganglia differentiation (sg) and lung mesenchyme (lm); (F) Rathke's pouch (rp) and facial mesoderm (fm); and (G) in nephrogenous mesenchyme surrounding the nephric ducts (nm). At HH stage 22 (H), cE13 expression is detected in the lateral regions of the maxilla primordia (lm), expression persists in the intersomitic region (ir) and in the anterior portion of the leg bud (lb). At HH stage 29 cE13 is observed in the developing eye (I and J); expression is detected in the lens periphery (lp) and ventral regions of the neural retina (nr). Abbreviations in white script: a, anterior; da, dorsal aorta; d, dorsal; ey, optic vesicle; nt, neural tube lumen; no, notochord; p, posterior; tr, trachea; v, ventral.



## 5.3 SPATIAL ANALYSIS OF E13 DURING MURINE EMBRYOGENESIS

### 5.3.1 Isolation of a murine E13 cDNA

In order to analyse whether mouse and chick E13 have conserved expression patterns, specific primers were designed to the identified mouse EST (Genbank accession number BE289531), and using RT-PCR a clone identical to BE289531 was generated (Chapter 2, section 2.13) from 8.5 and 9.5 days postcoitum (dpc) samples. The 477 bp fragment isolated by RT-PCR was cloned into a vector and used to synthesise a digoxigenin labeled anti-sense mRNA probe (mE13). Whole mount in situ hybridisations were carried out on mouse embryos ranging in age from 7.25 dpc to 11.5 dpc. The expression profile observed is depicted in Figure 5.4.

### 5.3.2 Murine E13 expression profile

E13 transcripts are first detected in the developing mouse embryo at the late primitive streak stage (7.25 dpc) are confined to a ring of neural ectodermal tissue and embryonic mesoderm surrounding the amnion of the embryo (Figure 5.4; panels A, B and C). By about 7.75 dpc, mE13 expression becomes localised to the anterior and posterior of the embryo adjacent to the future headfolds and tail bud respectively (Figure 5.4; panel D). By early somite stages, mE13 transcripts have been downregulated in the anterior and posterior of the embryo, but mE13 transcripts are expressed in cephalic mesenchymal tissue lateral to the open headfolds (Figure 5.4; panels E and H) and in the anterior region of the embryo that corresponds to the lateral plate mesoderm (Figure 5.4; panels E and I).

In the developing cephalic region of the mouse embryo a number of sites of mE13 expression are detected by whole mount in situ hybridisation. By 9.5 dpc mE13 transcripts are expressed along the anterior dorsal midline of the forebrain at a site that corresponds to the anterior midline groove (Figure 5.4; panel F), but expression is lost in this structure by 10.75 dpc (Figure 5.4; panel G). Later, at 10.75 dpc, mE13 expression is found within the telencephalic vesicle (Figure 5.4; panel G). At this stage, transcripts of mE13 are also observed in a number of different regions associated with the developing face, including the lateral nasal process adjacent to the naso-lacrimal groove that divides the lateral nasal process and maxillary

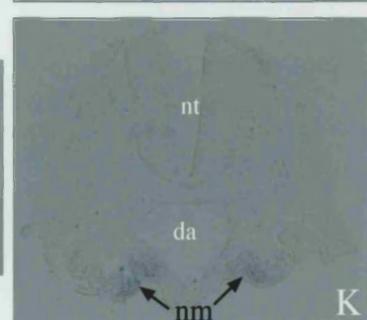
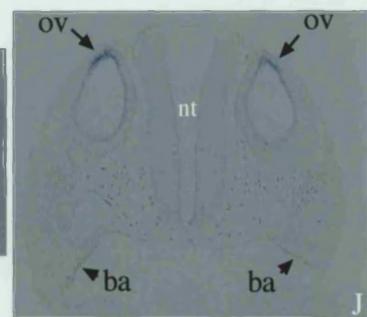
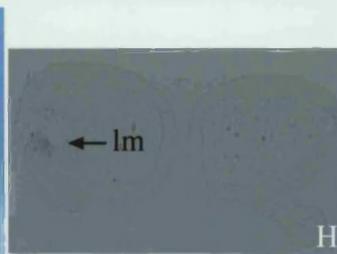
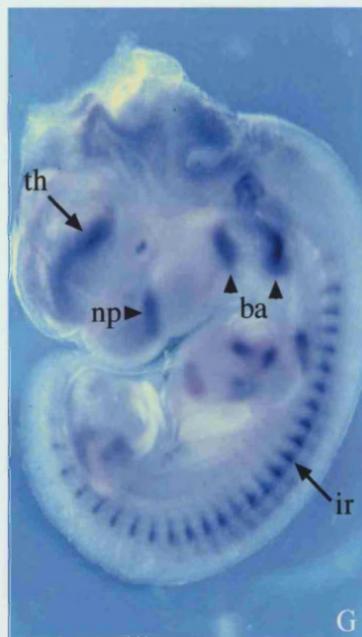
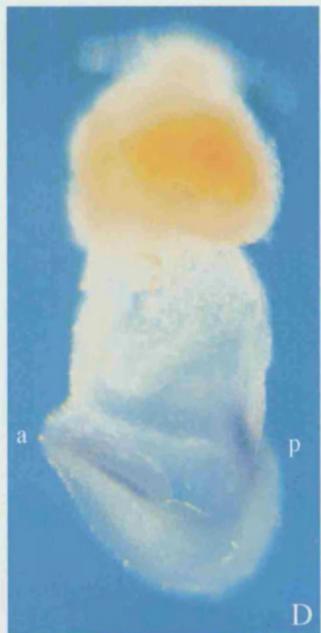
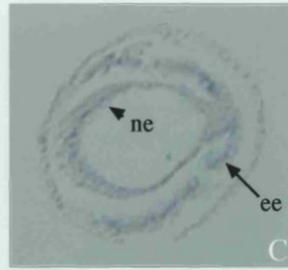
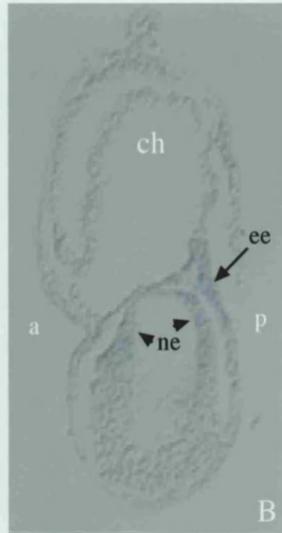
primordia of the first branchial arch (Figure 5.4; panel G). Cross sections through the branchial arches identify mE13 expression from 9.5 dpc (Figure 5.4; panel J), expression continuing in the posterior portion of the pharyngeal arch and the hyoid arch to at least 10.75 dpc (Figure 5.4; panel G). Histological sections through the otic vesicle show that mE13 is expressed in the dorsal epithelium of the otic vesicle at 9.5 dpc (Figure 5.4; panel J). Expression was apparent at later stages in the otic vesicle but this may be artifactual due to reagent trapping in the enclosed otic otocyst (Figure 5.4; panel G).

Other sites of murine E13 expression include the outflow tract region of the developing heart at 9.5 dpc (Figure 5.4; panel F) and nephrogenic mesenchyme adjacent to the dorsal aorta at 9.5 dpc (Figure 5.4; panels F and K). Transcripts are expressed in nephrogenic mesenchymal tissue until 10.75 dpc; expression at this stage is detected in the posterior nephrogenic mesenchyme of the embryo at about the axial level of somite 25 to 30 (Figure 5.4; panel G). Transcripts of mE13 are detected in intersomitic regions at 10.75 dpc (Figure 5.4; panel G) and are graded along the body axis, with highest expression rostrally, decreasing caudally (Figure 5.4; panel G). Murine E13 expression is also detected in the limb bud of the presumptive forelimb at 10.75 dpc but not in the less developed hindlimb (Figure 5.4; panel G).



#### Figure 5.4: Murine E13 expression profile

Expression of mE13 transcripts between 7.25 dpc and 10.75 dpc as detected by whole mount in situ hybridisation. A: Whole 7.25 dpc embryo; mE13 transcripts are detected in a ring around the allantois and in the chorion. B: Sagittal section through embryo (A) and C, transverse section of a similarly aged 7.25 dpc embryo. B and C: Expression is detected in the neural ectoderm (ne) and embryonic ectoderm (future head fold, ee). D: expression of mE13 is observed in the anterior and posterior regions of a 7.75 dpc embryo surrounding the headfolds and neural plate. E: Flat-mounted 8 dpc embryo, expression is observed in the tail region. H and I: cross sections show mE13 expression in headfold mesenchyme (hm) and in tail mesenchyme respectively. F: 9.5 dpc embryo, mE13 expression is detected in the anterior midline groove (mg) and the outflow tract of the heart (oft). J: Section through otic vesicle and branchial arch of 9.5 dpc embryo shown in F, note expression in otic vesicle (ov) and branchial arches (ba). K: Cross-section through the trunk of 9.5 dpc embryo, mE13 transcripts are detected in the nephrogenic mesenchyme (nm). G: 10.75 dpc embryo, expression is detected in the telecephalic hemisphere (th), lateral nasal process (np), branchial arches (ba) and intersomitic region (ir). Abbreviations in white script: a, anterior; ch, chorion; da, dorsal aorta; p, posterior; nt, neural tube lumen.



## 5.4 DISCUSSION

During chick and murine development, a number of tissues have been identified that express the E13 gene. Comparison of E13 transcript distribution in the chick with the mouse identified a number of domains of expression that are conserved between the two species. These include lateral plate mesoderm, the outflow tract of the heart, nephrogenic mesenchyme, otic vesicle, intersomitic region and several regions in the developing head. However, a number of E13 positive tissues are found to be different between chick and mouse including regions of the eye, branchial arches, sympathetic ganglia, lung mesenchyme and Rathke's pouch. It is possible however, that low levels of expression may not have been detected due to low sensitivity of the in situ hybridisation conditions utilised.

A number of BMP family members are expressed in varying domains in the developing chick and mouse embryo, some of which coincide with or are adjacent to E13 expressing tissues. Expression patterns of E13 compared with *BMP-2*, *BMP-4* and *BMP-7* are summarised in Table 5.1 (chick) and Table 5.2 (mouse). Moreover, amino acid sequence analysis of E13 (Chapter 4) has identified five cysteine-rich repeat motifs that in other molecules have been shown to interact with BMP family members, suggesting that E13 may interact with BMP molecules. To address the potential E13/BMP interaction, both biochemical and functional experiments have been undertaken, and these data are presented in Chapter 6. The implication(s) of these findings with respect to E13 expression profile is discussed in Chapter 7.

	cE13	<i>BMP-2</i>	<i>BMP-4</i>	<i>BMP-7</i>
Tail bud region (HH stage 8)	Lateral plate mesoderm & overlying ectoderm	Endoderm	Ectoderm	Ectoderm
Neural tube (HH stage 9+)	Dorsal r1/2 to r5/6 & trunk		Dorsal r3, r5 & trunk	Dorsal midline caudal to hindbrain
Otic cup (HH stage 13)	Posterior		Anterior & posterior	Dorsal & posterior
Heart (HH stage 13)	Outflow tract	Distal region of outflow tract	Myocardium overlying outflow tract	
Sympathetic Ganglia (HH stage 19)	Sites adjacent to dorsal aorta		Dorsal aorta	Dorsal aorta
Lung (HH stage 19)	Mesenchyme		Endoderm	
Rathke's Pouch (HH stage 19)	Epithelium		Ventral diencephalon	
Kidney (HH stage 19)	Nephrogenous mesenchyme		Surface ectoderm	
Maxillary primordia (HH stage 22)	Lateral edge of maxillary primordia	Medial maxillary epithelium	Maxillary primordia	
Limb (HH stage 22)	Anterior portion of the leg	AER & posterior portions	AER & anterior portions	AER & posterior portions
Eye (HH stage 29)	Ventral retina & equatorial lens	Dorsal & ventral retina	Dorsal & ventral retina	

Table 5.1: Summary of cE13, *BMP-2*, *BMP-4* & *BMP-7* transcript distribution during chick development from HH stage 8 to 29

	mE13	<i>BMP-2</i>	<i>BMP-4</i>	<i>BMP-7</i>
Neural stage (7.5 dpc)	Allantois, chorion & portion of primitive streak		Allantois, amnion & posterior primitive streak	
Heart (9.5 dpc)	Out flow tract	Atrioventricular canal	Myocardial layer of truncus arteriosus	Atrial & ventricular myocardium
Neural tube (9.5 dpc)	Anterior midline groove		Surface ectoderm overlying dorsal neural tube	Dorsal midline
Otic vesicle (9.5 dpc)	Dorsal region		Posterior margins	
Cephalic region (10.75 dpc)	Lateral nasal process & telecephalic hemisphere	Branchial arches	Distal ectoderm of processes	
Limb buds (10.75 dpc)	Domains of the forelimb	Posterior mesenchyme	Posterior & anterior mesenchyme	Limb bud mesenchyme

Table 5.2: Summary of mE13, *BMP-2*, *BMP-4* & *BMP-7* transcript distribution during murine development from 7.25 dpc to 10.75 dpc

## CHAPTER SIX

### FUNCTIONAL ANALYSES OF E13

#### 6.1 INTRODUCTION

Clone cE13 was originally identified during a large-scale in situ hybridisation screen of a subtracted chick hindbrain cDNA library via its restricted expression pattern at sites that correspond to pre-migratory neural crest (Chapters 3 and 4). Identification of a full-length chick E13 cDNA and sequence analysis of the predicted amino acid sequence identified several conserved features (Chapter 4). The cE13 gene encodes a protein with two different types of motifs: a von Willebrand type D (VWFD) domain and five cysteine-rich repeat (CR) motifs. VWFD domains are present in a number of molecules that form multimers and are found associated with the extracellular matrix such as von Willebrand Factor and Mucins (Sadler, 1998; Perez-Vilar and Hill, 1999), suggesting that E13 might function as a multimer and associate with the extracellular matrix. CR motifs are present in a number of BMP modulating molecules including Chordin (Sasai, *et al.*, 1994), Chordin Like (CHL: Nakayama, *et al.*, 2001) and Twisted gastrulation (Tsg: Mason, *et al.*, 1994).

Chordin is a BMP signalling antagonist that can bind and attenuate BMP-4 molecules via CR repeats number one and three. This direct interaction prevents BMP-4 from binding to its receptor and the induction of the BMP intracellular signalling cascade (Piccolo, *et al.*, 1996; Piccolo, *et al.*, 1997; Larrain, *et al.*, 2000). As cE13 encodes five CR repeats similar to Chordin then perhaps cE13 interacts with BMP molecules. In addition, chick and mouse E13 transcripts are expressed at a number of sites in the developing embryo that coincide with, or are adjacent to tissues that express BMPs (Chapter 5), suggesting that E13 is expressed at the correct time and location to interact with BMP molecules.

To test if chick E13 might function in similar ways to Chordin by affecting BMP function, several avenues have been investigated: generation of cell lines

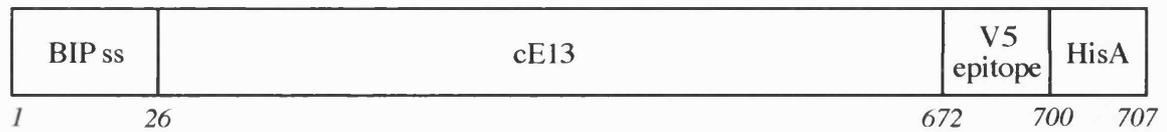
expressing a recombinant chick E13 protein to examine whether cE13 can directly bind BMP molecules; assays using *Xenopus* embryos to examine effects of BMP function; and *in ovo* overexpression in the chick to analyse the *in vivo* function of cE13.

## **6.2 GENERATION OF CELLS EXPRESSING RECOMBINANT CHICK E13 PROTEIN**

It was envisaged that stable cell line(s) expressing a recombinant form of the chick E13 protein could be used as a source of protein for various experiments. These included an antigen for antibody production, a source of protein for *in vitro* binding assays and for use in *in ovo* bead implantation experiments. These experiments require a functional protein, therefore for the production of heterologous chick E13 protein a eukaryotic expression system was chosen to maximise the chance of correct post-translational modification of the cysteine-rich cE13 protein. The *Drosophila* Expression System (DES; Invitrogen<sup>®</sup>) was chosen as it allows for the inducible expression and purification of heterologous proteins using Schneider 2 (S2) insect cells. To maximise the secretion of cE13 from a S2 expressing cell, the pMT/BiP/V5-HisA secretory construct was chosen due to a number of features. The vector contains a *Drosophila* secretory signal (BiP), a leader sequence that is more likely to be recognised and exported by the insect cells than the chick leader sequence, a V5 epitope to allow detection of the protein by Western analysis and a six histidine residue tag for purification of recombinant cE13 protein by metal-chelate affinity chromatography.

The full-length chick E13 cDNA was cloned into the pMT/BiP/V5-HisA construct by PCR (Chapter 2; section 2.8). The resulting pMT/BIP/cE13/V5-HisA construct has had the cE13 putative secretory signal (amino acids 1 to 32) and the endogenous cE13 stop codon (2163bp) removed, but cloned in-frame and downstream is a V5 epitope and poly (6) histidine tag (Figure 6.1, A). To confirm the cloning procedure had not introduced errors the entire construct was sequenced using gene specific primers and compared to the ORF of the full-length cE13 cDNA.

A



B

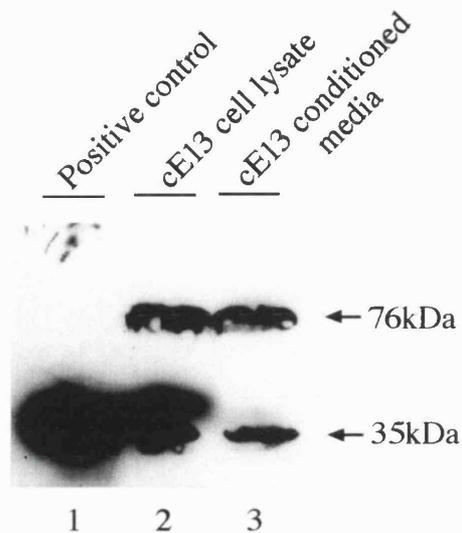


Figure 6.1: Construction and expression of recombinant chick E13 protein

A: Pictorial representation of the recombinant chick E13 protein (pMT/BIP/cE13/V5-HisA); BIP secretory signal (to optimise transport of protein from the S2 cell), cE13 amino acid sequence (from the signalase cleavage site to the last coding amino acid), V5 epitope (to allow detection by Western analysis) and HisA poly (6) histidine tag (for purification). Numbers in italics represent number of amino acid residues. B: Western blot analysis of stable lines expressing heterologous cE13 protein from; Lane 1, positive control (approximately 30 kDa); Lane 2, cell lysate and Lane 3, conditioned media.

Conceptual translation of the open reading frame of the pMT/BIP/cE13/V5-HisA construct predicts a recombinant cE13 protein of 707 amino acids with an unprocessed molecular weight of 78766Da (approximately 76 kDa when processed). In parallel with this cloning procedure the full-length zebrafish Chordin (Chordino; Gift from M. Hammerschmidt) cDNA was cloned in a similar strategy as cE13 into the pMT/BIP/V5-HisA vector to produce pMT/BIP/Chordin/V5-HisA (approximately 120 kDa when processed).

To test expression of the constructs, S2 cells were transiently transfected (calcium phosphate technique) and cell lysates and conditioned media were analysed for expression of recombinant protein by Western analysis using an anti-V5 HRP conjugated antibody. Expression of the pMT/BIP/cE13/V5-HisA construct identified two products, one of approximately 76 kDa and another of 35 kDa in the cell lysate and in conditioned media of transfected S2 cells (Figure 6.1; B). Stable lines expressing recombinant cE13 protein were established by co-transfection and stable integration of the pMT/BIP/cE13/V5-HisA construct and a Hygromycin antibiotic selection vector (pCoHYGRO) into the host cell's genome. Analysis of the amounts of heterologous cE13 protein produced by the stable cE13 S2 cell lines grown in serum containing or serum-free media showed that both forms of the recombinant cE13 protein are produced, albeit at levels less than cE13 transient transfections. Coomassie blue staining of cell lysates and conditioned media identified many other contaminating proteins present with the cE13 protein preparation. To remove these contaminating proteins the recombinant cE13 protein was purified by nickel-chelate affinity chromatography that was directed against the histidine tag, but this process resulted in poor protein yields. Sufficient heterologous cE13 protein for use as an antigen or in bead implantation experiments could not be purified using this strategy (data not shown).

### 6.3 *IN VITRO* BINDING OF cE13 & BMP-4 PROTEIN

Previously, *in vitro* binding assays have been undertaken to show direct interactions of a number of molecules with BMP-4; these include Chordin (Piccolo, *et al.*, 1996), CHL (Nakayama, *et al.*, 2001), Type IIA Procollagen (Zhu, *et al.*, 1999) and Tsg (Oelgeschläger, *et al.*, 2000). These binding experiments have been primarily undertaken by expressing the presumed BMP binding protein as a tagged recombinant protein in a cell line, and the resulting cell lysates or conditioned media containing the heterologous protein were used in immunoprecipitations to determine binding. To test if cE13, like Chordin, is capable of interacting with BMP-4 protein similar experiments were undertaken by immunoprecipitation of V5 tagged recombinant form of the chick E13 protein (as described above) and purified human BMP-4 protein (R&D Systems).

S2 cells in serum containing media were transiently transfected with either the cE13 or Chordin containing pMT/BIP/V5-HisA expression construct; prior to induction of the expression vector, the serum containing media was removed and replaced with serum-free media. Samples of cell lysates or conditioned media that contained the recombinant protein were mixed with 50 ng of BMP-4 protein and allowed to bind. The resulting complex was then precipitated with anti-BMP-4 antibody, pre-bound to protein A sepharose beads. Following washing to remove any non-specifically bound proteins, the immunocomplexes were subjected to PAGE and Western blotting analysis to check for the presence of V5 tagged cE13 or Chordin protein with a polyclonal anti-V5 HRP conjugated antibody (Figure 6.2). Analysis shows that the recombinant form of zebrafish Chordin protein from either cell lysate or conditioned media can bind to human BMP-4 protein (Figure 6.2; lanes 3 and 6). The full-length 76 kDa recombinant cE13 protein and smaller 35 kDa form, both present in the S2 cell lysate and conditioned media, are immunoprecipitated with recombinant human BMP-4 protein (Figure 6.2; lanes 4 and 7). Control reactions show that either cell lysate or conditioned media transfected with the pMT/BIP/V5-HisA vector is not immunoprecipitated with recombinant human BMP-4 protein (Figure 6.2; lanes 5 and 8).

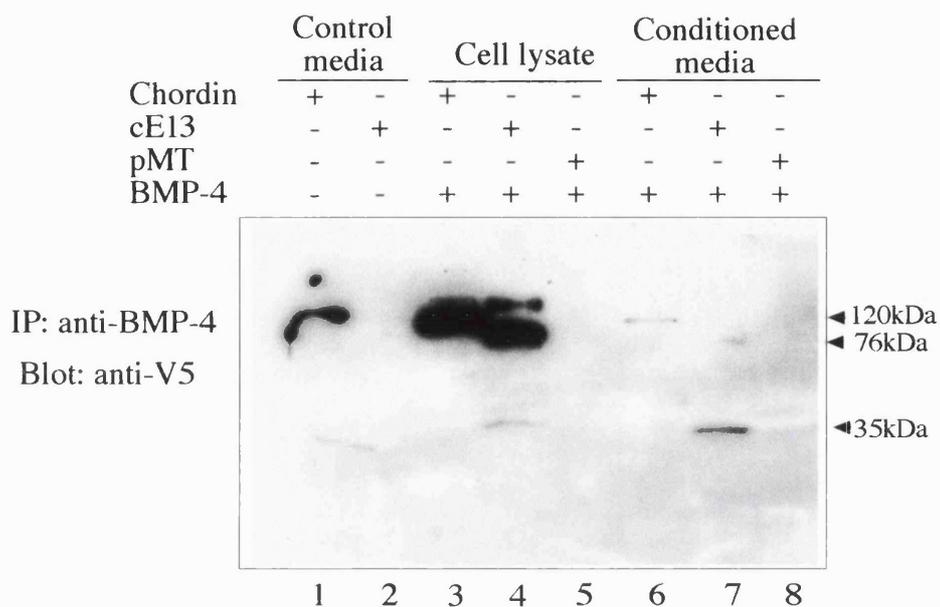


Figure 6.2: Direct interaction of cE13 with BMP-4

Immunoprecipitation/Western blot analysis of heterologous forms of chick E13 and zebrafish Chordin V5 tagged proteins with recombinant human BMP-4 protein. To a sample of cell lysate or conditioned media from Chordin or cE13 expressing S2 cells, 50ng of BMP-4 protein was added directly and incubated. The immunoprecipitation complex formed was precipitated with anti-BMP-4 antibody conjugated to protein A sepharose beads; the bound V5 tagged proteins were detected on Western blots by an anti-V5 HRP conjugated antibody. Chordin is co-precipitated with human BMP-4 in both the cell lysate and conditioned media fractions, lanes 3 and 6. Two fragments of cE13 are detected in both the cell lysate and conditioned media showing they interact with BMP-4, lanes 4 and 7. In control samples, cell lysate and conditioned media from pMT/BIP/V5-HisA transfected S2 cells do not precipitate BMP-4 protein, lanes 5 and 8. Lanes 1 and 2 are control samples of media from Chordin and cE13 expressing S2 cells respectively.

#### 6.4 OVEREXPRESSION OF cE13 IN *XENOPUS* EMBRYOS

During initial stages of *Xenopus* gastrulation *BMP-4* transcripts are expressed throughout the embryo (Dale, *et al.*, 1992), but as gastrulation precedes a unique population of cells form in a region of the mesoderm at the dorsal lip of the blastopore. This region, known as the organiser, secretes a number of BMP antagonists that diffuse away from the presumptive dorsal side of the embryo forming a concentration gradient of BMP inhibition (Chapter 1: Hemmati-Brivanlou and Thomsen, 1995; Holley, *et al.*, 1995; Dale and Jones, 1999). BMP molecules induce non-neural ventral cell fates within gastrulating tissue (Jones, *et al.*, 1992; Jones, *et al.*, 1996), and inhibition of BMP signalling by antagonists such as Chordin, Follistatin and Noggin allows neural induction by attenuating BMP signalling molecules in the dorsal portion of the embryo (Sasai, *et al.*, 1994; Hemmati-Brivanlou, *et al.*, 1994; Zimmerman, *et al.*, 1996).

The *Xenopus* embryo is amenable to study dorsal-ventral patterning because after the first embryonic division the future dorsal and ventral side of an embryo can be identified, enabling the targeted application of reagents to presumptive dorsal or ventral tissues. Injection of *Chordin* mRNA into ventral blastomeres of eight cell *Xenopus* embryos results in embryos with partial secondary axis or a reduction in structures posterior to the head, phenotypes characteristic of dorsalisation (Sasai, *et al.*, 1994). Injection of *Noggin* mRNA into ventral blastomeres results in embryos with severely truncated tails and enlarged head structures, a phenotype consistent with *Noggin* dorsalisating ventral tissues (Smith and Harland, 1992). Conversely, overexpression of *BMP-4* in dorsal regions by injection of *BMP-4* mRNA into dorsal blastomeres inhibit the formation of dorsal and anterior neural cell types, suggestive of a ventralised phenotype (Jones, *et al.*, 1992; Jones, *et al.*, 1996). Therefore, to test if chick E13 is able to mediate BMP signalling, cE13 mRNA was injected into either dorsal or ventral blastomeres of developing *Xenopus* embryos to determine if dorsal-ventral patterning can be disrupted.

The full-length chick E13 cDNA was directionally cloned into the expression vector p $\Delta$ CS2+ to produce p $\Delta$ CS2cE13. The pCS2+ vector has been extensively used in zebrafish and *Xenopus* expression assays because synthetic mRNA generated from this vector has increased *in vivo* message stability (Rupp, *et al.*, 1994). Nucleotide sequencing using gene specific primers and sequence analysis of the

p $\Delta$ CS2cE13 was undertaken to determine correct cloning of the construct, and *in vitro* translation of sense cE13 mRNA generated by *in vitro* transcription of the p $\Delta$ CS2cE13 expression construct confirmed that as expected a product of 71 kDa is produced (Figure 6.3; panel A). To enable control experiments to be undertaken, a full-length chick *BMP-4* cDNA (gift from Randy Johnson) was cloned into the p $\Delta$ CS2+ vector and sequenced to determine correct cloning, and a full-length *Xenopus Noggin* cDNA in the pCS2+ expression vector (gift from Nobue Itasaki) was used to generate sense mRNA for injections. Synthesised sense mRNAs were co-injected with a lineage tracer (FIDx) into either the ventral-marginal or dorsal-marginal zone of four cell staged *Xenopus* embryos that were allowed to develop to Nieuwkoop stage 27 (Nieuwkoop and Faber, 1967).

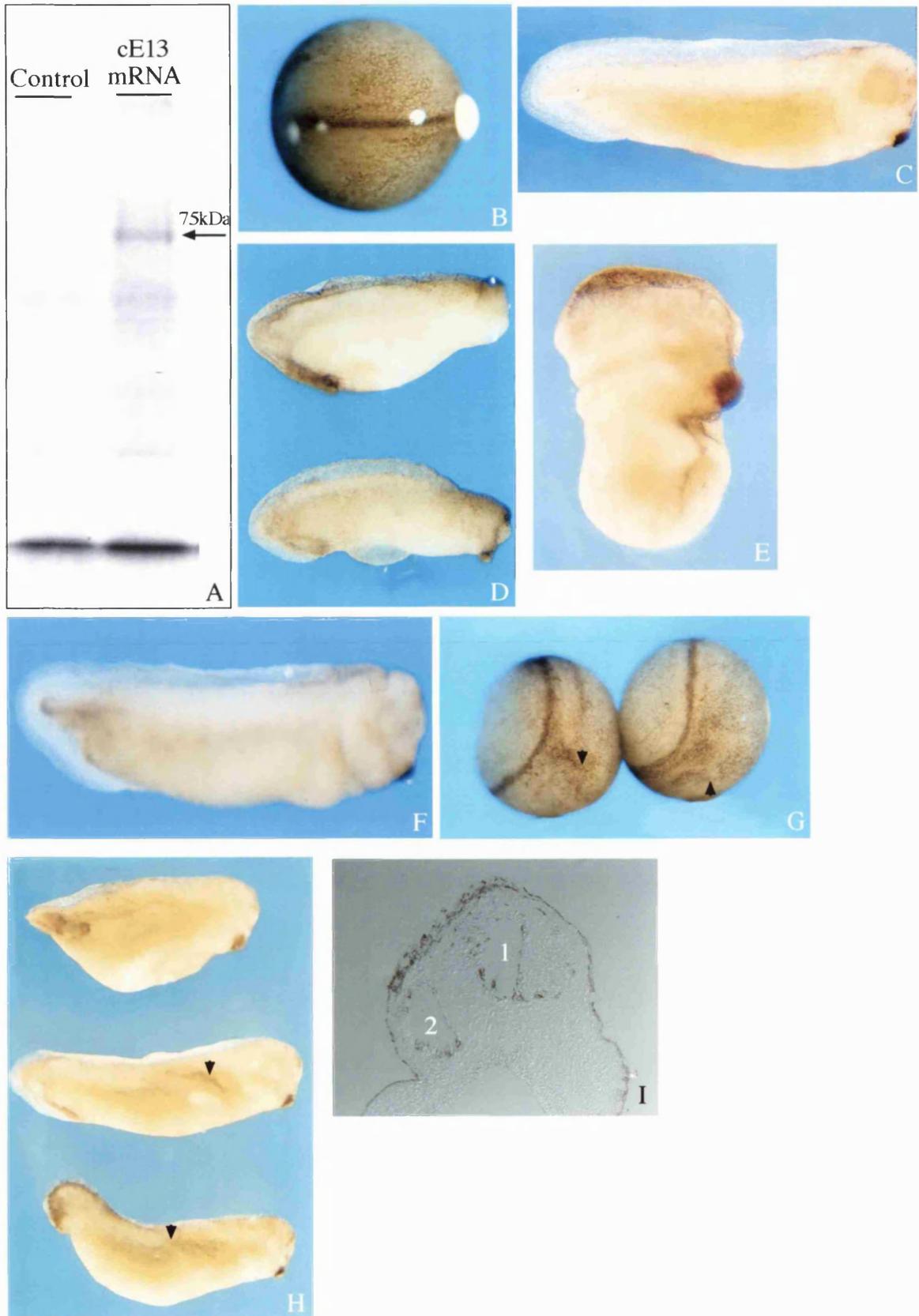
Visual analysis of embryos revealed that injections of 500 pg of *BMP-4* mRNA (n = 39) into dorsal blastomeres at the four cell stage causes a decrease or loss of anterior head structures in 50% of injected embryos' this is consistent with a ventralised phenotype (Figure 6.3; panel D: also see; Dale, *et al.*, 1992; Jones, *et al.*, 1992). Consistent with published results, injections of 25 pg of *Xenopus Noggin* mRNA (n = 34) into ventral blastomeres at the four cell stage resulted in 90 % of the embryos with severely truncated tail and enlarged head structures in 90 %, characteristic of a dorsalised phenotype (Figure 6.3; panel E: Smith and Harland, 1992; Sasai, *et al.*, 1994).

Injections of 750 pg sense cE13 mRNA (derived from p $\Delta$ CS2cE13 construct) into dorsal blastomeres (n = 45) does not disrupt the development of embryos (Figure 6.3; panels F). Embryos injected ventrally with 750 pg of cE13 mRNA (n = 69) exhibited a decrease in body length in 31% of embryos and in some injected embryos a partial secondary axis was induced (36%: Figure 6.3; panels G and H). The partial secondary axis observed in some cE13 mRNA injected embryos lacked any anterior structures such as eyes and cement glands. Histological sectioning identifies a smaller second neural epithelium is formed but no notochord is present (Figure 6.3; panel I). The absence of a notochord is common in manipulated *Xenopus* embryos and does not negate the classification of the secondary axis (Steinbeisser, *et al.*, 1993). The dorsalised phenotype observed in embryos ventrally injected with 750 pg of cE13 mRNA were similar, but not as severe as embryos ventrally injected with 25 pg of *Xenopus Noggin* mRNA.



Figure 6.3: Chick E13 mRNA dorsalises *Xenopus* embryos when injected ventrally

A: Auto-radiograph image of an *in vitro* translation of cE13 mRNA derived from pΔCS2cE13 expression vector and negative control. B and C: Un-injected wild type *Xenopus* embryos at stage 14 and 27 of development respectively. Stage 27 *Xenopus* embryos injected; dorsally with 500 pg of Chick *BMP-4* mRNA (D), or ventrally with 25 pg of *Xenopus Noggin* mRNA (E) at the four cell stage. Embryos injected dorsally at 4 cell stage with 750 pg of cE13 mRNA have no noticeable effect on development, (F), whereas ventral injections of 750 pg of cE13 mRNA can reduce the length of the embryos A-P axis (H), or induce partial secondary axis (arrow heads in panels G and H, stage 14 and 27 respectively). Cross-section through the trunk of an embryo injected ventrally with 750 pg of cE13 mRNA with partial secondary axis confirms the presence of the primary neural epithelium (1) and an ectopic second neural epithelium (2).



## 6.5 XENOPUS ANIMAL CAP ASSAYS

To further investigate possible roles of chick E13 on BMP signalling *Xenopus* animal cap explant assays were employed. *Xenopus* ectoderm tissue isolated from the animal pole of late blastula staged embryos develops as non-neural ectoderm, this is because tissue isolated prior to organiser formation has not been exposed to neuralising signals such as Chordin or Noggin. The non-neural cell fate can be challenged with the introduction of various neuralising factors such as Noggin or Chordin (Lamb, *et al.*, 1993; Sasai, *et al.*, 1995). In ectodermal explants, the BMP signalling antagonists attenuate the endogenous BMP molecules allowing neural induction and the expression of neural specific genes that can be analysed by RT-PCR using primers-pairs specific for neural markers.

One cell stage *Xenopus* embryos were injected at the animal pole with varying amounts of cE13 mRNA (100 pg - 4 ng) and allowed to develop to blastula stage 8 when animal caps were cut and isolated. These isolated ectodermal explants were allowed to develop until whole sibling embryos reached the early tail bud stage (stage 20) and then RNA was extracted and cDNA synthesised. The induction of different tissue types was assessed by radioactive PCR using a battery of primer pairs for various tissue specific markers. These included the pan-neural marker *NCAM* (Neural Cell Adhesion Molecule: Kintner and Melton, 1987), the anterior forebrain neural marker *BF-1* (Brain Factor-1: Bourguignon, *et al.*, 1998), and the posterior midbrain/anterior hindbrain marker *En-2* (Engrailed-2: Hemmati-Brivanlou and Harland, 1989). Muscle-specific actin was also employed to determine if the neuralisation of ectoderm explants was direct and no contaminating mesoderm tissue was present (Hemmati-Brivanlou and Melton, 1994). Elongation Factor-1 alpha (*EF-1 $\alpha$* ) primers were used to allow semi-quantitative comparisons of the amounts of isolated mRNA; the number of *EF-1 $\alpha$*  transcripts are similar in all cells during early *Xenopus* embryogenesis (Krieg, *et al.*, 1989). These experiments were replicated three times.

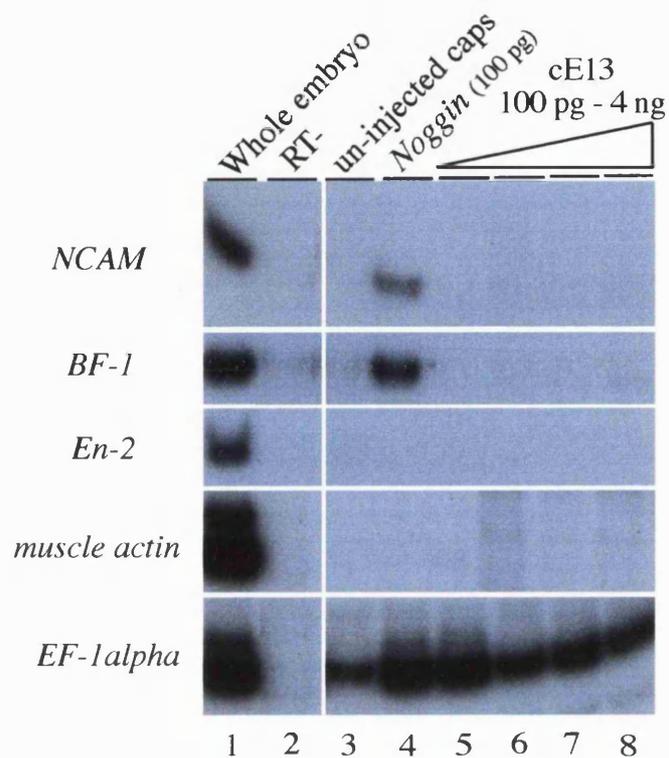


Figure 6.4: Chick E13 mRNA does not induce neural markers in *Xenopus* ectoderm explants

Animal caps were excised from injected *Xenopus* embryos at stage 8, cultured until stage 20 and assayed for the expression of marker mRNAs by RT-PCR. Lanes contained: 1, RNA extracted from whole embryos; 2, RT-PCR control; 3, un-injected caps; 4, 100 pg of *Noggin* mRNA; 5, 100 pg; 6, 500 pg; 7, 1 ng; 8, 4 ng of cE13 mRNA. Note; *Xenopus* *Noggin* mRNA induces the general neural marker *NCAM* and anterior neural marker *BF-1*. The mesodermal marker muscle actin has not been induced. Increasing amounts of cE13 mRNA does not induce expression of any of the neural markers. Elongation factor-1 alpha (*EF-1 $\alpha$* ) was used to assess the relative amounts of recovered mRNA.

### 6.5.1 Injection of cE13 mRNA

To determine if cE13 can induce neural tissue directly, in a manner similar to known BMP antagonists (Noggin and Chordin: Lamb, *et al.*, 1993; Sasai, *et al.*, 1995; Sasai, *et al.*, 1996), cE13 mRNA was injected into one cell *Xenopus* embryos at varying concentrations (100 pg to 4 ng); these embryos were allowed to develop before animal caps were cut, isolated and cultured as described above. The cultured explants were then analysed for the induction of neural tissues using the neural markers *NCAM*, *BF-1* and *En-2* (Figure 6.4).

In the control experiments, RNA isolated from un-injected whole embryos expressed all markers assayed for (Figure 6.4; lane 1), and in the RT-PCR control and un-injected cap control, no neural markers were induced (Figure 6.4; lanes 2 and 3 respectively). Injection of 100 pg of *Noggin* mRNA induces the neural markers *NCAM* and *BF-1*, but as expected does not induce the midbrain/hindbrain neural marker *En-2* (Figure 6.4; lane 4). The expression of neural markers was a direct result of *Noggin* mRNA as the mesodermal marker muscle actin was not induced, confirming that the neuralisation of ectoderm was direct and no contaminating mesoderm tissues were present. Chick E13 mRNA (generated from the p $\Delta$ CS2cE13 construct) injected at increasing concentrations from 100 pg to 4 ng does not induce any of the neural specific markers *NCAM*, *BF-1* or *En-2* (Figure 6.4 lanes 5 to 8). The detection of similar levels of *EF-1 $\alpha$*  RNA present in each sample indicates that similar amounts of mRNA was recovered in each pool.

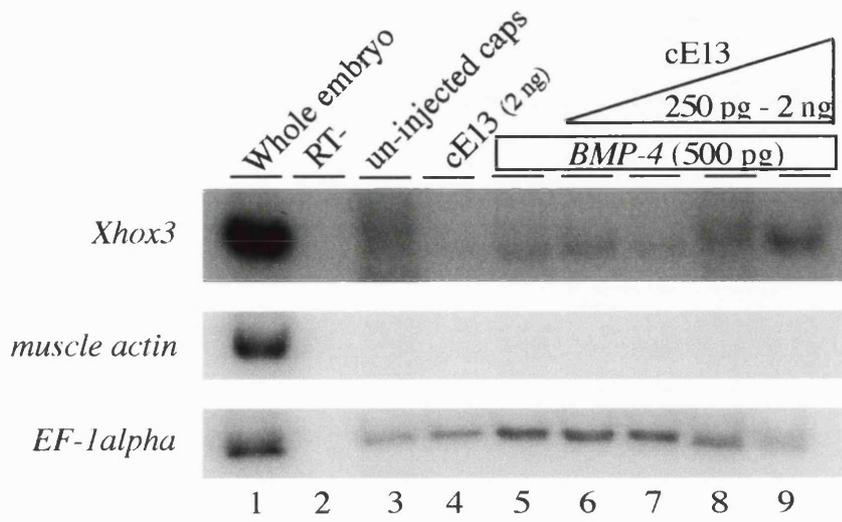
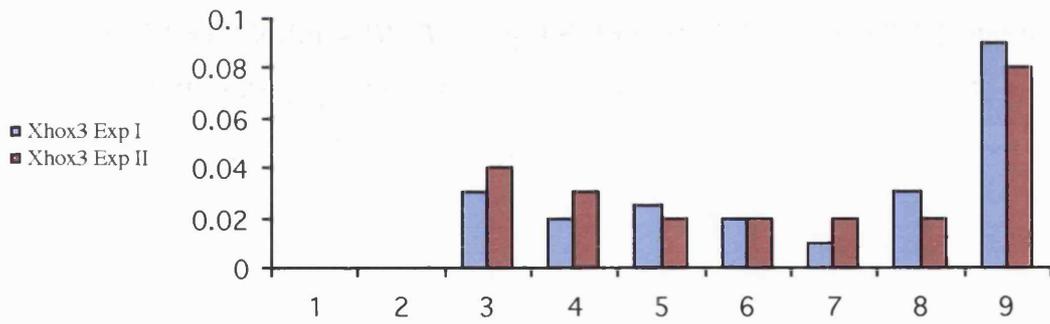
### 6.5.2 Co-injection of cE13 and *BMP-4* mRNA

The *Drosophila* homologue of cE13 (Chapter 4), Crossveinless 2 (*Cv-2*) is required for high levels of BMP-like signalling suggesting that it potentiates BMP-like signalling (Conley, *et al.*, 2000). To determine if cE13 promotes BMP signalling in a manner similar to *Cv-2* levels of *Xhox3* activation, an indirect downstream target of *BMP-4* (Ruiz i Altaba and Melton, 1989; Dale, *et al.*, 1992; Hemmati-Brivanlou and Thomsen, 1995), were assessed using the *Xenopus* animal cap assay. *Xhox3* expression is detected in RNA isolated from whole *Xenopus* embryo (Figure 6.5; A, lane 1) where it is most highly expressed in posterior and ventral mesoderm (Ruiz i Altaba and Melton, 1989). As expected, whole un-injected embryos expressed all markers and no expression of any marker was detected in the RT-PCR negative control (Figure 6.5; A, lanes 1 and 2 respectively). Residual expression of *Xhox3* was detected in un-injected animal caps and at similar levels in caps injected with 2 ng of cE13 mRNA (Figure 6.5; A, lanes 3 and 4 respectively). The expression of *Xhox3* remains relatively constant with the injection of 500 pg of *BMP-4* (Figure 6.5; A, lane 5), similarly, co-injections of 500 pg of *BMP-4* and increasing amounts of cE13 mRNA from 250 pg to 1 ng, has little effect on the level of *Xhox3* expression (Figure 6.5; A, lanes 6 to 8). Co-injection of 500 pg of *BMP-4* and 2 ng of cE13 mRNA elevates the expression of *Xhox3* relative to the RNA loading control *EF-1 $\alpha$*  (Figure 6.5; A, lane 9). To determine the level of *Xhox3* expression relative to the amount of mRNA isolated from each sample, band intensities were quantified using a Phosphoimager. Quantification of the bands show that 500 pg of *BMP-4* mRNA plus 2 ng of cE13 mRNA elevates the expression of *Xhox3* in isolated animal caps, approximately four times higher than 500 pg of *BMP-4* mRNA alone (Figure 6.5; B).



Figure 6.5: Chick E13 mRNA increases the expression of a downstream BMP target

The co-injection of cE13 and *BMP-4* mRNA can increase the expression of *Xhox3*, a downstream target of BMP signalling. A: animal caps were excised from injected embryos at stage 8, cultured until stage 20 and assayed for the expression of marker mRNAs by RT-PCR. Lanes contained: 1, RNA extracted from whole embryos; 2, RT-PCR control; 3, un-injected caps; 4, 2 ng of cE13 mRNA; 5, 500 pg of *BMP-4* mRNA; 6, 500 pg of *BMP-4* + 250 pg of cE13 mRNA; 7, 500 pg of *BMP-4* + 500 pg of cE13 mRNA; 8, 500 pg of *BMP-4* + 1 ng of cE13 mRNA; 9, 500 pg of *BMP-4* + 2 ng of cE13 mRNA. Note; the levels of *Xhox3* expression when 2 ng of cE13 or 500 pg of *BMP-4* mRNA is injected is greater than in un-injected caps. Also note; relative to the loading control (*EF-1 $\alpha$* ), co-injection of 500 pg of *BMP-4* mRNA and increasing amounts of cE13 mRNA (up to 1 ng) has no effect on *Xhox3* expression. Relative intensities of the *Xhox3* bands were measured and standardised relative to the *EF-1 $\alpha$*  loading control. B: Bar chart to showing the relative intensities of *Xhox3* expression.

**A****B**

## 6.6 OVEREXPRESSION OF CHICK E13 IN THE DORSAL NEURAL TUBE

The function of cE13 *in vivo* in the developing chick embryo was investigated by overexpression. In the chick hindbrain *BMP-4* transcripts are expressed in two foci of expression centred in dorsal r3 and r5 and the majority of neural crest cells that form in r3 and r5 are eliminated by a BMP-4 induced, *msx2* mediated, cell death pathway (Graham, *et al.*, 1993; Graham, *et al.*, 1994; Takahashi, *et al.*, 1998). These published data, coupled with the findings that cE13 and *BMP-4* expression patterns coincide in the dorsal hindbrain (Chapter 5), and that cE13 can interact and modulate BMP-4 signalling, suggested that there could be effect(s) on neural crest cell development when cE13 is overexpressed in the chick hindbrain.

The electroporation technique has been used successfully to investigate the function(s) of a number of genes (Itasaki, *et al.*, 1999; Inoue and Krumlauf, 2001; Kos, *et al.*, 2001). The electroporation technique passes a pulse of electric current across the embryo, and as DNA has a negative charge any DNA within the electrical field moves towards the cathode. If the DNA is an expression construct present in the lumen of the neural tube then it will transfect one side of the neural tube with the other side forming a control. The p $\Delta$ CS2cE13 expression construct was injected *in ovo* into the neural tube lumen at the level of the prospective hindbrain in HH stage 8-/8 embryos. Transfected embryos were allowed to develop for approximately 12 hours to HH stage 10+ before being harvested, and fixed at a time just as neural crest cells are initiating migration from r4. To determine if the p $\Delta$ CS2cE13 expression construct could be expressed *in vivo* it was electroporated into chick embryos that were allowed to develop and then fixed, as described above. The transfected embryos were then assayed for expression of cE13 transcripts by whole mount *in situ* hybridisation using a cE13 riboprobe (Chapter 5); cE13 transcripts were detected on the transfected side of embryos demonstrating that the p $\Delta$ CS2cE13 expression construct can be expressed *in vivo* (Figure 6.6; panel A).

Analysis of electroporated embryos requires a knowledge of what portion of the neural tube has been transfected, and therefore a full-length lacZ reporter construct (cloned into the pCS2+ expression construct) was co-transfected with p $\Delta$ CS2E13. Subsequent staining for LacZ activity allowed detection of the anterior and posterior limits of transfection and therefore of the domain of cE13

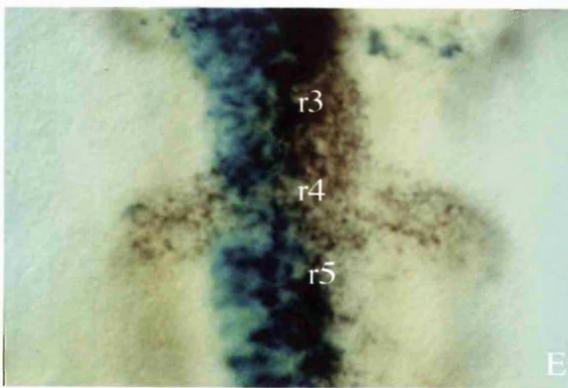
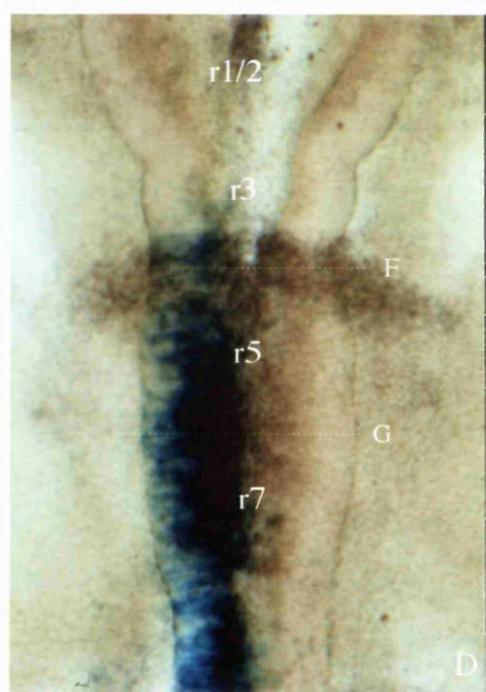
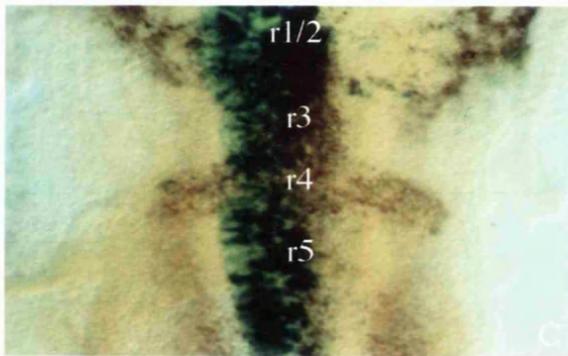
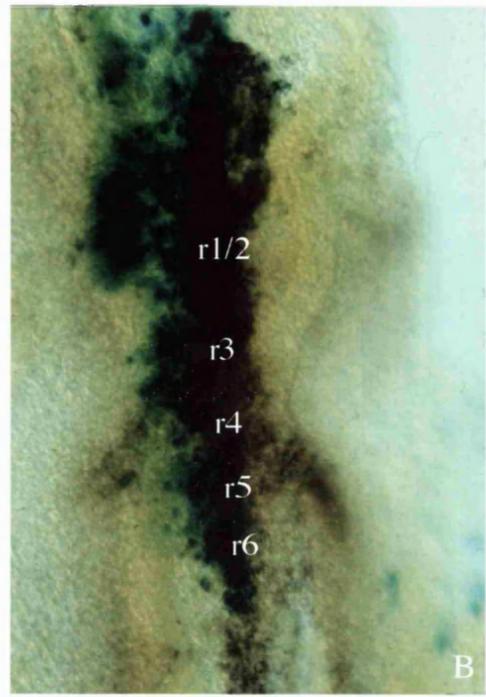
overexpression. The pre-migratory and post-migratory neural crest markers *Slug* (Nieto, *et al.*, 1994) and *AP-2* (Shen, *et al.*, 1997) were employed as markers to analyse what effect cE13 overexpression has on the migration of cranial neural crest cells.

Analysis of cE13 electroporated embryos (n = 18) using *Slug* as a neural crest marker, identified one embryo with less r4 derived migratory neural crest cells on the transfected side compared to the non-transfected side (Figure 6.6; panel B). However, in all other embryos transfected with the p $\Delta$ CS2cE13 expression construct and analysed using the neural crest cell marker *Slug*, had no reproducible affect on migratory neural crest cell from the hindbrain (Figure 6.6; panel C). Analysis of embryos transfected with the cE13 expression construct using *AP-2* as a neural crest cell marker (n = 20) identified five embryos that exhibited a phenotype with varying degrees of severity (the most pronounced is depicted in Figure 6.6; panel D). These five embryos exhibited a decrease in the amount, or a delay in the migration of *AP-2* expressing neural crest cells from rhombomere 4. Cross-sections at the level of r4 identify that *AP-2* positive migratory r4 neural crest cells have not migrated as far laterally from the dorsal midline on the transfected side than the non-transfected (Figure 6.6; panels D and F). Cross-sections of the same embryo further caudal to r4 at around the axial level of r6, identifies an increase in the number of pre-migratory *AP-2* expressing neural crest cells on the transfected side compared to the non-transfected side (Figure 6.6; panel G). This phenotype was not detected in the majority of embryos that had been electoporated with the p $\Delta$ CS2cE13 expression construct when analysed using the *AP-2* ribroprobe as a neural crest cell marker (a representative embryo is depicted in Figure 6.6; panel E).



Figure 6.6: Overexpression of cE13 in the hindbrain region of the chick neural tube

A: HH stage 11 chick embryo that has been electroporated with the p $\Delta$ CS2cE13 expression construct at HH stage 8 and allowed to develop to stage 11; the embryo has been assayed for cE13 expression by in situ hybridisation, note asymmetric distribution of cE13 in situ signal. B, C, D and E: images of flat mounted chick hindbrain regions of four embryos, rhombomeres (r) are labeled. Embryos were electroporated at HH stage 8-8 with expression vectors p $\Delta$ CS2cE13 and pCS2LacZ. Embryos were allowed to develop to HH stage 11- and then fixed, a lacZ stain performed (turquoise colour), followed by whole mount in situ hybridisation using either *Slug* (panels B and C) or *AP-2* (panels D to G) as neural crest cell markers (Purple/brown colour). B: Embryo exhibiting the most pronounced phenotype identified using *Slug* as a riboprobe; note the decrease in amount of *Slug* expressing r4 derived neural crest cells on the electroporated side. C: Representative image of a cE13 electroporated embryo analysed using *Slug*; no change in *Slug* positive migratory neural crest cells. D: Embryo exhibiting the most pronounced phenotype using *AP-2* as a riboprobe; embryo exhibits a decrease in *AP-2* positive r4 migratory neural crest cells. E: Representative phenotype, the amount of *AP-2* positive neural crest cells migrating for r4 are similar on the transfected side to the non-transfected side. F and G: Transverse sections through embryo (D) as indicated with dashed lines. In cross-section through r4 (F), neural crest cells have not migrated as far laterally on the electroporated side as on the non-transfected side. Further caudally at approximately r6 (G), there are more pre-migratory neural crest cells on the electroporated side than the non-electroporated side.



## 6.7 DISCUSSION

### 6.7.1 Chick E13 protein interacts with human BMP-4 protein

The chick E13 protein includes five cysteine-rich repeat (CR) motifs. Other proteins that contain these CR motifs include Chordin, Type IIA procollagen, CHL and Tsg, all of which have been shown to directly bind to BMP molecules (Piccolo, *et al.*, 1996; Zhu, *et al.*, 1999; Oelgeschläger, *et al.*, 2000; Nakayama, *et al.*, 2001). BMPs have many important roles throughout development (Hogan, 1996) and as cE13 has been shown to be expressed with, or adjacent to BMP expressing tissues (Chapter 5), it was decided to determine if cE13 was capable of interacting with BMP-4. To achieve this *Drosophila* S2 cells were transfected with an inducible expression construct containing the chick E13 cDNA.

Two forms of the recombinant cE13 protein were detected in the cell lysate and conditioned media of pMT/BIP/cE13/V5-HisA expressing S2 cells: the expected 76 kDa cE13 protein and a 35 kDa product. The smaller protein represents a cleaved form of the recombinant cE13 protein that corresponds to a carboxyl portion of the protein as the V5 tag is at this terminus. *In silico* analysis of the recombinant cE13 protein suggests that the cE13 protein is cleaved after the fourth CR motif and before the beginning of the fifth CR motif. At present it is not known if this cleaved form of cE13 is a result of specific or non-specific proteolytic degradation that will produce either a functionally active or inactive cE13 protein. To determine if this cleavage occurs *in vivo*, peptide antibodies have been designed to recognise the cE13 protein; one against the amino-terminus upstream of the proposed cleavage site and the other against the carboxyl-terminus that will target the 35 kDa cleaved product. At the time of writing antibodies are not yet available.

One way to show direct interactions of proteins is by *in vitro* binding assays. The approach taken to determine if cE13 interacts with BMP-4 was similar to those undertaken in identifying other BMP binding proteins. Cell lysates or conditioned media from pMT/BIP/cE13/V5-HisA transiently transfected S2 cells were mixed with recombinant BMP-4 protein and immunoprecipitated. The results show that a recombinant form of the chick E13 protein can bind to human BMP-4 protein.

BMP signalling antagonists can physically interact with more than one BMP family member (shown by immunoprecipitation); for example Chordin can form

complexes with BMP-4 or BMP-2 (Piccolo, *et al.*, 1996), and Noggin can bind BMP-4, BMP-2 or to a lesser extent to BMP-7 (Zimmerman, *et al.*, 1996), albeit by a different binding site on BMP-4 than Chordin. Further investigation is required to determine if cE13 interacts with other BMP family members. Interestingly, as well as the full-length cE13 protein the 35 kDa cE13 fragment, that corresponds to a COOH-terminus cleaved product of the cE13 protein, can also associate with BMP-4. This interaction could be direct as the cleaved product of cE13 contains one CR motif (CR5); *in vitro* studies have shown that isolated CR motifs from Chordin can bind directly to BMP-4 (Larrain, *et al.*, 2000).

#### 6.7.2 Whole *Xenopus* embryo injections suggest that chick E13 has a similar activity to BMP signalling antagonists

From the binding experiment chick E13 protein has been shown to interact with BMP-4, but from these data it is not known if the resulting protein complex promotes or inhibits the activity of the BMP-4 molecule. To test this, *Xenopus* embryos have been injected with chick E13 mRNA into either dorsal or ventral blastomeres and allowed to develop before being visually assessed for a phenotype. Dorsal injections of cE13 mRNA has no effect on embryonic development, but ventral injections result in embryos with a reduction in body axis length and in a proportion the formation of a partial secondary axis. These phenotypes are similar to the phenotype observed when BMP signalling antagonists such as *Noggin* or *Chordin* are injected ventrally (Smith and Harland, 1992; Sasai, *et al.*, 1994), suggesting that cE13 functions as an antagonist of BMP signalling. However, although the cE13 dorsalisated phenotype is similar to the dorsalisated *Noggin* phenotype it is not as severe, even though thirty times more cE13 mRNA was injected than *Noggin* mRNA. One explanation could be that the *Noggin* mRNA used in the injections was generated from a different expression construct than cE13, therefore the stability of the *Noggin* mRNA *in vivo* may be greater than the cE13 mRNA. Alternatively, cE13 protein may be less potent or have a different activity compared with *Noggin* protein.

### 6.7.3 The antagonistic function of Chick E13 on BMP signalling is indirect

The assays discussed above were undertaken in whole *Xenopus* embryos, and from these results it is not known if the dorsalising effect observed is direct or as a result of indirect interactions with other molecules present in the ventral domain of an injected embryo. To address this issue the *Xenopus* animal cap assay was used by injecting cE13 mRNA into the one cell stage embryo and allowing it to develop until blastula stage before cutting ectoderm caps and culturing them in isolation. If cE13 is a direct antagonist of BMP signalling like Noggin, then analysis of the explants cell fate by RT-PCR would detect the induction of neural markers such as *NCAM* or *BF-1*. These experiments show that cE13 mRNA cannot induce neural cell fates in ectoderm explants and therefore the BMP antagonistic function observed in whole *Xenopus* injections may be a result of indirect interactions with other molecule(s) that are not present in ectoderm explants.

*Xenopus* Kielin is the most similar protein to cE13 that has been identified to date (Chapter 4). Kielin is comprised of 27 CR motifs and a C-terminal VWFD domain (Matsui, *et al.*, 2000), compared to the 5 CR motifs and a C-terminal VWFD domain coded by cE13 (Chapter 4). As these two molecules are composed of the same types of domains then perhaps they might have similar functions or properties. Experiments to investigate the role of *Xenopus* Kielin demonstrate that it can dorsalise ventral marginal zone explants in a manner similar to a BMP antagonist, but overexpression of Kielin in ectodermal explants does not induce expression of neural markers (Matsui, *et al.*, 2000). This suggests that cE13 has a similar biochemical function as Kielin; both molecules can apparently attenuate BMP-4 signalling, although since neither molecule can induce neural tissue, this may be an indirect or partial antagonism.

### 6.7.4 Chick E13 mRNA can promote the expression of downstream BMP targets

The *Drosophila* homologue of cE13, Crossveinless 2 (*Cv-2*), is required for high levels of BMP-like signalling in the cross veins of the developing wing (Conley, *et al.*, 2000) raising the possibility that cE13 has a similar agonistic activity on BMP signalling. To test this hypothesis, the *Xenopus* animal cap assay was used to co-inject chick *BMP-4* mRNA and cE13 mRNA to detect if the expression levels of a downstream BMP target were affected. *Xhox3* was used as an indirect downstream

target of BMP-4 signalling (Dale, *et al.*, 1992; Hemmati-Brivanlou and Thomsen, 1995), and if cE13 is capable of promoting BMP signalling then co-injections of cE13 and *BMP-4* mRNAs will increase levels of *Xhox3* expression. These data show that when *BMP-4* (500 pg) and cE13 (2 ng) mRNA are co-injected the level of *Xhox3* activation increases; therefore, cE13 increases the expression of downstream components of the BMP signalling cascade, suggesting that cE13 can potentiate BMP-4 signalling.

Data from the whole *Xenopus* embryo injections suggest that cE13 may function as a BMP antagonist, whereas data from *Xenopus* animal cap assays suggest that cE13 is functioning as a BMP agonist. One possibility is that cE13 has dual roles and is able to directly promote and inhibit BMP signalling. Interestingly, a BMP binding molecule that contains two partial CR motifs called Twisted gastrulation (Tsg) can both antagonise and promote BMP signalling (Mason, *et al.*, 1994; Oelgeschläger, *et al.*, 2000; Chang, *et al.*, 2001). Tsg activity is dependent on the presence or absence of the metalloprotease Xolloid that can cleave Chordin at two sites when it is bound to a BMP molecule (Piccolo, *et al.*, 1997). In the absence of Xolloid, Tsg acts as a BMP antagonist by binding to BMP-4 (via its two partial CR motifs) when Chordin is bound, stabilising the BMP-4/Chordin complex and preventing BMP-4 from interacting with its receptor (Ross, *et al.*, 2001; Chang, *et al.*, 2001). When Chordin is cleaved by Xolloid, Tsg functions as a BMP agonist by competing with the two cleaved Chordin fragments preventing them from binding to and inactivating the BMP-4 molecule, enabling the active BMP-4 molecules to bind to its receptor (Oelgeschläger, *et al.*, 2000). It is therefore possible that the activity of cE13 is different in whole *Xenopus* embryos injections compared to the *Xenopus* animal cap assays as there may be modifying cofactors present in the whole embryo that are absent in the mesoderm explants. These co-factors could cleave the cE13 protein thereby affecting its function. Indeed, two cleaved forms of the cE13 protein have been identified *in vitro* and if this occurs *in vivo* then it is important to investigate how the cleavage affects cE13 activity.

#### 6.7.5 Function of Chick E13 in the dorsal neural tube

To investigate the function of cE13 *in vivo*, chick E13 was overexpressed in the neural tube of developing chick embryos using the electroporation technique.

Since cE13 is expressed in the dorsal neural tube at sites of pre-migratory neural crest cells (Chapters 3 and 5), this will lead to more widespread, persistent and higher levels of cE13 expression. Neural crest cells migrating from the hindbrain are separated by neural crest free regions adjacent to r3 and r5, and one mechanism that has been proposed to divide the migratory neural crest cells into these three separate streams is the BMP-4/*msx2* mediated cell death of neural crest cells (Lumsden and Graham, 1996). Apoptosis occurs in the dorsal neural tube, with raised levels over r2/3 and r5, in a region that coincides with the expression of a homeobox containing gene *msx2*. Ectopic expression of the *msx2* gene can eliminate all migratory neural crest cells from the dorsal neural tube, suggesting that *msx2* can mediate apoptotic elimination of pre-migratory neural crest cells from r3 and r5 (Takahashi, *et al.*, 1998). BMP-4 expression is focussed in dorsal r3 and r5 (Graham, *et al.*, 1993), and has been shown to induce *msx2* in explant cultures and therefore can induce the apoptotic elimination of pre-migratory neural crest cells from r3 and r5 (Graham, *et al.*, 1994).

As previously discussed, cE13 has been shown to modulate BMP signalling. If cE13 promotes BMP signalling then one can predict that the overexpression of cE13 within the hindbrain region would increase the activity of BMP-4 and in turn the expression of *msx2*. The promotion of *msx2* expression would result in an increase in the levels of apoptosis, ultimately resulting in a decrease in the number of migratory neural crest cells from r4. Conversely, if cE13 inhibits BMP function, the levels of apoptosis would decrease resulting in an increase in migratory neural crest cells that would normally have been eliminated. Overexpression of cE13 by *in ovo* electroporation resulted in a proportion of embryos with a decrease or delay in neural crest cells migrating from r4, when assayed by *in situ* hybridisation using *Slug* or *AP-2* as neural crest cell markers. These data are consistent with cE13 promoting BMP signalling, but due to the relatively small number of the embryos exhibiting a decrease in the number of neural crest cells migrating from r4, this cannot be firmly concluded. Reasons for the inconsistent results could be technical limitations of the experiment and/or relate to the function of cE13.

When using the electroporation technique to overexpress a gene, the location and levels of expression varies between each electroporated embryo. This is because the site and amount of construct transfected into the cells of the neural epithelium

cannot be controlled and therefore every transfected embryo will overexpress cE13 at different sites and at different concentrations, thus producing variable results. A possible reason for the overexpression of cE13 not effecting neural crest cell migration is because the amount and location of endogenous cE13 protein in the dorsal neural tube is sufficient to fulfill its function(s), and overexpressing or widening the domain of cE13's function in the neural tube would have no effect.

## CHAPTER SEVEN

### GENERAL DISCUSSION

#### 7.1 PROPOSED MODEL OF E13 FUNCTION

From the data presented in this thesis and from published literature, a hypothesis has been formed as to the possible function of cE13. BMP molecules are diffusible having effects at regions distant from their site of synthesis (Jones, *et al.*, 1996), therefore it is possible that cE13 tether and localise BMP molecules at sites where high levels of BMP signalling are required, acting as an anchor of BMP molecules.

Sequence analysis has shown that cE13 contains a VWFD domain that is found in a number of extracellular associated proteins, molecules such as von Willebrand Factor and members of the mucin protein family (Sadler, 1998; Perez-Vilar and Hill, 1999). Therefore, the VWFD domain could associate with the extracellular matrix leaving the predicted BMP binding CR motifs to protrude into the extracellular space. Here they could bind to BMP molecules present in the extracellular space, thus preventing the BMP molecules from diffusing and localising the BMP signalling molecule. Co-injection of cE13 and *BMP-4* mRNA in *Xenopus* ectodermal explants elevates the expression of *Xhox3*. Applying the proposed model to this result suggests that cE13 lessens the diffusion of BMP-4 protein from the caps into the surrounding environment; cE13 therefore retains the BMP-4 protein in the cap where it is capable of inducing the expression of downstream components of its signalling pathway. Further investigation is required to address this issues; this is discussed in section 7.3.2 (i).

BMP-4 molecules can bind directly to the extracellular matrix by associating with heparan sulphate proteoglycans via a three amino acid motif at its N-terminus (Ohkawara, *et al.*, 2002). Moreover, the signalling range and activity of BMP-4 molecules are also regulated during its maturation by the processing of its pro-domain by endoproteases such as Furin (Constam and Robertson, 1999; Cui, *et al.*,

2001). These data suggest that diffusion of BMP signalling molecules is dependent on a number of events; our hypothesis proposes that cE13 is another such factor that restricts the diffusion of BMP molecules, thus increasing BMP signalling within the E13 domain of expression.

Comparative analysis of both chick and mouse E13 transcript distribution with the published expression profile of a number of BMP family members (Chapter 5), identify that sites of E13 expression coincide with BMP expression profiles or at sites adjacent to BMP expressing tissues. Discussed below is an overview of chick and mouse E13 expression profiles and their temporal and spatial relationship with BMP family members in a number of embryonic tissues, and E13's possible function based on the proposed model.

#### 7.1.1 Expression during neural induction

A number of BMP family members play an integral role during chick neural plate formation. *BMP-2*, *BMP-4* and *BMP-7* are all expressed at early stages of neural induction in the non-neural ectoderm in distinct domains surrounding the future neural plate (Streit, *et al.*, 1998; Streit and Stern, 1999). Chick E13 transcripts are first detected in the developing chick embryo at HH stage 8, in the lateral plate mesoderm and ectoderm surrounding the regressing primitive streak; this expression domain corresponds to gastrulating tissue around the tail bud region. At this stage *BMP-2*, *BMP-4* and *BMP-7* are expressed in partially overlapping domains at the lateral edges of the neural plate and at lower levels in the non-neural ectoderm and lateral mesoderm (Streit, *et al.*, 1998; Streit and Stern, 1999) (Figure 7.1; A). During this period, BMP-4 is required to generate ventral (extraembryonic and lateral plate) mesoderm (Streit and Stern, 1999). In the proposed model, cE13 would bind and localise BMP-4 molecules in presumptive ventral mesoderm, elevating BMP levels in this tissue and preventing their diffusion into dorsal mesoderm fated territories. At later stages of neural induction (e.g. HH stage 10), BMPs are not as well characterised, but *BMP-4* is reported to be strongly expressed in lateral plate mesoderm (Schmidt, *et al.*, 1998) at sites that correlate with cE13 expression. At these stages however, the expression domain of *BMP-4* extends further anteriorly in mesoderm and is expressed at later developmental stages than cE13, suggesting that cE13 function would be to localise and promote BMP-4 signalling in lateral plate

mesoderm in a sub-domain of BMP-4 expressing tissue, in the posterior of the embryo.

Transcripts of murine E13 are first detected at 7.25 dpc, and this expression pattern correlates with *BMP-4* expression in the allantois (Jones, *et al.*, 1991). Further analysis of mE13 expression is required to establish if mE13 is expressed at earlier stages of gastrulation and if it is, whether the expression pattern coincides with BMP expression domains. At the onset of neurulation (7.75 dpc), mE13 is expressed in anterior and posterior regions of the neural plate, being rapidly downregulated as the headfolds form. At this stage, murine *BMP-4* is expressed in the allantois, amnion and posterior primitive streak (Jones, *et al.*, 1991), correlating with mE13 expression in the allantois and regions of the primitive streak (Figure 7.2; A).

#### 7.1.2 Chick E13 is expressed in the dorsal neural tube during the initiation of neural crest cell migration

Neural crest cells are induced at the border between the neural and non-neural ectoderm, at the lateral edges of the neural folds (Baker and Bronner-Fraser, 1997; LaBonne and Bronner-Fraser, 1999; Nieto, 2001). In chick, once the folds have fused at the midline the epithelial pre-migratory neural crest cells become detached from the neural tube in a wave from anterior to posterior along the developing embryo. These motile mesenchymal neural crest cells migrate laterally away from the neural tube along specific routes to a number of different locations in the developing embryo, giving rise to many different derivatives (Noden, 1988; Stemple and Anderson, 1992; Krull, 2001).

Detection of cE13 transcripts in the dorsal neural tube suggests they are both temporally and spatially restricted to regions of pre-migratory cranial, and trunk neural crest cells. The most pronounced domain of cE13 expression in pre-migratory neural crest cells is detected in the hindbrain (r1/2 to r5/6), but expression in a subset of cells extends rostrally into the midbrain and posterior forebrain. Chick E13 expression in regions anterior to the hindbrain occurs at a time after the onset, but prior to the end, of neural crest cell migration from these axial levels. The dynamic expression pattern exhibited by cE13 coupled with comparative analysis of its expression patterns with a number of neural crest cell markers, suggests that cE13 is

expressed in a specific population of pre-migratory neural crest cells. In the hindbrain, cE13 expression is downregulated (but not lost) in the dorsal neural tube as neural crest cells initiate their migration. As cE13 expression is maintained at low levels in the dorsal neural tube, after the initial wave of neural crest cell migration, then perhaps cE13 has a role during the emigration of subsequent migratory populations of neural crest cells from many different axial levels.

At HH stage 11, expression of cE13 is detected in regions lateral to the midbrain and posterior forebrain; this expression pattern suggests that cE13 may be expressed in a subset of migrating neural crest cells.

(i) Possible relationship of cE13 with BMP-4

Neural crest cells migrate from the hindbrain in a restricted pattern resulting in neural crest cell free regions adjacent to rhombomeres (r) 3 and r5. These neural crest free zones divide the migrating neural crest cells into three streams; neural crest cells from r1 and r2 migrate predominantly into the first branchial arch (BA1), r4 neural crest cells populate BA2, and neural crest cells from r6 migrate to BA3/4 (Lumsden, *et al.*, 1991; Lumsden and Krumlauf, 1996; Krull, 2001). Although all rhombomeres produce neural crest cells (Birgbauer, *et al.*, 1995; Kulesa and Fraser, 1998), r3 and r5 contribute significantly less neural crest to the migratory streams than neighbouring even numbered rhombomeres (Graham, *et al.*, 1993; Graham, *et al.*, 1994; Takahashi, *et al.*, 1998). The relatively few neural crest cells that are produced by r3 and r5 migrate either rostrally or caudally joining neighbouring migratory crest streams (Kulesa and Fraser, 1998), avoiding paraxial exclusion zones in the mesoderm lateral to r3 (Farlie, *et al.*, 1999). One mechanism proposed to establish the separated migratory neural crest streams is programmed cell death of neural crest cells in r3 and r5 (review see: Graham, *et al.*, 1996; Lumsden and Graham, 1996). Apoptosis occurs throughout the dorsal neural tube, but with raised levels over r2/3 and r5, in a region that coincides with the expression of the homeobox containing gene *msx2* (Graham, *et al.*, 1993). Ectopic expression of the *msx2* gene in the dorsal neural tube results in the elimination of migratory neural crest cell at all axial levels that overexpress *msx2*, suggesting that this gene mediates the apoptotic elimination of cranial neural crest from r3 and r5 (Takahashi, *et al.*, 1998). *BMP-4* is also expressed in dorsal r3 and r5 (the distribution of BMP-4

protein is not known) and can induce *msx2* expression in rhombomere explant cultures, suggesting that BMP-4 induces *msx2* and ultimately apoptotic elimination of neural crest cells from these rhombomeres (Graham, *et al.*, 1993; Graham, *et al.*, 1994). Noggin, a BMP antagonist (Zimmerman, *et al.*, 1996), is expressed in dorsal r4 and early r4 migratory neural crest cells and acts to protect neural crest cells from BMP-4 mediated cell death in this rhombomere (Smith and Graham, 2001). Chick E13 transcripts coincide with *BMP-4* in r3 and r5 (Figure 7.1; B), therefore since cE13 can interact with BMP-4 molecules and promote BMP-4 signalling then perhaps cE13 indirectly modulates apoptosis in these rhombomeres. BMP-4 molecules that are secreted into the extracellular spaces in r3 and r5 could be sequestered by the cE13 protein, retaining them near to the dorsal midline where the BMP-4 protein would have its effect on neural crest cell development (see section 7.3.2; (iii)).

In the trunk, *BMP-4* is expressed uniformly along the dorsal neural tube whereas Noggin is expressed in a gradient from high caudal to low rostral (Sela-Donenfeld and Kalcheim, 1999). Noggin expression is highest opposite presomitic mesoderm and is rapidly downregulated as the somites form. The transition of high Noggin (BMP-4 inhibited) pre-migratory neural crest cells to low (BMP-4 activated), triggers neural crest cell migration (Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000). In the dorsal neural tube of the trunk, cE13 transcripts correlate with *BMP-4* expression at the time of neural crest cell emigration. Therefore, cE13 would localise BMP-4 protein in the dorsal neural tube where it is required to initiate neural crest migration.

(ii) Murine E13 is not expressed at sites of pre-migratory neural crest

Murine E13 transcripts are detected in the dorsal neural tube along the midline of the forebrain at 9.5 dpc, however its spatial and temporal expression pattern is not consistent with a role in neural crest cell migration (Serbedzija, *et al.*, 1992). Indeed, mE13 expression at 9.5 dpc coincides with the recently formed anterior midline groove and may therefore have a role in the subdivision of the prosencephalon.



Figure 7.1: Cartoon of chick E13, *BMP-2*, *BMP-4* & *BMP-7* transcript distribution during a number of developmental stages

Cartoon depicting the distribution of chick E13, *BMP-2*, *BMP-4* and *BMP-7* transcript in: A, tail bud region of a HH stage 8 embryo; B, dorsal neural tube of a HH stage 9+ embryo; C, otic cup of a HH stage 13 embryo; D, at sites of sympathetic ganglia formation and dorsal aorta of a HH stage 19 embryo; and E, nephrogenic mesenchyme of a HH stage 19 embryo. Abbreviations: a, anterior; d, dorsal; p, posterior; r, rhombomere; sc, spinal cord; v, ventral.

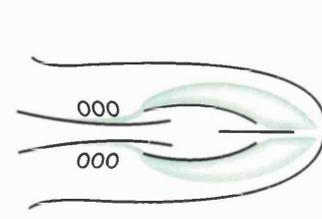
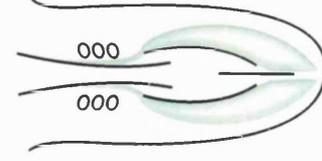
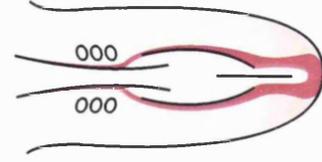
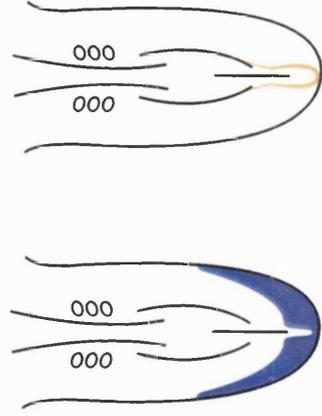
**CE13**

**BMP-2**

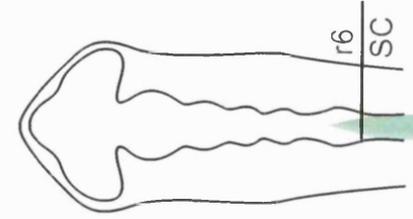
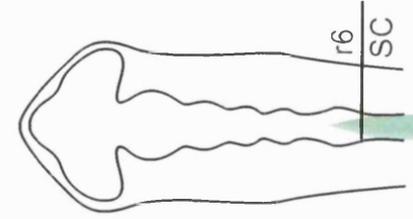
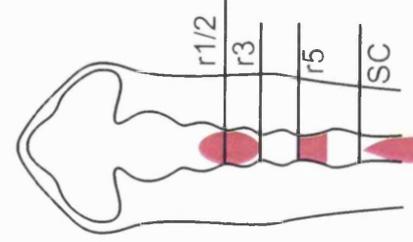
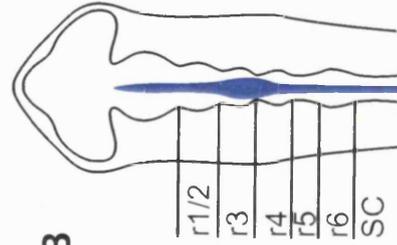
**BMP-4**

**BMP-7**

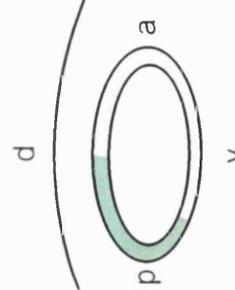
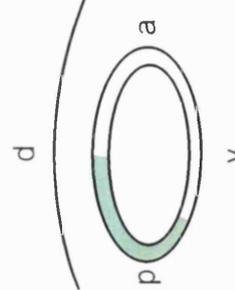
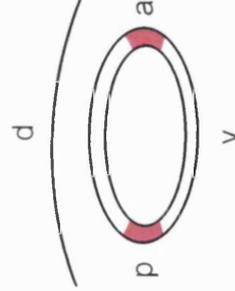
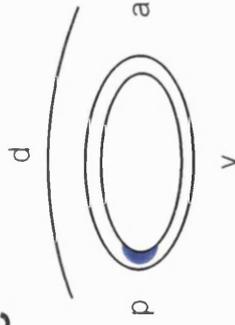
**A**



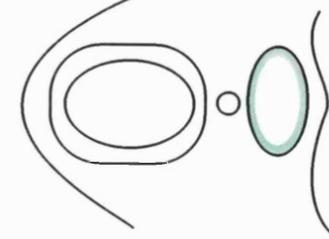
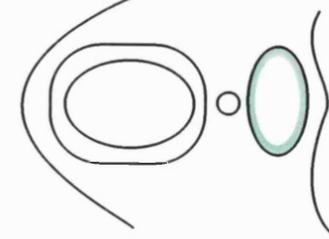
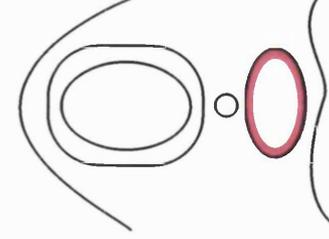
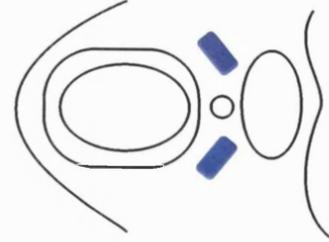
**B**



**C**



**D**



**E**

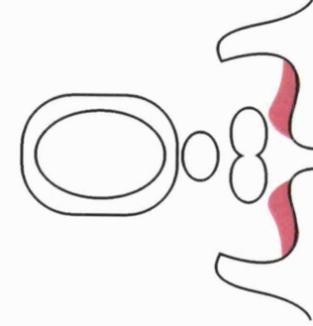
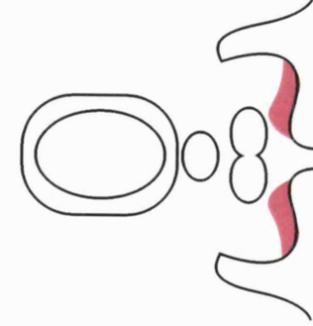
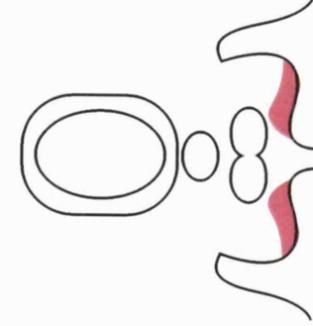
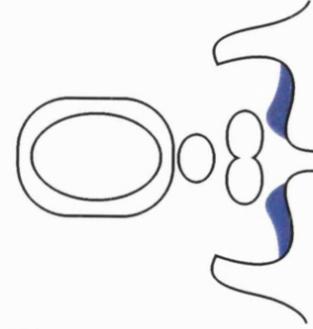




Figure 7.2: Cartoon of murine E13, *BMP-4* & *BMP-7* transcript distribution during a number of developmental stages

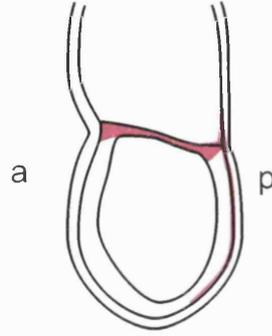
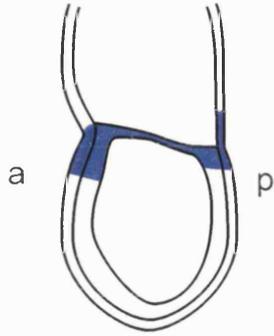
Cartoon depicting the distribution of murine E13, *BMP-4* and *BMP-7* transcripts at: A, neural stage 7.5 dpc mouse embryo; B, locations in or adjacent to the dorsal neural tube of a 9.5 dpc embryo; C, otic vesicle of a 9.5 dpc embryo. Note, *BMP-4* expression illustrated is as reported by Dudley and Robertson (1997). Abbreviations: a, anterior; d, dorsal; p, posterior; v, ventral.

*mE13*

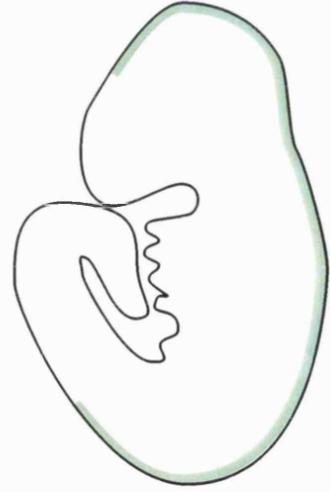
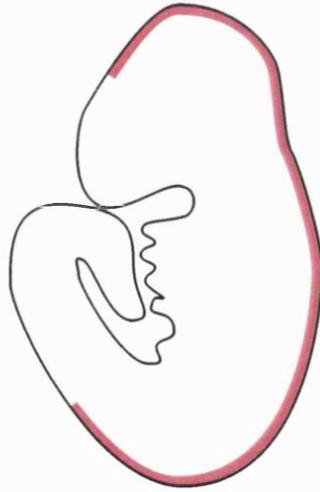
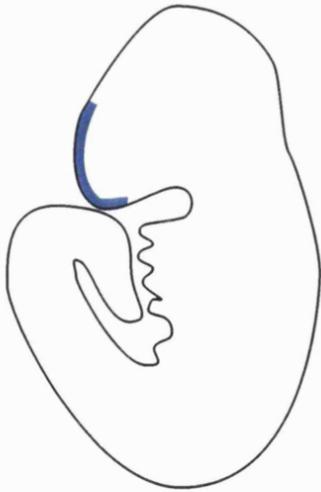
*BMP-4*

*BMP-7*

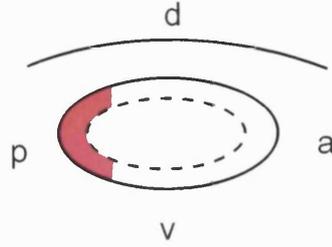
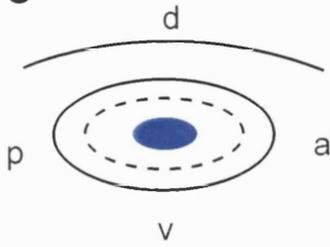
**A**



**B**



**C**



(iii) Molecular cues that govern neural crest migration differ between chick & mouse

The expression pattern of chick and mouse E13 suggest the role of chick E13 during neural crest cell development is not conserved in the mouse embryo. Some molecular cues that govern neural crest development in the chick and mouse appear to be different; one molecule that does not have a conserved expression pattern between the two species is *BMP-4*. Chick *BMP-4* is expressed in dorsal r3 and r5 in the hindbrain, and induces two foci of pre-migratory neural crest cell death, thereby depleting the number of migratory neural crest cells from r3 and r5 (Graham, *et al.*, 1993; Graham, *et al.*, 1994). In the murine embryo however, the expression pattern of *BMP-4* at the time of neural crest cell emigration (8.5 dpc) is different to that in the chick (Dudley and Robertson, 1997; Trainor, *et al.*, 2002b). Murine *BMP-4* transcripts are localised to the surface ectoderm and neural folds in the forebrain/midbrain region, but no foci of *BMP-4* expression in dorsal r3 or r5 is reported. Since neural crests migrate from the murine hindbrain in three separate streams then BMP-4 function appears to differ between these two species. Another BMP family member, BMP-7, exhibits an expression pattern that is not conserved between chick and mouse; at 9.5 dpc, *BMP-7* is expressed in the dorsal regions of the diencephalon (Lyons, *et al.*, 1995), in a region caudal to the expression domain of mE13 (Figure 7.2; B), whereas in the chick *BMP-7* transcripts are restricted to the dorsal neural tube caudal to the hindbrain (Figure 7.1; B: Dudley and Robertson, 1997).

### 7.1.3 Expression in the outflow tract of the developing heart

BMPs have a number of roles during heart development including the induction of cardiac myogenesis (Schultheiss, *et al.*, 1997), formation of the primitive heart (Shi, *et al.*, 2000) and its maturation (Allen, *et al.*, 2001). During the period of heart septation, cE13 expression (HH stage 13-26) coincides with two BMP family members; *BMP-2* is expressed in the distal region of the outflow tract from HH stage 16 and *BMP-4* is expressed in the myocardium overlying the outflow tract by HH stage 18 (Allen, *et al.*, 2001). Loss of BMP-2 and BMP-4 function in the developing chick outflow tract by the misexpression of their specific antagonist Noggin (Zimmerman, *et al.*, 1996), results in abnormal development of the outflow

tract (Allen, *et al.*, 2001). During this period of heart septation, migratory neural crest cells enter and populate the developing outflow tract (Lyons, 1996; Lough and Sugi, 2000). These data suggest that BMP-2 and BMP-4 signalling is required for the migration of neural crest cells into the developing outflow tract. Expression of cE13 in the outflow tract of the heart is adjacent to *BMP-4* expressing tissue, suggesting that cE13 could bind and localise the diffusing BMP-4 protein in the outflow tract promoting BMP-4 signalling in this region.

During murine heart development, *BMP-2* is expressed in pro-myocardium and is required for normal heart development because the *BMP-2* null mutants exhibit cardiac defects (Zhang and Bradley, 1996). At 8.5 dpc, *BMP-4* transcripts are detected predominantly in the wall of the thoracic body adjacent to the pericardial cavity; at the same stage, *BMP-7* transcripts are expressed in myocardial tissue of both the atrial and ventricular chambers (Dudley and Robertson, 1997). These data suggest *BMP-4* and *BMP-7* have a role during the early stages of murine heart development. At around 9.5 dpc the time at which mE13 is expressed in the outflow tract, *BMP-7* continues to be expressed throughout the heart whereas *BMP-2* and *BMP-4* are expressed in the atrioventricular canal and truncus arteriosus respectively (Lyons, *et al.*, 1990; Jones, *et al.*, 1991). These data suggest mE13 is expressed at the right time and correct location to promote BMP-4 signalling in the developing outflow tract of the heart.

#### 7.1.4 E13 expression coincides with BMP family members that are required for inner ear development

In the developing chick embryo, a number of molecules have been identified with roles in inner ear development (Torres and Giraldez, 1998) including two members of the BMP family (*BMP-4* and *BMP-7*) whose expression patterns overlap with cE13 (Figure 7.1; C). *BMP-4*, one of the earliest markers for sensory inner ear development, is first expressed at the medial and posterior margins of the otic placode and as the placode deepens, become localised to the rim of the otic cup (Wu and Oh, 1996). Chick E13 is expressed in a posterior region of the otic cup, overlapping with a posterior region of *BMP-4* expression (Wu and Oh, 1996). Within the otic cup, *BMP-7* is expressed prior to and exhibits a more extensive expression pattern than *BMP-4*, with expression becoming localised to the dorsal and posterior

regions of the otic cup at the onset of cE13 expression in this structure (Oh, *et al.*, 1996; Mowbray, *et al.*, 2001). Ectopic expression of Noggin arrests the development of the semi-circular canals (Gerlach, *et al.*, 2000), suggesting that BMP-4 is required for the development of inner ear structures. In the proposed model, chick E13 would localise BMP-4 protein at the posterior portion of the otic cup elevating the concentration of active BMP-4 protein and therefore BMP-4 signalling required for the development of the semi-circular canals.

Murine *BMP-4* is expressed in the posterior margins of the otic cup, once the otocyst has formed (at the time that coincides with mE13 expression) *BMP-4* transcripts remain focused in the same posterior region (Morsli, *et al.*, 1998). The semicircular canals form from the posterior portion of the otic vesicle suggesting that BMP-4 has a functional role in this process (Fekete, 1996; Kil and Collazo, 2001). Murine E13 and *BMP-4* transcripts do not coincide in the developing otocyst (Figure 7.2; C), but until a direct comparison is made it is not possible to determine if *BMP-4* and mE13 sites of expression abut one another that would suggest possible interactions.

#### 7.1.5 Chick E13 is expressed adjacent to the BMP expressing dorsal aorta at sites of sympathetic ganglia formation

BMP-4 and BMP-7 proteins are synthesised by dorsal aortic tissue and diffuse to locations adjacent to the dorsal aorta where they have their effect. Overexpression of *BMP-4* induces neurotransmitter molecules such as Phox2a, a transcription factor involved in adrenergic differentiation (Pattyn, *et al.*, 1997; Patzke, *et al.*, 2001). Ectopic application of recombinant BMP-4 and BMP-7 proteins enhance formation of primary sympathetic neurons *in vivo* (Reissmann, *et al.*, 1996), and inhibition of BMP signalling by Noggin prevents the formation of adrenergic sympathetic neurons (Schneider, *et al.*, 1999). In the chick embryo, cE13 is expressed at sites adjacent to the dorsal aorta that correspond to sites of neural crest cells differentiating to form sympathetic ganglia. In the proposed model, chick E13 protein would localise the diffusing BMP-4 molecule at sites where it is required for sympathetic ganglia differentiation, thereby increasing the concentration of active BMP-4 protein at sites of sympathetic ganglia differentiation and preventing the continued diffusion and dilution of the BMP signalling molecules.

#### 7.1.6 Potential role of E13 in the development of the chick respiratory system

The rudimentary chick respiratory tract forms between two and four days of development (Warburton, *et al.*, 2000), sectioning identified expression of cE13 in the mesoderm surrounding the early lung buds at about HH stage 19. In the mouse, the proximal-distal axis of the developing lung is regulated by BMP-4 (Bellusci, *et al.*, 1996), but in the chick, only the expression pattern of *BMP-4* is reported (Sakiyama, *et al.*, 2000). At HH stage 20, *BMP-4* is expressed in mesoderm adjacent to the developing bronchus endoderm in tissues that coincide with cE13 expression. Therefore if chick BMP-4 function in the lung is the same as the mouse, and since cE13 can bind and promote BMP-4 signalling, then the function of cE13 in this tissue would be to modulate the proximal-distal morphogenesis of the developing chick lung.

#### 7.1.7 E13 is expressed in a number of domains during chick and mouse cephalic development

The development of the chick face relies on the outgrowth of several primordia that consist of mesenchymal tissue surrounded by a layer of epithelium. The growth of the primordial buds is dependent on epithelial to mesenchymal interactions. A number of signalling molecules have been identified that exhibit restricted domains of expression and include members of the BMP family (Francis-West, *et al.*, 1998). At HH stage 16/17, *BMP-4* transcripts are detected in epithelial tissue that overlies the bulges of mesenchyme (that develop into the maxillary primordia) and only become detectable in the mesenchyme of the maxillary primordia at HH stage 24 (Francis-West, *et al.*, 1994; Barlow and Francis-West, 1997). At HH stage 16/17, *BMP-2* is expressed in a small region of mesenchyme within the emerging maxillary primordia, and at later stages (HH stage 20/24) is expressed in the medial portion of the maxillary epithelium (Francis-West, *et al.*, 1994; Barlow and Francis-West, 1997). Both BMP-2 and BMP-4 are required for the outgrowth and patterning of the facial primordia, since ectopic application of Noggin protein attenuates the two signalling proteins causing abnormal craniofacial development (Barlow and Francis-West, 1997). Chick E13 expression coincides with

*BMP-2* and *BMP-4* but is restricted to a sub-domain of the BMP expressing tissue. Therefore, since cE13 can interact with *BMP-4* molecules it may localise *BMP-4* signalling molecules, thus promoting *BMP-4* signalling in or adjacent to a sub-domain of BMP expressing mesenchymal tissue at the lateral edges of the maxillary primordial.

In the mouse, two BMP family members are reported to be expressed in the developing murine face (Jones, *et al.*, 1991; Kanzler, *et al.*, 2000). *BMP-4* is expressed in the distal regions of the facial processes (Jones, *et al.*, 1991) and *BMP-2* in a number of restricted domains of expression within the face (Lyons, *et al.*, 1990). Inhibition of *BMP-2* and *BMP-4* by a viral form of Noggin results in loss of migratory cranial neural crest cells and *BMP-2*<sup>-/-</sup> embryos lack branchial arches and migratory neural crest cells that normally populate these arches (Zimmerman, *et al.*, 1996; Kanzler, *et al.*, 2000). These data implicate *BMP-2* with a role in cranial neural crest development, but do not provide any information that can be used to predict mE13 function in the branchial arches. Similar to chick, mE13 expression in the branchial arches at the time of facial primordia outgrowth suggests mE13 may localise and promote BMP signalling in regions of the developing murine face.

Inductive tissue interactions between the diencephalon and Rathke's pouch are required for differentiation and maturation of a number of pituitary cell lineages (review: Treier and Rosenfeld, 1996). A number of signalling molecules are expressed by the ventral diencephalon including *BMP-4* (Jones, *et al.*, 1991; Takuma, *et al.*, 1998). Chick *BMP-4* is required for the formation of Rathke's pouch as blocking of BMP signalling by Noggin protein prevents pituitary gland development (Treier, *et al.*, 1998). Since cE13 can interact and promote *BMP-4* signalling, the proposed model suggests that cE13 sequesters and localises *BMP-4* molecule diffusing from the ventral diencephalon to Rathke's pouch where BMP signalling is required for the formation and development of the pituitary gland.

#### 7.1.8 Chick E13 is co-expressed with *BMP-4* in nephrogenic mesenchyme

The nephric duct is the first structure to differentiate from the intermediate mesoderm and is required for all subsequent urino-genital development, but despite its essential role during kidney morphogenesis, nephric duct formation remains

poorly characterised (Vize, *et al.*, 1997; Kuure, *et al.*, 2000). The mesenchymal to epithelial conversion required to form the nephric duct is dependent upon interactions with the overlying surface ectoderm (Obara-Ishihara, *et al.*, 1999). Chick *BMP-4* is expressed in the surface ectoderm and can substitute for the surface epithelium when removed, providing evidence that it is required for nephric duct formation (Obara-Ishihara, *et al.*, 1999). Chick E13 is expressed in the intermediate mesoderm adjacent to the surface ectoderm, surrounding the *BMP-4* induced differentiating nephric ducts (Figure 7.1; E). Therefore based on the proposed model, cE13 would bind *BMP-4* molecules in the intermediate mesoderm promoting *BMP-4* signalling and nephric duct formation.

Many BMPs (*BMP-4*, *BMP-5* and *BMP-7*) are expressed during murine embryonic kidney development (Hogan, 1996; Godin, *et al.*, 1999). At 10.5 dpc *BMP-4* is expressed in stromal mesenchymal cells surrounding the mesonephric (Wolffian) duct, and unlike the homozygous *BMP-4* mutant mice, heterozygous *BMP-4* mice are viable and exhibit kidney anomalies (Miyazaki, *et al.*, 2000). The *BMP-4*<sup>+/-</sup> kidney phenotype can be rescued by the application of recombinant *BMP-4* protein, suggesting that *BMP-4* is required for kidney morphogenesis (Miyazaki, *et al.*, 2000). *BMP-7* expression is first reported in the uretheric bud (an outgrowth of the Wolffian duct) at 11.0 dpc (Godin, *et al.*, 1998), a developmental period after the first detectable expression of mE13 in the pronephros. Null *BMP-7* mutants survive to birth but die soon after due to severe kidney defects, suggestive of the role of *BMP-7* in propagating kidney morphogenesis as mesenchymal cells fail to become epithelised during development (Dudley, *et al.*, 1995; Jena, *et al.*, 1997; Godin, *et al.*, 1998). In the developing pronephros mE13 expression is transient and does not coincide with any reported BMP expression domain, therefore although mE13 may have a role during the differentiation of the intermediate plate mesoderm and/or the induction and formation of the pronephros, it is unlikely that it interacts with BMP family members for which expression patterns have been reported.

#### 7.1.9 Expression of E13 is restricted to the intersomitic region

Somites form either side of the neural tube along the trunk in an anterior to posterior direction along the developing embryo, and later differentiate to form the vertebral column, ribs and all skeletal muscle of the body (Christ and Ordahl, 1995;

McGrew and Pourquie, 1998; Christ, *et al.*, 2000). The morphological and molecular analysis of the development of the vertebrae column is well documented, but little is reported about the function of the intersomitic region during the period in which chick and mouse E13 are expressed. Recently, Scleraxis, a basic helix-loop-helix transcription factor has been identified and characterised in chick; at HH stage 22, it is expressed in intersomitic mesoderm (Schweitzer, *et al.*, 2001), in a pattern similar to cE13. Scleraxis is a highly specific marker for tendons and ligaments, suggesting that E13 may be expressed in an early cell populations that later form connective tissues.

#### 7.1.10 Chick E13 is expressed in the lens and retina of the developing eye

After induction of the optic placode a series of morphological events occur that ultimately result in the formation of the eye, these include changes in cell shape, an increase in the rate of cell proliferation and localised programmed cell death (Saha, *et al.*, 1992; Chow and Lang, 2001). *BMP-4* and *BMP-7* transcript distributions have been characterised and are expressed in a number of tissues during chick eye development (Francis-West, *et al.*, 1994; Trousse, *et al.*, 2001). *BMP-4* is expressed in the prospective dorsal neural retina after optic vesicle invagination, where it regulates cell proliferation in the dorsal portion of the developing chick optic cup by mediating apoptosis (Trousse, *et al.*, 2001). At later stages of eye development, at the time cE13 is expressed in ventral regions of the neural retina (HH stage 29), *BMP-2* and *BMP-7* are expressed in boundary tissue between the dorsal and ventral portions of the retina (Belecky-Adams and Adler, 2001). At the same developmental stage, cE13 expression is restricted to the ventral portion of the neural retina, but until a direct comparison is made it is not possible to determine if these sites of expression are close enough to suggest a possible interaction.

#### 7.1.11 E13 is expressed in developing limbs

As the limbs develop a series of patterning signalling pathways generate the dorso-ventral, proximo-distal and antero-posterior axes (Tickle, 1995). At early stages of limb development, *BMP-2*, *BMP-4* and *BMP-7* are all expressed along the apical ectodermal ridge (AER) and at a number of other sites: *BMP-2* is expressed in

the posterior portion of the limb bud; *BMP-4* is expressed in an anterior region of the limb bud and *BMP-7* is expressed predominantly in the posterior limb bud, but also in an anterior region (Francis, *et al.*, 1994; Schweitzer, *et al.*, 2001). At HH stage 22, expression of cE13 is detected in an anterior region of the developing leg bud coinciding predominantly with *BMP-4*'s anterior site of expression, but also with the weak anterior expression domain of *BMP-7*. Chick E13 was not detected in the AER, suggesting that the function of cE13 with respect to *BMP-4* (and possibly *BMP-7*) is localised to the mesenchyme of the developing limb bud. Interestingly at HH stage 22, chick E13 transcripts are only detected in the developing leg and not the wing, suggesting that cE13 function differs in the early development of the leg and the wing but further investigation is required to confirm this observation.

The limb primordia of the mouse form between 9 and 9.5 dpc (forelimb) and 9.5 and 10 dpc (hindlimb). Murine E13 transcripts are detected in the developing forelimb at 10.75 dpc and therefore mE13 is not required for the induction, formation or initial outgrowth of the limb buds. Transcripts of mE13 are first detectable in the developing limbs during the formation of the stylopod, autopod and zeugopod and during these stages *BMP-2*, *BMP-4* and *BMP-7* are expressed in the developing joints and footpads (Luo, *et al.*, 1995; Lyons, *et al.*, 1995). A more detailed analysis of mE13 expression is required at earlier stages of limb outgrowth to determine the exact timing of the onset of E13 expression, and their temporal and spatial relationship with BMP family members.

## 7.2 EXPLANATION OF APPARENT ANTAGONISTIC EFFECT OF cE13

The cleaved cE13 protein product detected in cells expressing recombinant cE13 protein corresponds to the carboxyl terminus portion of the cE13 protein. If the proposed model is correct and cE13 is cleaved *in vivo*, then the cleaved cE13 protein may have a different function to its non-cleaved form. As a full-length protein, cE13 could bind and localise BMP molecules, but once cleaved at the predicted site between CR4 and CR5 the four CR motifs of the amino-terminal portion of the cE13 protein would be released into the extracellular space where they may have a different effect on BMP signalling. Injection of cE13 mRNA into whole *Xenopus* embryo suggests that cE13 is an agonist of BMP signalling whereas overexpression of cE13 in *Xenopus* ectodermal explants suggests that cE13 does not directly inhibit BMP signalling. The difference in these results could be explained by the different activities of the full-length and cleaved forms of the cE13 protein. Perhaps in whole *Xenopus* injections the cE13 protein is cleaved and the amino portion of the protein may have an agonistic activity on BMP signalling. However, in *Xenopus* ectodermal explant assays the cE13 protein is not cleaved and thus the full-length cE13 protein localises BMP molecules thereby promoting BMP signalling.

Another potential explanation is that if cE13 sequesters BMPs then injection of cE13 mRNA into whole *Xenopus* may have an indirect effect on BMP signalling resulting in a phenotype suggestive of a BMP antagonist. If cE13 binds and sequesters active BMP molecules at sites away from BMP molecules normal site(s) of action, then cE13 could have an apparent inhibitory effect on BMP signalling. Indeed this may explain the dorsalisated phenotype that occurs following the overexpression of BMP in the *Xenopus* embryo, but to confirm this the location of cE13 mRNA injection and ectopic expression in the embryo with relation to the phenotype needs to be investigated.

## 7.3 FINAL PERSPECTIVES

### 7.3.1 Identification of further genes expressed in the developing chick hindbrain

The screening method employed to identify genes that are expressed in developing neural tissues has proved successful. To date, less than a quarter of the subtracted chick hindbrain cDNA library has been screened by *in situ* hybridisation and yet has identified 39 clones with restricted expression patterns in developing cranial neural tissues. It can be predicted that between 100 and 200 clones with regionalised domains of expression remain to be identified in the library. The identification of further molecules would provide a more comprehensive view of the molecular events that accompany the formation of the hindbrain, and thus an extension of the screen has been initiated in the laboratory. To optimise the efficacy of this extended screen a more targeted approach will be taken: the library will be arrayed and hybridised with the 445 clones that were isolated during this project. Positively hybridising clones will be identified and removed from further analysis, thereby reducing the number of whole mount *in situ* hybridisation reactions and streamlining the screening procedure.

Another extension to the screening project would be to investigate the function of all of the unknown genes that have been identified during this project. The majority of the ESTs identified during this screen do not share any significant homology to any known sequences in public domain databases, and the design of experiments to directly address their function within the chick embryo was difficult. However, the overexpression and 'knockdown' of a genes function are two techniques that could be used to investigate the role of unknown genes. *In ovo* electroporation is a technique that enables easy and efficient overexpression of a gene within the chick neural tube and neural crest cells (Itasaki, *et al.*, 1999; Inoue and Krumlauf, 2001). To extend this project into a functional screen by overexpressing each gene, full-length cDNAs corresponding to the clones identified during this screen could be re-cloned into expression vectors and introduced into the neural tube by electroporation. A more feasible technique that is available to investigate the function of the isolated clones could utilise the antisense morpholino technology (Summerton and Weller, 1997; Heasman, *et al.*, 2000). Antisense morpholinos inhibit protein synthesis as they prevent the ribosomal complex from

translating the mRNA by binding to complementary sites in the 5' UTR or surrounding the initiation codon of the targeted endogenous mRNA. Morpholinos have been used primarily in *Xenopus* and zebrafish (Heasman, *et al.*, 2000; Nasevicius and Ekker, 2000), but two recent publications report their use in the chick embryo to perturb gene function (Tucker, 2001; Kos, *et al.*, 2001). The design of morpholinos relies on knowing the sequence surrounding the start codon, and this is now becoming easier due to the increasing amounts of sequence being deposited in EST and genomic databases.

### 7.3.2 Further investigation into the function of E13

Bone morphogenetic proteins (BMPs) are expressed at a number of different tissues at many stages during embryological development where they modulate a variety of events (Hogan, 1996; Mehler, *et al.*, 1997). The BMP antagonist Chordin is a well characterised modulator of BMP signalling (Sasai, *et al.*, 1994; Piccolo, *et al.*, 1996), but recent advances have identified a number of molecules such as Xolloid, Cerberus and Tsg that impose a further level of control on BMP signalling (Piccolo, *et al.*, 1997; Piccolo, *et al.*, 1999; Oelgeschläger, *et al.*, 2000; Chang, *et al.*, 2001). The identification of E13 and the data presented in this thesis demonstrate E13 is expressed at the correct time and location to interact with BMP molecules (Chapter 5) and indeed E13 has been shown to bind to BMP-4 and can modulate a downstream target of BMP signalling (Chapter 6).

#### (i) Does E13 associate with the extracellular matrix and localise BMP molecules?

As discussed in Chapter 6, the proposed model for the role of E13 is to localise BMP molecules at sites where high levels of BMP signalling are required. To do this it has been reasoned that the E13 protein may interact with the extracellular matrix (ECM), preventing it from diffusing from its site of expression and acting as a molecular anchor of BMP-4 molecules (Chapter 6). Experiments in the laboratory are currently underway to determine if cE13 interacts with the ECM and if it can localise BMP molecules. To determine if E13 can bind to the ECM, a tagged recombinant cE13 protein is being expressed in a mammalian cell line. Chick E13 expressing cells will be separated from the ECM; the cells and ECM fractions will be assayed for the presence of cE13 protein by Western analysis (as previously

demonstrated by other ECM binding proteins such as the proto-oncogene *int-1* and the glycoprotein Wnt11 (Bradley and Brown, 1990; Christiansen, *et al.*, 1996)). To determine if cE13 can localise BMP-4 molecules and restrict their domain of activity, an experiment is planned similar to one described by others utilising injected *Xenopus* animal caps (Gurdon, *et al.*, 1994; Jones, *et al.*, 1996). Injected ectodermal explants expressing either BMP-4 or BMP-4 and cE13 will be juxtaposed with un-injected ectoderm explants and incubated for approximately 4 hours before being fixed and analysed. The range of BMP-4 activity with and without cE13 will be analysed by determining the induction of downstream components of the BMP signalling cascade (*Xhox3*) (Hemmati-Brivanlou and Thomsen, 1995) in the adjacent non-injected tissue. If cE13 localises BMP-4 then the domain of *Xhox3* expression will be less in the *BMP-4* and cE13 injected caps than in *BMP-4* injected caps.

(ii) Is E13 protein cleaved *in vivo*?

The expression of recombinant cE13 protein in *Drosophila* Schneider 2 insect cells identified a full-length and cleaved form of the chick E13 protein. This observation requires further investigation to determine if this processing occurs *in vivo*. To address this issue two peptide antibodies have been designed; one against the amino portion and the other against the carboxyl portion of the cE13 protein, either side of the predicted cleavage site. These peptides are being utilised to raise two antisera that will be used to detect the cE13 protein in a number of different experiments. These will include Western analysis of whole embryo extracts to determine if cE13 is cleaved *in vivo*, and *in situ* immunochemistry experiments to determine the distribution of cE13 protein *in vivo*.

If the cE13 protein is cleaved *in vivo* then the two forms of the cE13 protein may have different activities within the developing embryo. If the full-length cE13 protein can localise BMP-4 via binding of the VWFD domain to extracellular matrix, cleavage of the protein may release BMP-4/cE13 complexes. To investigate this possibility experiments will use truncated forms of cE13 in different assays. These assays include overexpression of truncated cE13 mRNAs in developing *Xenopus* embryos and in animal caps expressing BMP-4, to determine if different portions of the cE13 protein have different effects on BMP signalling. Other experiments may include the production of truncated tagged cE13 proteins for use in binding assays to

determine what portion(s) of the cE13 protein is required to interact with BMP molecules.

(iii) Functions of cE13 *in vivo*

Analysis of embryos overexpressing cE13 in the dorsal neural tube has focused on identifying changes in neural crest cell migration using migratory neural crest cell markers. Chick E13 has been shown to modulate downstream targets of BMP-4, and therefore it will be interesting to investigate if the levels of expression of other components of the BMP signalling pathway are affected when cE13 is overexpressed *in vivo*. BMP-4 induces apoptotic elimination of neural crest cells in r3 and r5 (Graham, *et al.*, 1993; Graham, *et al.*, 1994), therefore it would be interesting to investigate if there is a change in the level of cell death when cE13 is overexpressed by using either acridine orange, nile blue or the TUNEL assay (Grasl-Kraupp, *et al.*, 1995) to detect cells undergoing apoptosis. Another more quantitative assay to determine if the overexpression of cE13 effects neural crest cell development would be to electroporate cE13 into the developing chick neural tube, followed by neural tube explant cultures to determine if there are changes in the timing of neural crest cell migration or in the number of migratory neural crest cells.

A more informative approach to elucidate the function of cE13 may be loss of function experiments. One technique that may enable loss of function or transcript knock-down experiments are morpholino antisense oligomers, as previously described (Summerton and Weller, 1997; Nasevicius and Ekker, 2000; Tucker, 2001; Kos, *et al.*, 2001). As the sequence surrounding the initiation codon of both the chick and zebrafish E13 has been identified antisense morpholinos can be designed against these sequences. The production of E13 peptide antibodies will be a useful tool to determine the efficacy of the antisense morpholinos as the levels of E13 protein can be detected.

Another avenue of investigation that has been initiated in the laboratory is to generate a null mutation of the E13 gene in the mouse. As described in Chapter 4, a partial mouse E13 EST has been identified and this sequence will be used to identify genomic clones that are required for the targeted disruption of the mE13 gene. This knock-out will potentially provide a very useful tool in elucidating the function of E13.

(iv) Does E13 affect the specification of dorsal neuronal cell types in the neural tube?

One aspect of BMP signalling that has not been investigated during this project is the role of BMP signalling in the establishment of dorsal neuronal cell types in the neural epithelium. The specification of neuronal cell types in caudal regions of the neural tube is dependent on the cells position within the dorsoventral axis (review: Briscoe and Ericson, 2001). Recent evidence suggests that two opposing signalling mechanisms are responsible for the specification of the neuronal cell type. Sonic hedgehog (Shh) is expressed ventrally with a graded signalling activity from high ventral to low dorsal, inducing ventral neuronal cell types (Marti, *et al.*, 1995; Hammerschmidt, *et al.*, 1997; Briscoe and Ericson, 1999). Opposing this ventral signalling mechanism are the dorsal BMPs that are expressed in the surface ectoderm and dorsal neural tube (review see: Mehler, *et al.*, 1997). Although the dorsal to ventral distribution of BMP proteins have not been investigated in the neural tube, BMPs are proposed to diffuse away from the dorsal midline establishing a graded signal from high dorsal to low ventral and have been shown to induce dorsal and intermediate neuronal cell types (Nguyen, *et al.*, 2000). The expression pattern of cE13 in the dorsal neural tube is transient coinciding with the onset of neural crest cell migration and during this period BMPs have a number of roles during emigration of these cells (Chapter 1: Graham, *et al.*, 1994; Sela-Donenfeld and Kalcheim, 1999). If cE13 acts as a molecular anchor for BMP molecules then perhaps cE13 localises BMP family members expressed by pre-migratory neural crest cells, thus preventing ventral diffusion of these BMPs that could affect dorsal ventral patterning of neuronal cell types. Therefore, ectopic expression of cE13 in the neural tube is predicted to affect the formation of the different neuronal cell types along the dorso-ventral axis. Further to this, a collaboration with a colleague in the department (J. Briscoe) has been initiated to determine if the overexpression of cE13 affects the specification of neuronal cell types along the dorsal to ventral axis in the developing chick embryo.

The identification of E13 and the experiments that have been presented in this thesis represent a significant first step in elucidating the modulating function of cE13 on BMP signalling. Clearly, further investigation is required to fully understand the role of E13 during embryonic development and its relationship with BMPs.

## APPENDIX A

### GENERAL SOLUTIONS:

DepcPBS	0.001 % (v/v) Depc and 1X PBS in DDW and autoclaved.
LB (Broth)	1 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl pH 7.4.
LB-agar	L-Broth supplemented with 1.5 % (w/v) bacto-agar.
PBS	Prepared by adding phosphate buffered saline tablets (Oxoid BR14a) to DDW and autoclaving.
PBTw	0.1 % (v/v) Tween20 in 1X PBS DDW
PBTx	0.1 % (v/v) TritonX100 in 1X DepcPBS.
RT-PCR-loading dye	80 % (v/v) Formamide, 1 mM EDTA, 0.1 % (w/v) bromophenol blue, 0.1 % (w/v) xylene cyanal
Xgal stain solution	5 mM $K_3Fe(CN)_6$ , 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ , 2 mM $MgCl_2$ , 0.01 % (w/v) sodium deoxycholate, 0.02 % (w/v) NP40, 1 mg/ml Xgal in DepcPBS.

### ELECTROPHORESIS BUFFERS:

TBE	0.089 M Tris Borate, 0.002 M EDTA pH 8.0.
TAE	0.04 M Tris Acetate, 0.02 M EDTA pH 8.0.
10X Load Buffer	2.5 % (w/v) Ficoll 400, 0.042 % (w/v) xylene cyanol, 10 % (w/v) bromophenol blue.

## PROTEIN BIOCHEMICAL REAGENTS:

Blocking Solution	5 % (w/v) Skimmed milk powder (Marvel), 0.2 % (v/v) Tween20 in PBS.
Coomassie stain	0.25 % (w/v) Coomassie brilliant blue, 50 % (v/v) methanol, 10 % (v/v) glacial acetic acid.
Gel destain	10 % (v/v) methanol, 7 % (v/v) glacial acetic acid.
PLC Buffer	50 mM Hepes (pH7.5), 150 mM NaCl, 1.5 mM MgCl <sub>2</sub> , 1.5 mM CaCl <sub>2</sub> , 0.1 % (w/v) CHAPS, 0.1 % (w/v) Octylglucoside, 1 % (v/v) TritonX100, 10 % (v/v) Glycerol, 1 mM EGTA, 10 mM NaPi, 100 mM NaF, 1x Protease Inhibitors, 0.5 % (w/v) BSA in DDW.
SDS running buffer	0.075 M Tris base, 0.192 M glycine, 0.1 % (w/v) SDS, pH 8.3.
SDS sample buffer	80 mM Tris-HCl, 10 % (v/v) glycerol, 50 mM 2 β-mercaptoethanol, 0.01 % (w/v) bromophenol blue, pH 6.8.
Transfer Buffer	25 mM Tris-base, 0.1 M glycine, 20 % (w/v) methanol, 0.05 % (v/v) SDS, pH 7.4.

## XENOPUS SOLUTIONS:

10X NAM	110 mM NaCl, 2M KCl, 1 mM Ca(NO <sub>3</sub> ) <sub>2</sub> , 1 mM MgSO <sub>4</sub> , 0.1 mM EDTA.
10 % NAM (500 ml)	5 ml 10X NAM, 10 ml 0.1 M sodium phosphate (pH7.4), 2.5 ml 10 mg/ml gentamycin.
75 % NAM (500 ml)	37.5 ml 10XNAM, 10 ml 0.1 M sodium phosphate (pH7.4), 5 ml 0.1 M NaHCO <sub>3</sub> , 2.5 ml 10 mg/ml gentamycin.
2 % cysteine hydrochloride pH7.9 - 8.1 (200 ml)	4.4 g L-cysteine hydrochloride monohydrate, 1.33 - 1.36 g NaOH, H <sub>2</sub> O to 200 ml.
10X MEM salts	1 M MOPS, 20 mM EDTA, 10 mM MgSO <sub>4</sub> .
MEMFA	1X MEM salts, 3.7 % (v/v) formaldehyde

## PLASMID VECTORS:

pBluescript II KS (+/-)	(Stratagene)
pSport1	(GibcoBRL)
pCS2+	(Stratagene)
$\Delta$ pCS2+	(Dr. Christiansen, NIMR)
pcDNA3.1(-)/Myc-HisA	(Invitrogen)
pMT/BIP/V5-HisA	(Invitrogen)

## APPENDIX B

In situ hybridisation program for the InsituPro in situ machine (AbiMed) that is based on 'Protocol Four' as previously described (Xu and Wilkinson, 1998). Program has been designed to process typically 3 pre-hybridised embryos (in 'medium' sized sample tubes) up to and including the final antibody wash (embryos are removed from the machine prior to the colour reaction). Commands 1 to 31 are as recognised by the in situ machine, text in brackets is a brief description of the command.

- 1 SetMultCon                    x0000000  
(heat block off)
- 2 Rinse                        5000 / 5000  $\mu$ l  
(rinse/wash pipetter with 5000  $\mu$ l of Depc DDW)
- 3 SetMultCon                    x1000001  
(heat block on, set to 60 °C)
- 4 Wait                         5 min  
(pause for 5 minutes to allow samples to reach hybridisation temperature)
- 5 Incubate 16 h 400 Probe-SAMPLE\_A  
(400  $\mu$ l of hybridisation solution /riboprobe added to samples and incubated for 16 hours)
- 6 Incubate 30 min 300 E-SAMPLE\_A  
(300  $\mu$ l of hybridisation solution added to each sample and incubated for 30 minutes)
- 7 Incubate 30 min 300 E-SAMPLE\_A  
(300  $\mu$ l of hybridisation solution added to each sample and incubated for 30 minutes)
- 8 Incubate 30 min 300 E-SAMPLE\_A  
(300  $\mu$ l of hybridisation solution added to each sample and incubated for 30 minutes)
- 9 Incubate 30 min 300 E-SAMPLE\_A  
(300  $\mu$ l of hybridisation solution added to each sample and incubated for 30 minutes)
- 10 Incubate 30 min 400 B-SAMPLE\_A  
(400  $\mu$ l of hybridisation solution:MABT (1:1) added to each sample and incubated for 30 minutes for 30 minutes)
- 11 Incubate 30 min 400 B-SAMPLE\_A  
(400  $\mu$ l of hybridisation solution:MABT (1:1) added to each sample and incubated for 30 minutes for 30 minutes)
- 12 SetMultCon                    x0000000  
(heat block off)

- 13 Wait 30 min  
(heat block cools to room temperature)
- 14 Incubate 10 min 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT buffer added to each sample and incubated for 10 minutes)
- 15 Incubate 10 min 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT buffer added to each sample and incubated for 10 minutes)
- 16 Incubate 10 min 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT buffer added to each sample and incubated for 10 minutes)
- 17 WaitForKey  
(program pause to allow fresh blocking solution to be inserted into the machine)
- 18 Incubate 2 h 400 C-SAMPLE\_A  
(400  $\mu$ l of blocking solution added to samples and incubated for 2 hours)
- 19 WaitForKey  
(program pause to allow fresh blocking solution + antibody to be inserted into the machine)
- 20 Incubate 4 h 400 D-SAMPLE\_A  
(400  $\mu$ l of blocking solution/antibody added to samples and incubated for 4 hours)
- 21 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 22 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 23 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 24 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 25 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 26 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 27 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 28 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 29 Incubate 1 h 400 A-SAMPLE\_A  
(400  $\mu$ l of MABT added to samples and incubated for 1 hour)
- 30 Rinse 5000 / 5000  $\mu$ l  
(rinse/wash pipetter with 5000  $\mu$ l of Depec DDW)
- 31 < PRG-END >  
(end of program)

## APPENDIX C

Amino acid alignments of clones identified in the in situ hybridisation screen (Chapter 3) using the BLOSUM30 ClustalW algorithm (Thompson, *et al.*, 1994). Identical amino acid residues are indicated with asterisks and conserved amino acid substitutions are indicated by a decimal point, dashes indicate spaces inserted by the alignment programme to optimise alignments.

(i) Amino acid alignment of the entire coding region of clone 0A11 (chick eIF2 $\alpha$  kinase) with the rat PKR protein and rat HRI protein. The 4 amino acid motif boxed in grey hi-lights the conserved catalytic domain of eIF2 $\alpha$  kinase and HRI (Chen, *et al.*, 1991).

```

eIF2 $\alpha$ K 1 MWR---GREVPPR-----AAAHRPPPAIQFPEESPEPRFDE---SDVPAELRVANGSQ 47
PKR    1 MASDTPGFYVDKLN-----KYSQIHVKVYIYKEISVTGPPHRRRFTFQVIIIEEREFPEGE 55
HRI    1 MLG---GGSVDGERDTDDDAAGAVAAPPAIDFPAEVS DPKYDE---SDVPAELQVFKEPL 54
      *   *   *                               *   *   *   *   *   .

eIF2 $\alpha$ K 48 KFNFTSTIQNQLLLVSLLEHLCHMYTHNPVHSMCLFRILRQAFTRTGLLSPFAFCDEFS 107
PKR    56 GRSKQEAKNNAAKLAVEILDNENKVDS----HTDASEQGLIEG-NYIGLVNSFAQ-KEN- 108
HRI    55 QQPTFPFLVANQLLLVSLLEHLSHVHEPNPLHSKQVFKLLCQTFIKMGLLSSFTCSDEFS 114
      * * . * . . . * . * . * . * . *

eIF2 $\alpha$ K 108 TVRLQHNRAITELMKAANRQVLNGELD-NGDSRAIGEKEVLLEAQTSTRYLNEFDEIARLG 166
PKR    109 ---LPVNFELCDPDSQLPHRFIC-KCKIGQTTYGTGFGANKKEAKQLAAKNAYQKLSEKS 164
HRI    115 SLRLHHNRAITHLMRSAKERVRQDPCQDNSYMQKIRSREIALEAQTSTRYLNEFEELAILG 174
      * * . . . . * . * . * . * .

eIF2 $\alpha$ K 167 KGGYGQVYKVRNKLDGQFYAIKKINIKKATRRDCMKVLEVKVLAGLQHPNIVGYHTAWM 226
PKR    165 PSKTGFVTSLSDFSSSSSITSNSASQSASGRD-----FEDIFMNGLREKRKSG----- 213
HRI    175 KGGYGRVYKVRNKLDGQHYAIKKILIKSATKTDCMKVLEVKVLAGLQHPNIVGYHTAWI 234
      * * . * . * * . * . * . *

eIF2 $\alpha$ K 227 EHVQTACPKDKTVLKLQSLPLEQES-GD-DHCHIQSVESGSSIIIFADLTSQEEKSGDSTC 284
PKR    214 ----VKVPSDDVLRNKYTLDDRFSK--D-----FEDIEEIGSGGFGQVFKAKHR-IDGKT 261
HRI    235 EHVHVLQPQDRVPIQLPSLEVLSEHEGDRNQGGVKDNESSSIIIFAELTPEKENPLAESD 294
      * * . * * * * * .

eIF2 $\alpha$ K 285 LRNPDGESVQNMDVRNDFTNNSKEICMKPNRCELPIELQEDSDSSVDCRSTDLKHDSY 344
PKR    262 YAIKR-----ITYNTKKAKR-----EVQALAE LNHAN-----IVQYRVCW 296
HRI    295 VKNEN-----NNLVSYRANLVIRSSSESESSIELQEDGLN-----ESPLRPVVKH 339
      . * . .

eIF2 $\alpha$ K 345 DPPCSLDLDASAGSKSCTEECSGNDVALCGEFEVEYRLMLYIQMQLCEL-SLWDWIAARN 403
PKR    297 E---GEDYDYP-----E---NSTN--GDTSTRYKTRCLFIQMEFCDKGTQQWLEKRN 341
HRI    340 QLPLGHSSDVEG-NFTSTDESEEDNLLNLGQTEARYHMLHLHIQMQLCEL-SLWDWIAERN 397
      * * * * * * * * * * * *
  
```







(vi) Amino acid alignment of clone 1D10 with human HRI protein

```
1D10    1          LDIQCEEMNPSRWAEELLSTMKSCSTIRLDDCNLSSSNCKDLSSIIHTNPSXXX  53
RAI     1  MSLDIQSLDIQCEELSDARWAEELLPLLQQCQVVRLLDDCGLTEARCKDISSALRVNPALAE  60
          ***** . . ***** .. * .*****.*. .***.** ..**

1D10    54  XXXXXXXXGDAGIEYLCKGLLTPSCSLQKLWLQNCNLTSASCETLRVLSAQPSLTELHV  113
RAI     61  LNLRSNELGDVGVHCVLQGLQTPSCKIQKLSLQNCCLTGAGCGVLSSTLRRTLPTLQELHL  120
          ** * . . .** ***** .***.*****.* * * * * * * .*. * **

1D10    114  GDNKLGTAGVKVLCQGLMNPCKLQKLQLEYCELTADIVEALNAALQAKPTLKELT  169
RAI     121  SDNLLGDAGLQLLCEGLLDPQCRLEKLQLEYCSLSAASCEPLASVLRRAKPDFKELTVSNN  180
          ** * * * . . .**.*. * .*.***** * * * * * * .*.*** **
```

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# Molecular control of neural crest formation, migration and differentiation

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Induction, migration and differentiation of the neural crest are crucial for the development of the vertebrate embryo, and elucidation of the underlying mechanisms remains an important challenge. In the past year, a novel signal regulating the formation of neural crest cells has been identified, and advances have been made in uncovering roles for bone morphogenetic protein signals and for a transcription factor in the onset of neural crest migration. There have been new insights into the migration and plasticity of branchial neural crest cells. Important progress has been made in dissecting the roles of bone morphogenetic protein, Wnt and Notch signalling systems and their associated downstream transcription factors in the control of neural crest cell differentiation.

## Addresses

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## Abbreviations

A–P	anteroposterior
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
ET	endothelin
ngn	Neurogenin
TH	tyrosine hydroxylase

## Introduction

Neural crest cells are induced at the dorsolateral edge of the neural plate; from there, they delaminate and migrate along specific routes to many destinations in the vertebrate embryo. These cells differentiate into a wide variety of cell types, including neurons and glial cells of the peripheral nervous system, melanocytes, smooth muscle, and cartilaginous and skeletal elements in the head [1]. The remarkable migratory behaviour and diversity of cell types that the neural crest forms raises the challenge of identifying the mechanisms that underlie their induction, migration and guidance, and that control their differentiation at appropriate locations. In this review, we will discuss progress in the identification of molecular mechanisms that control neural crest development, focusing on advances that have been made subsequent to excellent reviews published in 1999 [2,3].

## Induction

Grafting experiments in the chick have shown that interactions between embryonic non-neural ectoderm (presumptive epidermis) and neural plate induce the formation of neural crest cells at their interface, and that each of these tissues contributes to the neural crest [4,5]. The

ability to respond to inductive signals is not restricted to the presumptive epidermis, since during a restricted period of competence, extra-embryonic ectoderm also forms neural crest when transplanted into the midbrain [6]. A number of signals have been implicated in the formation of the neural crest, including members of the Wnt, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families [5,7–9]. BMPs and FGFs also have an earlier role in establishment of the neural plate border [10], suggesting that these signals control a series of patterning events at the interface of neural and non-neural ectoderm.

Recently, a novel extracellular glycoprotein — Noelin-1 — has been implicated in neural crest development. Noelin-1 is expressed at the lateral edges of the neural plate and, later, in migratory neural crest cells [11\*\*]. Overexpression of Noelin-1 by retroviral infection of the neural tube results in prolonged neural crest cell production and migration. Furthermore, overexpression prolongs the period during which the neural crest can be regenerated after dorsal neural tube ablation. These findings suggest that Noelin-1 maintains the competence of neural epithelial cells to form neural crest.

## Initiation of migration

Following their induction in the dorsolateral neural tube, neural crest cells undergo an epithelial to mesenchymal transition and begin to migrate. Recent evidence suggests that, in addition to being involved in the induction of neural crest, BMPs are required for the initiation of migration of trunk neural crest [12\*\*]. In the chick embryo, BMP-4 is expressed uniformly along the dorsal neural tube, and one of its antagonists, Noggin, is expressed in the dorsal neural tube in a high caudal to low rostral gradient. Ectopic expression of BMP-4 causes premature migration of neural crest cells, whereas ectopic application of Noggin results in a delay in migration [12\*\*]. As neural crest emigration occurs in a wave, from rostral to caudal, these findings suggest that BMP-4 and Noggin act antagonistically to regulate delamination and migration of trunk neural crest cells. The effects of driving ectopic Noggin expression in transgenic mice suggest that BMPs are also required to form migratory cranial neural crest, but it is currently unclear whether this effect of Noggin reflects a role of BMPs in neural crest induction or migration, or both [13].

A key issue is the identity of the targets of BMP signalling that underlie the epithelial to mesenchymal transition and migratory behaviour of neural crest cells. The earliest known response to neural crest induction is expression of *Slug* or *Snail*, members of the *Snail* family of zinc finger transcription factors [14,15]. Treatment of chick [14] or *Xenopus* [16]

embryos with antisense oligonucleotides to *Slug* mRNA results in an inhibition of cranial neural crest cell migration. By testing the effects of expressing fusions with transcriptional repressor or activator domains, it was shown that *Xenopus* Slug protein normally acts as a repressor [17\*]. In an analogous manner to *Drosophila* Snail, Slug may downregulate genes whose expression needs to be excluded from neural crest and have an indirect role in the upregulation of genes in the neural crest. On the basis of these results, a fusion protein was designed that acts as a hormone-inducible dominant negative inhibitor of Slug and Snail protein function. This reagent enables temporal control of the inhibition of function, and was used to demonstrate a requirement for Slug/Snail in the expression of *Twist*, which encodes a specific marker of neural crest; a later role in controlling neural crest cell migration was also indicated [17\*].

Recent work [18\*\*] has demonstrated that mouse Snail binds to the promoter and represses expression of the *E-cadherin* gene, which encodes a cell adhesion molecule expressed in a number of epithelial tissues, including the epidermis, but not in mesenchymal cells. Following expression of *Snail* in epithelial cell lines, *E-cadherin* is downregulated and the cells become mesenchymal and migratory. Furthermore, a striking correlation was found between the expression of *Snail* and the invasiveness of cell lines and tumours [18\*\*]. These data suggest that Snail-mediated repression is an important mechanism for the induction of an epithelial to mesenchymal transition in cells expressing *E-cadherin*. As *E-cadherin* is not expressed in the neural plate or premigratory neural crest, it is likely that other cadherins may be a target of Snail in this tissue.

These findings raise the question as to whether *Snail/Slug* may be a primary target of inductive signals that lies upstream of all genes required for early steps of neural crest development. A candidate downstream gene is the GTP-binding protein *rhoB*, which is induced by BMP signalling and is required for the epithelial to mesenchymal transition of premigratory neural crest [19]. However, whereas *Noggin* inhibits *rhoB* expression in presumptive trunk neural crest in chick embryos, *Slug* expression is not affected [12\*\*]. A similar lack of correlation with *Slug* regulation occurs for *cadherin-6B* expression in presumptive neural crest [12\*\*]. It seems, therefore, that *rhoB* and *cadherin-6B* gene expression is either independent of *Slug* or requires a BMP-induced transcription factor that collaborates with *Slug*. Consistent with this picture, ectopic expression of *Slug* leads to an increase in the number of neural crest cells, but this only occurs at the normal location of neural crest formation [9,17\*], providing evidence that targets of inductive signals in addition to *Slug/Snail* are required to generate neural crest.

### Guidance and plasticity of branchial neural crest

There is an important link between the guidance and differentiation of neural crest cells, since in some cases specified cells are targeted to the correct destinations, while in others cells migrate to sites where they encounter

inductive signals. A number of cues have been identified that guide neural crest cells, including molecules that provide a substrate for migration, and others that delimit pathways by acting as repellents [20–22]. Work in the past year has given a new picture of the migratory pathways and plasticity of cranial neural crest cells.

Branchial neural crest cells migrate from specific sets of hindbrain segments (rhombomeres) into specific branchial arches, where they differentiate to form distinct bones and cartilage in each arch. It is believed that the guidance of neural crest cells into appropriate branchial arches is crucial for patterning, on the basis of evidence that premigratory branchial neural crest is committed to a specific anteroposterior (A–P) identity controlled by *Hox* gene expression [23]. In the chick and mouse, branchial neural crest cells migrate into the first, second and third branchial arches in three streams separated by gaps adjacent to rhombomeres r3 and r5. All rhombomeres produce neural crest [24], but r3 and r5 contribute fewer neural crest cells [25] that migrate rostrally or caudally to join the streams arising from r2, r4 and r6 [26]. This pattern of migration is due to paraxial exclusion zones in the mesoderm adjacent to r3 and r5 [27]. Unexpectedly, it was found that a null mutation of the mouse *ErbB4* neuregulin receptor results in aberrant migration of neural crest cells into the paraxial mesoderm adjacent to r3 [28\*\*]. Since *ErbB4* is expressed in r3/r5, and wild-type neural crest cells migrate adjacent to r3 in a mutant host, it is unlikely that *ErbB4* has a direct role in guidance. Rather, these findings suggest that the hindbrain patterns the adjacent mesoderm such that it excludes neural crest cells adjacent to r3, and this patterning indirectly requires *ErbB4* function.

The separation of streams of branchial neural crest cells by paraxial exclusion zones in the chick suggested that different mechanisms might operate than those seen in *Xenopus*, where these streams are adjacent. *In ovo*, however, time-lapse movies of chick embryos have now revealed that following their initial separation adjacent to the neural tube, the second- and third-arch neural crest streams contact each other in more ventral regions [29\*\*]. These findings raise the possibility that interactions between neural crest streams may restrict intermingling, similar to the situation in *Xenopus* embryos [30]. However, such restrictions are not absolute, since a few cells cross between the streams of neural crest in the chick [29\*\*], which begs the question of the fate of such cells.

Initial work suggested that branchial neural crest cells maintain A–P identity and *Hox* expression following transplantation such that they instead migrate into a more posterior or anterior branchial arch [31,32]. However, other studies suggested that neural crest cells can be more plastic in their A–P identity [33–35]. A potential caveat to the transplantation experiments is that if large groups of cells are moved, they might maintain their identity due to mutual interactions. Indeed, recent experiments in which small numbers of cells are transplanted in the mouse have shown that branchial neural crest cells lose their A–P identity

(marked by expression of a transgenic *Hox* gene reporter) when they migrate into a different branchial arch from their normal target [36\*\*]. Branchial arch mesoderm was found to provide permissive signals for the maintenance of *Hox* gene expression that had been specified in premigratory neural crest, but was not able to reprogramme cells to a new identity [36\*\*]. Therefore, rather than an irreversible specification of the A–P identity of neural crest prior to migration, a pre-pattern is established that requires migration into an appropriate environment to be maintained. This may enable the maintenance of appropriate domains of A–P specification despite some intermingling between neural crest streams.

### Differentiation

Much progress has been made in the formulation of increasingly detailed maps of neural crest lineages, and in the identification of extracellular signals and transcription factors that influence neural crest differentiation [37–39]. In the past year, there have been new insights into the roles of several classes of signalling molecules and downstream effectors in the differentiation of melanocytes and in several neural lineages, including autonomic and sensory neurons and glia.

### Melanocytes

*In vivo* fate maps and *in vitro* analyses of restrictions to neural crest cell lineages suggest that the decision to differentiate to form pigment cells (melanocytes) or neural derivatives is made early during migration. In the zebrafish, medially located cranial neural crest cells are induced by local Wnt-1 and Wnt-3A signals to form pigment cells, whereas lateral cells that are further from the Wnt signals form neurons [40]. Genetic screening in the zebrafish for mutations affecting pigmentation has identified a gene, *nacre*, that encodes a basic helix-loop-helix (bHLH) protein related to the product of the *microphthalmia* gene, which is required for development of all pigment cells in the mouse [41\*\*]. *Nacre* is essential for differentiation of melanophores, one of the three pigment cell lineages present in zebrafish, and overexpression of *nacre* switches cells to a melanophore fate [41\*\*]. These findings raised the question as to whether *nacre* is a target of Wnt signals. By mapping the promoter region of the *nacre* gene, it has now been shown that Tcf/Lef (T cell transcription factor/lymphoid enhancer factor) binding sites are present that mediate responses to Wnt signalling and are involved in the regulation of *nacre* expression [42\*]. The first steps of a pathway that leads from an inductive signal to a pigment cell fate are thus emerging.

### Autonomic nervous system

The autonomic nervous system comprises three components — the sympathetic, parasympathetic and enteric — all derived from the neural crest. BMP-4 and BMP-7 are expressed in the dorsal aorta adjacent to the sympathetic ganglia and induce sympathetic neurogenesis [43,44]. When BMP activity is blocked *in vivo* by ectopic expression of Noggin, sympathetic neurons fail to form as demonstrated by the loss of expression of terminal noradrenergic marker genes such as *tyrosine hydroxylase* (*TH*) [45\*]. Downstream

effectors of BMPs in the sympathetic pathway include the bHLH proneural gene *Mash1* and the genes encoding the transcription factors *Phox2a* and *Phox2b*. *Mash1* expression is induced by BMPs in neural crest cultures [46] and reduced in sympathetic ganglia following the inhibition of BMP function *in vivo* [45\*]. Loss of *Mash1* function *in vivo* leads to a loss of adrenergic neurons [47,48]. *Phox2a* is upregulated by *Mash1* [48] and has been shown recently to be sufficient to induce the formation of sympathetic neurons *in vivo* [49\*\*], although loss of *Phox2a* function results in the absence of only a subset of peripheral ganglia [50]. BMP induction of *Phox2a* and *TH* expression (via *Mash1*) can be enhanced by low level activation of cAMP signalling in neural crest cultures, whereas higher levels of cAMP have a negative effect on their expression, which suggests that cAMP signalling may act as a binary regulator of sympathoadrenal development [51\*\*]. *Phox2b* expression also relies on BMP signalling [45\*], and targeted disruption of the *Phox2b* gene has demonstrated that it is required for the upregulation of *Phox2a* and for the maintenance of *Mash1* expression, and is essential for the generation of all autonomic ganglia [52\*\*]. *dHAND* and *eHAND* are other bHLH transcription factors whose expression in peripheral ganglia is found exclusively in sympathetic neurons [53]. *eHAND* expression is lost in *Mash1* mutant mice [54] and both *dHAND* and *eHAND* are required for the production of catecholaminergic neurons [55].

The enteric nervous system comprises two layers of interconnected ganglia in the gut wall and is derived from vagal and sacral neural crest [56]. Part of the enteric nervous system comprises a sympathoenteric lineage, which express the receptor tyrosine kinase c-Ret, as well as *Mash1* and *TH*. These cells are lost in c-Ret [57] and *Mash1* deficient mice [58], suggesting critical roles in enteric neurogenesis, and c-Ret is also required as a survival factor for undifferentiated precursors [59]. Similarly, intestinal ganglia are absent in mice deficient for a ligand of c-Ret, glial-cell-derived neurotrophic factor (GDNF) [60,61], which promotes the proliferation of enteric neuron and glial precursors *in vitro* [62]. This activity is antagonised by endothelin-3 (ET-3) [63,64], which thus maintains a population of precursors by inhibiting GDNF-induced neuronal differentiation in the gut. Inactivation of ET-3 [65], or its receptor, endothelin receptor B [66,67], results in loss of both enteric neurons and melanocytes, pointing to a role of ET-3 signalling in more than one neural crest cell lineage. Furthermore, other endothelins regulate other aspects of neural crest development, since mutation of the endothelin receptor A or its ET-1 ligand leads to disruption in patterning of neural crest derivatives during craniofacial development [68].

### Sensory neurons

Sensory neurons constitute the remainder of the peripheral nervous system and are specified by a different set of cues to those utilised by autonomic neurons. The bHLH proteins Neurogenin (*ngn*)-1 and -2 are expressed in the neural crest precursors of sensory neurons, and loss of function of either results in the loss of distinct sublineages

of sensory neurons [69,70,71\*\*]. Intriguingly, although *ngn1* can compensate for the loss of *ngn2* function in the specification of large diameter neurons, this involves the specification of separate precursors rather than an overlapping function within the same cell population [71\*\*]. Ectopic expression of *ngn1* in migratory neural crest cells biases their distribution to the sensory ganglia, and in mesoderm results in the expression of both pan-neuronal and sensory-specific markers [72\*]. NeuroD and other transcription factors expressed in the sensory precursors are downstream of *ngn* function and are required for the generation of specific subsets of sensory neurons [3]. Recently, a population of dividing sensory neuronal precursor cells that express *ngn2* have been identified in neural tube explants that are insensitive to conversion to an autonomic fate by BMP2, suggesting that these neural precursors are committed to a sensory fate [38]. This helps to explain how some neural crest cells can encounter strong autonomic inducing factors during their migration yet still develop into sensory neurons, but the mechanism that induces this commitment is at present unknown.

### Glial cells

The peripheral ganglia contain glial support cells that differentiate after the neuronal component. Previous work has revealed an important role of neuregulin and the ErbB3 receptor, which heterodimerises with ErbB2 to allow signal transduction. Early migrating neural crest cells express ErbB2 and respond to neuregulin *in vitro* by differentiating into glia at the expense of neurons [73], whereas mice lacking functional ErbB3 lack Schwann cells [74]. It has recently been demonstrated that activation of Notch signalling in the neural crest leads to a decrease in the expression of early neuronal markers and an increase in the number of glial cells [75\*\*]. Conversely, inhibition of Notch signalling leads to an increase in the number of cells in the peripheral ganglia expressing early neuronal markers [75\*\*]. This effect of Notch signalling is apparently dominant to the neuronal inducing effect of BMP2 and exerts itself in a transient manner. These results help to explain how a multipotent population of neural crest cells can form both neurons and glia sequentially, even when they are exposed to strong neuronal inducing factors.

### Conclusions

Impressive progress continues to be made in the identification of extracellular signals and transcription factors that control the formation, migration and differentiation of neural crest cells. The finding that the same signals, such as BMPs, control multiple aspects of neural crest development raises the important general question of how a signal controls different responses at different stages. In view of the key role of Notch in the control of differentiation in many tissues, it seems very likely that further roles of Notch in modulating responses to other signals will be uncovered in the neural crest. Another important theme that is emerging from work on the neural crest and other tissues is a wider degree of plasticity in cell fate, such as A-P specification, than had

previously been appreciated. Owing to recent advances in *in vivo* time-lapse imaging, the highly dynamic nature of cell movements is being directly visualised, revealing that some migrating cells enter territory inappropriate for their state of specification. It is likely that local signals regulating cell fate (or cell death) has an important role in maintaining patterns of A-P specification as well as cell differentiation. Furthermore, *in vivo* observations of the dynamic contacts between neural crest cells, and between these cells and their neighbours, highlight that much remains to be understood regarding the mechanisms that underlie guided migration.

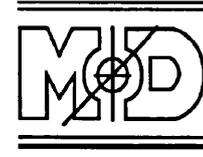
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## Screening from a subtracted embryonic chick hindbrain cDNA library: identification of genes expressed during hindbrain, midbrain and cranial neural crest development

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### Abstract

The vertebrate hindbrain is segmented into a series of transient structures called rhombomeres. Despite knowing several factors that are responsible for the segmentation and maintenance of the rhombomeres, there are still large gaps in understanding the genetic pathways that govern their development. To find previously unknown genes that are expressed within the embryonic hindbrain, a subtracted chick hindbrain cDNA library has been made and 445 randomly picked clones from this library have been analysed using whole mount in situ hybridisation. Thirty-six of these clones (8%) display restricted expression patterns within the hindbrain, midbrain or cranial neural crest and of these, twenty-two are novel and eleven encode peptides that correspond to or are highly related to proteins with previously uncharacterised roles during early neural development. The large proportion of genes with restricted expression patterns and previously unknown functions in the embryonic brain identified during this screen provides insights into the different types of molecules that have spatially regulated expression patterns in cranial neural tissue. Published by Elsevier Science Ireland Ltd.

**Keywords:** Hindbrain; Subtractive hybridisation; In situ hybridisation; Chick; Rhombomere; Neural crest; Midbrain; Isthmus; cDNA library

### 1. Introduction

During early vertebrate development, the hindbrain is transiently divided into a series of units called rhombomeres (r) along the antero–posterior (A–P) axis. The early segmental patterning of the rhombomeres is reflected at later stages of head development in the pattern of neuronal differentiation and also in the specification of the branchial neural crest that migrates into the branchial arches (for reviews see Lumsden and Krumlauf, 1996; Wilkinson, 1995).

Some molecules with known roles in hindbrain segmentation and patterning have been identified, including transcription factors such as members of the HoxA, B and D clusters, Krox20 and *kreisler* and other proteins such as the Eph receptor tyrosine kinases and their ligands the ephrins (Lumsden and Krumlauf, 1996). With the exception of *kreisler*, which was identified in a mouse mutant with disrupted hindbrain segmentation posterior to r3 (Cordes and Barsh, 1994), all of these genes have been recognised as having potential roles during hindbrain development by

virtue of their restricted expression within particular rhombomeres.

*Hox* gene expression in the hindbrain occurs in a nested set of domains with anterior limits at the position of rhombomere boundaries (see Lumsden and Krumlauf, 1996). Evidence from null mouse mutants (Chisaka and Capocchi, 1991; Gavalas et al., 1997; Gendron-Maguire et al., 1993; Rijli et al., 1993; Studer et al., 1996) suggests that *Hox* genes have roles in specifying and maintaining the A–P identity of rhombomeres and neural crest. *Krox20* expression is restricted to r3 and r5 (Wilkinson et al., 1989) and targeted deletions of this gene demonstrate that it is required for the maintenance of these two rhombomeres (Schneider-Maunoury et al., 1997, 1993). In the mouse, *kreisler* is expressed in r5 and r6 (Cordes and Barsh, 1994) and in *kreisler* homozygous mutants there is a loss of overt segmentation posterior to r3 which can be attributed to the complete loss of r5 (Manzanares et al., 1999). Several members of the Eph receptor tyrosine kinase family are expressed at high levels in r3 and r5 in territories adjacent to that of their respective ephrin-B ligands in even numbered rhombomeres (Wilkinson, 2000). Blocking of Eph receptor tyrosine kinase function and mosaic activation of both Ephs and ephrins in vivo have implicated both these

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proteins in the restriction of cell intermingling between rhombomeres (Xu et al., 1995, 1999).

Anterior rhombomeres are also influenced by signals from the isthmus constriction which is found between the midbrain and r1. Fibroblast Growth Factor (*Fgf*)-8 is expressed in the isthmus (Crossley and Martin, 1995) and ectopic application of FGF8 protein in ovo mimics the function of the isthmus in anteriorising the hindbrain (Irving and Mason, 2000). Expression domains of *Otx2* in the forebrain and midbrain (Bally-Cuif et al., 1995) and *Gbx2* in the hindbrain (Shamim and Mason, 1998) meet at the isthmus and experimental evidence suggests that both these genes are required for the correct spatial expression of *Fgf8* and specification of the isthmus organiser (Acampora et al., 1997; Wassarman et al., 1997).

Cranial neural crest cells migrate from the neural tube in three streams separated by neural crest free regions adjacent to r3 and r5. The origin of this segmented pattern of cranial neural crest migration has been linked to the segmental expression of several molecules in the hindbrain. These include the neuregulin receptor ErbB4 which is expressed in r3 and r5 and patterns the adjacent cranial mesoderm to create an exclusion zone for migrating crest cells (Golding et al., 2000), and BMP4 and *Msx2*, both of which are expressed in r3 and r5 at the time of crest cell emergence from the neural tube and underlie the apoptotic elimination of many neural crest cells from these rhombomeres (Graham et al., 1994; Takahashi et al., 1998).

A number of other genes have also been identified whose expression is restricted to specific regions along the A–P axis of the hindbrain and midbrain during early embryonic development but for most, their functions remain less well understood. These encode a variety of molecules including transcription factors of the *Irx* (Goriely et al., 1999), *Sax* (Schubert et al., 1995), *Hlx* (Fjose et al., 1994), *Her/Hes* (Lobe, 1997; Pasini et al., 2001) and *Ash* (Allende and Weinberg, 1994) families; secreted/cell surface proteins such as WNT8C (Hume and Dodd, 1993), *FJX1* (Ashery-Padan et al., 1999), cadherins (Inoue et al., 1997) and collapsins (Shepherd et al., 1996); transmembrane receptors such as FGF receptors (Thisse et al., 1995; Walshe and Mason, 2000); molecules involved in retinoid signalling including Cellular Retinoic Acid Binding Proteins (CRABPs) (Lyn and Giguere, 1994; Maden et al., 1991) and Retinoic Acid Receptors (RARs) (Mollard et al., 2000); other nuclear receptors such as seven-up related proteins (Fjose et al., 1993) and other factors that act as specific antagonists such as follistatin (Graham and Lumsden, 1996) and *sprouty2* (Chambers et al., 2000).

Despite the progress made in dissecting the genetic pathways involved in the early development of the vertebrate hindbrain and cranial neural crest, large gaps in current knowledge still exist. The purpose of this study is to identify novel candidate genes with potential roles in early cranial neural development. Numerous methods can be used to detect genes involved in the development of particular

tissues. These include screens for phenotype such as genetic screens (Driever et al., 1996; Haffter et al., 1996; Nusslein-Volhard and Wieschaus, 1980) and functional screens (Smith and Harland, 1991) or screens that assess gene expression patterns and utilise techniques such as differential display (Chambers et al., 2000; Liang and Pardee, 1992); microarrays (DeRisi et al., 1996; Iyer et al., 1999); subtractive hybridisation (Diatchenko et al., 1996; Harrison et al., 1995) and in situ hybridisation (Gawantka et al., 1998). This latter group of expression pattern screening methods reveal candidate genes that are subsequently analysed for function.

We have chosen to combine the construction of a cDNA library from chick embryonic hindbrains with a large-scale in situ hybridisation screen. We also generated a pre-streak stage chicken embryo cDNA library and used this in a subtractive hybridisation to generate a subtracted hindbrain cDNA library which is significantly enriched for genes expressed in the hindbrain and depleted of ubiquitously expressed genes. Four hundred and forty-five random clones from the subtracted hindbrain library were tested by whole mount in situ hybridisation. Thirty-six clones display highly restricted expression patterns within the hindbrain, midbrain or cranial neural crest. Sequence analysis of these 36 clones reveal that three correspond to genes with established functions during early cranial neural development. A further 10 encode homologues of proteins with no previously recognised roles in early neural development and 23 encode novel proteins. This study provides groundwork for a global understanding of segment diversity and early hindbrain development.

## 2. Results

### 2.1. Generation and characterisation of a subtracted hindbrain cDNA library

Due to the comparatively large size of the chick embryonic hindbrain, it was possible to gather enough material to construct a conventionally cloned cDNA library which circumvents the need for a PCR based amplification step which can introduce bias and generate artefacts (Smith and Gridley, 1992). In order to increase the efficiency of screening by in situ hybridisation, ubiquitously expressed genes were removed from this hindbrain library by a subtractive hybridisation technique. We reasoned that embryos isolated prior to the formation of Koller's sickle ('pre-streak' embryos) would be a good choice to use as driver for this subtractive hybridisation as this tissue has yet to initiate embryonic differentiation (Izpisua-Belmonte et al., 1993).

The subtracted library was generated by hybridising single-stranded, sense, tracer DNA derived from the hindbrain cDNA library to anti-sense biotin labelled mRNA derived from the 'pre-streak' library. Biotin tagged tracer-

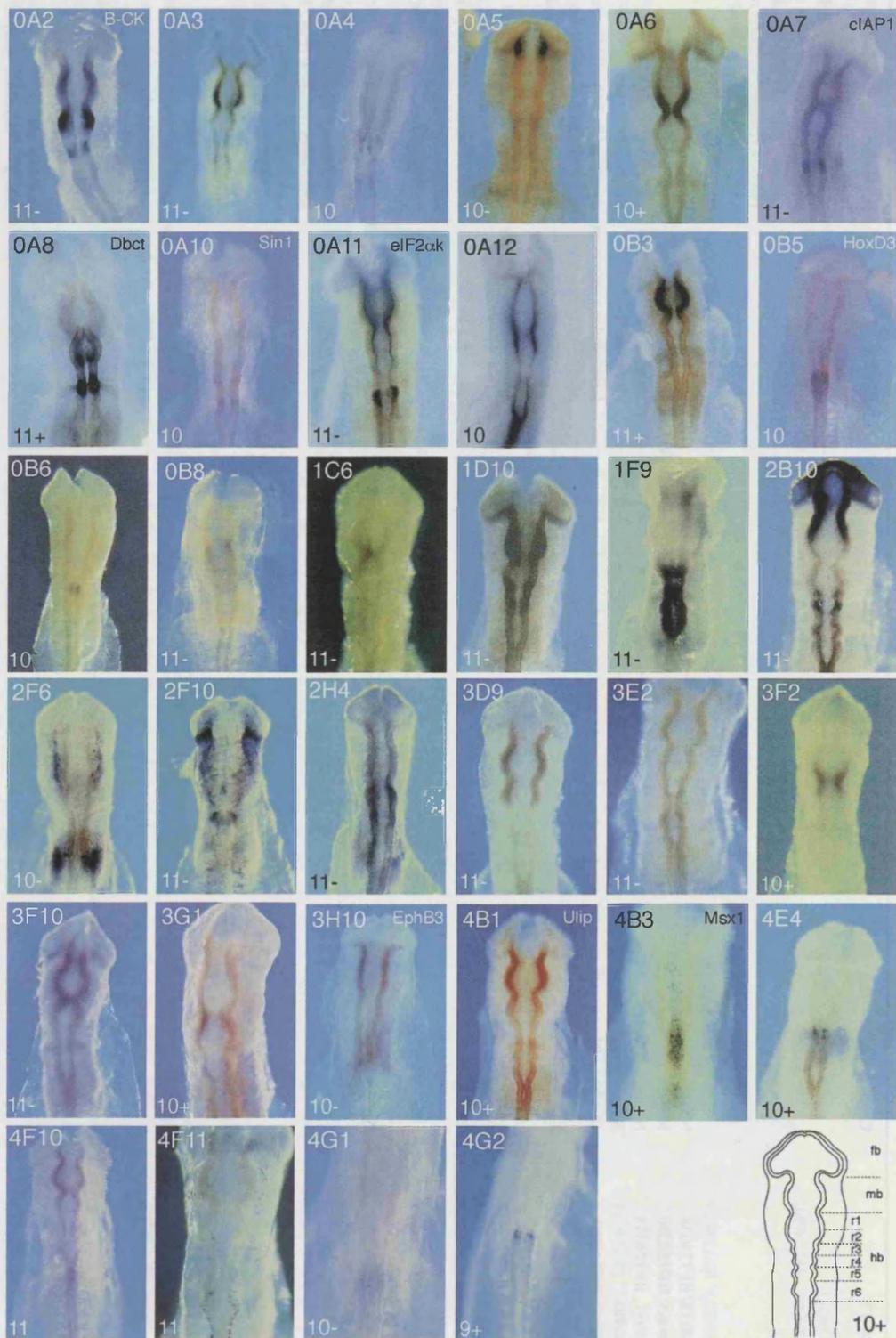


Fig. 1. Patterns of gene expression in cranial neural tissues of clones identified during this screen. In the upper left hand corner of each panel appears the clone identification number. The age of the embryo (in HH stage) is indicated in the lower left-hand corner of each photograph. The identity of clones (when known) is indicated in the upper right hand corner of each panel. The line drawing at the bottom right hand corner of the figure shows gross cranial neural tube architecture at HH10+. r, rhombomere; mb, midbrain; fb, forebrain; hb, hindbrain.

Table 1  
Summary of clones identified with restricted expression domains within cranial neural tissues in the chick<sup>a</sup>

Clone ID	Genbank accession numbers	Similarity; e value	Expression in ...											Protein type	Functions/comments
			fb	mb	r1	r2	r3	r4	r5	r6	sc	nc			
0A2	BF724032	Chick B-CK; e = 0.0	+/-	++	++	+	+/-	++	-	+	-	-	-	Enzyme	Brain specific, cytoplasmic isoform of creatine kinase. Transfers phosphoryl groups from creatine phosphate to ADP to generate ATP (Prichard et al., 1983). Expressed in foetal rat brain in dividing astrocytes (Manos and Bryan, 1993)
0A3	BF724033, BF724034	None	+/-	++	+/-	-	+	-	+	-	-	-	Unknown	Unknown	
0A4	BF724035, BF724036	None	-	+	+	-	+	-	+	-	-	-	mb,r1,r2	Unknown	
0A5	BF724037, BF724038	None	-	++	-	-	+/-	-	-	-	-	-	Unknown	Unknown	
0A6	BF724039	None	-	++	-	-	-	-	-	-	-	-	Unknown	Unknown	
0A7	BF724040, BF724041	Chick cIAP1; e = 0.0	-	+	++	+	+	+	++	+	+	-	Intracellular component of signalling cascade	Recruited to TNF $\alpha$ R1/2 after ligand binding in association with TRAFs (Shu et al, 1996) and leads to NF- $\kappa$ B dependent transcriptional activation of initiator caspases (Wang et al., 1998). Can also bind directly to and inhibit effector caspases leading to inhibition of apoptosis (Roy et al, 1997).	
0A8	BF724042, BF724043 AF330009	Human Dbct; e = 0.0	-	-	-	++	-	++	-	-	-	r4	Cytoskeleton associated protein	Mutations in human Dbct lead to Type I lissencephaly which is characterised by abnormal cortical lamination (Gleeson et al., 1998; desPortes et al., 1998). The Dbct protein binds to tubulin and stabilises microtubules (Taylor et al., 2000).	
0A9	BF724044	None	-	-	-	-	-	+	-	-	-	-	Unknown	Unknown	
0A10	BF724045, BF724046	Chick Sin1; e = 0.0	-	-	-	-	+	-	+	-	-	-	Intracellular component of signalling cascade	Yeast Sin1 interacts with Sty1 stress activated protein kinase and is phosphorylated following stress in a Sty1 independent manner (Wilkinson et al., 1999). Human Sin1 (JC310) is capable of rescuing a temperature sensitive RAS2 <sup>val19</sup> mutation when overexpressed in <i>S. cerevisiae</i> . (Collicelli et al., 1991)	

Table 1 (continued)

Clone ID	Genbank accession numbers	Similarity; e value	Expression in ...										Protein type	Functions/comments
			fb	mb	r1	r2	r3	r4	r5	r6	sc	nc		
0A11	BF724047, BF724048 AF330008	Mouse HRI; e-159	+	+	-	-	-	-	+	-	-	-	Serine/threonine kinase	Highly related to HRI (Chen et al, 1991); PKR (Meurs et al, 1990) and PEK (Shi et al, 1998). These all specifically phosphorylate eIF2 $\alpha$ and prevent translational initiation in eukaryotes.
0A12	BF724049, BF724050	None	-	+	++	+/-	-	+/-	+	+	+	-	Unknown	Unknown
0B3	BF724051, BF724052	Human L15344; e-120	-	++	-	-	-	-	-	-	-	-	Unknown	Unknown - L15344 identified through genome sequencing
0B5	BF724053	Chick HoxD3; e = 0.0	-	-	-	-	-	-	++	+	+/-	-	Transcription factor	Homeobox containing transcription factor known to be expressed caudally from r5, HoxB3 <sup>-/-</sup> embryos display defects in the IXth cranial nerve (Manley and Capecchi, 1997).
0B6	BF724054, BF724055	None	-	-	-	-	++	-	-	-	-	-	Unknown	Unknown
0B8	BF724056, BF724057	None	-	+	+	-	-	-	-	-	-	-	Unknown	Unknown
1C6	BF724058, BF724059	None	-	+	+	-	-	-	-	-	-	-	Unknown	Unknown
1D10	BF724060, BF724061	Human RI/RAI; e-53	+/-	++	+/-	+/-	++	+/-	+/-	+/-	-	-	Specific antagonist	RAI previously isolated from adult brain - binds to and inhibits ribonuclease (Nadano et al., 1994)
1F9	BF724062, BF724063	None	-	+	-	++	++	++	++	++	-	-	Unknown	Unknown
2B10	BF724064, BF724065	None	++	++	-	+	++	-	-	-	-	-	Unknown	Unknown
2F6	BF724066, BF724067	None	-	-	-	-	-	-	-	-	-	mb	Unknown	Unknown
2F10	BF724068, BF724069	None	-	-	-	-	-	-	-	-	-	panmig	Unknown	Unknown
2H4	BF724070, BF724071	None	+/-	+/-	++	+	+	+/-	+/-	+/-	+/-	-	Unknown	Unknown
3D9	BF724072, BF724073	None	++	++	++	-	-	-	-	-	-	-	Unknown	Unknown
3E2	BF724074, BF724075	Human G2; e-73	-	-	-	-	+	-	+	-	-	-	Unknown	Unknown - 'G2' identified through genome sequencing
3E11	BF773561, BF773562	None	-	-	-	-	+	-	-	-	-	hb	Unknown	Unknown
3F2	BF724076	None	+	+	-	-	-	-	-	-	-	-	Unknown	Unknown
3F10	BF724077, BF724078	Mouse SPDSY; e-33	-	++	-	-	-	-	-	-	-	-	Enzyme	SPDSY enzyme involved in polyamine synthesis in the production of spermidine (Korhonen et al., 1995)
3G1	BF724079, BF724080	None	-	-	+	-	+	-	-	-	-	-	Unknown	Unknown
3H10	BF724081	Chick EphB3; e = 0.0	-	+	++	+	+	+	+	-	-	-	Receptor tyrosine kinase	Eph receptor tyrosine kinase known to be expressed in the hindbrain. Ephs act to restrict cell intermingling between forming rhombomeres (see Wilkinson, 2000).

Table 1 (continued)

Clone ID	Genbank accession numbers	Similarity; e value	Expression in ...											Protein type	Functions/comments
			fb	mb	r1	r2	r3	r4	r5	r6	sc	nc			
4B1	BF724082, BF724083 AF330010	Mouse ULIP4; e-84	-	++	+	+/-	-	+	+/-	+	+	r4	Enzyme	Represents a new ULIP protein. This family includes unc-33 which when mutated leads to aberrant <i>C. elegans</i> axonal projections (Hedgecock et al., 1987); TOAD-64 which is upregulated during rat neurogenesis (Minturn et al., 1995) and Ulip which is phosphorylated in response to NGF and is found in growth cones in mouse brain (Byk et al., 1996)	
4B3	BF724084	Chick Msx1; e = 0.0	-	-	-	-	-	-	-	-	-	premig	Transcription factor	Homeobox containing transcription factor expressed in many tissues including neural crest (Robert et al., 1989). <i>Msx1</i> <sup>-/-</sup> mice display midline and other craniofacial abnormalities (Satoka and Maas, 1994). <i>Msx1</i> expression regulated by FGFs and BMPs but subsequently functions upstream of these (see Peters and Balling, 1999)	
4E4	BF724085, BF724086	None	+	-	-	-	++	-	+/-	+/-	+/-	-	Unknown	Unknown	
4F10	BF724087, BF724088	None	-	+	+/-	-	-	-	-	-	+	-	Unknown	Unknown	
4F11	BF724089, BF724090	Human CB1; e-77	-	+/-	+/-	+/-	-	-	+/-	+/-	+/-	-	Transmembrane protein	Central cannabinoid receptor CB1 modulates activity of adenylate cyclase, Ca <sup>2+</sup> and K <sup>+</sup> channels and stimulates the MAPK pathway (see Howlett, 1998). <i>CB1</i> <sup>-/-</sup> mice are viable but have reduced locomotor activity (Zimmer et al., 1999)	
4G1	BF724091, BF724092	None	-	-	-	-	-	-	+	-	-	-	Unknown	Unknown	
4G2	BF724093	None	-	-	-	-	++	-	-	-	-	-	Unknown	Unknown	

<sup>a</sup> Similarities refer to the best hit following database searching using the BLAST algorithm. The 'e value' denotes the degree of similarity with a value of 0 indicating a direct match. Levels of gene expression are denoted as '++' representing high levels of expression followed in decreasing order by '+' and '+/-'. '-' denotes expression below the level of detection. Abbreviations: fb, forebrain; mb, midbrain; r, rhombomere; sc, spinal cord; nc, neural crest; panmig, pan migratory neural crest; premig, premigratory neural crest.

driver hybrids were removed by binding to streptavidin and the resultant tracer-specific, single-stranded DNA was converted to double-stranded DNA to produce the subtracted hindbrain cDNA library. The number of independent clones in the original amplified hindbrain library was  $5 \times 10^5$  clones. Following subtraction and the removal of empty vector background, the size of the library was  $2.5 \times 10^3$  clones, which indicates that 99.5% of clones were removed during the subtraction procedure.

The success of the subtraction procedure was assessed by screening  $2 \times 10^4$  clones from both the unsubtracted and subtracted hindbrain libraries by colony screening for the ubiquitously expressed gene *GAPDH* (Panabieres et al., 1984) and two genes expressed in the embryonic hindbrain *EphB3* (Sajjadi and Pasquale, 1993) and *HoxA3* (Saldivar et al., 1996). In the unsubtracted library, 240 colonies hybridised with *GAPDH* compared to 0 positively hybridising colonies following subtraction. Prior to subtraction, four and 16 colonies hybridised with *EphB3* and *HoxA3* probes respectively whereas after subtraction these figures rose to 43 and 30 indicating between a 2- to 10-fold enrichment for these genes within the library. This indicates that the subtraction procedure has successfully eliminated some ubiquitously expressed genes and significantly enriched for other genes which are normally expressed within the hindbrain.

In order to calculate the complexity of the subtracted library,  $1.2 \times 10^3$  clones were randomly picked and gridded onto agar plates. Radioactively labelled probes were generated from nine other randomly selected clones from the library and individually used to screen the gridded library. The number of positively hybridising colonies per probe ranged from 0 to 2, with an average number of 0.6 per  $1.2 \times 10^3$  colonies. This indicates that a particular gene is likely to be represented once in every  $2 \times 10^3$  clones.

## 2.2. Identification of clones displaying restricted expression patterns within the developing brain

In order to identify genes with restricted domains of expression within developing cranial neural tissues, we assessed the expression patterns of randomly chosen clones from the subtracted hindbrain library using whole mount in situ hybridisation of chicken embryos. To analyse expression during both early and late hindbrain development, a range of at least three embryos (HH 9 to 12) were analysed for each probe. The expression of hindbrain segmentation genes such as *Krox20* first reach detectable levels at the beginning of this period and continue to be expressed throughout this time (Irving et al., 1996).

Thirty-six of the 445 clones (8%) tested in this manner displayed expression patterns that were restricted to parts of the midbrain, hindbrain or cranial neural crest arising from these regions (Fig. 1 and Table 1). Three hundred and forty (76%) of the clones either exhibited non-regionalised expression in the embryo or were exclusively expressed at

uniform levels throughout the neural tube (not shown). Signal was undetectable in 69 (16%) of the clones screened (not shown), which may reflect either low levels of transcript expression or insensitivity of the probe under the hybridisation conditions utilised. In cranial neural tissue, a range of expression patterns were identified, with some clones expressed in broad domains, for example within the whole hindbrain (clone 1F9) or midbrain (clone 0B3), others in specific rhombomeres (e.g. clones 0A7, 0A11) and others in specific populations of cells within particular rhombomeres (e.g. clones 0B6, 4E4 and 4G2). Some clones are expressed at the isthmus between the midbrain and r1 (e.g. 0A6, 0B8, 1C6, 3F2 and 3G1) or in migrating neural crest cells (e.g. 2F10).

Clones exhibiting restricted expression patterns in cranial neural tissues were sequenced from both ends and conceptual translations in all six reading frames of these expressed sequence tags (ESTs), were compared to public domain protein databases. Expected (e) values lower than  $10^{-6}$  were taken to represent sequence similarity and these values are listed in Table 1 for each clone. Three of the clones exhibiting regionalised neural expression (3H10, 0B5 and 4B3) encode molecules with previously demonstrated expression or function during early cranial neural development: the receptor tyrosine kinase *EphB3* (Sajjadi and Pasquale, 1993; Wilkinson, 1995), and the homeobox containing transcription factors *HoxD3* (Manley and Capecchi, 1997; Searcy and Yutzey, 1998) and *Msx1* (Feledy et al., 1999; Yokouchi et al., 1991) suggesting that the screening method can identify genes of functional importance.

Nine of the 36 clones with regionally restricted expression patterns are identical to, or closely related to genes/proteins with previously characterised expression or functions in tissues other than early embryonic neural tissue. These cover a range of molecules including enzymes, membrane bound receptors, intracellular components of

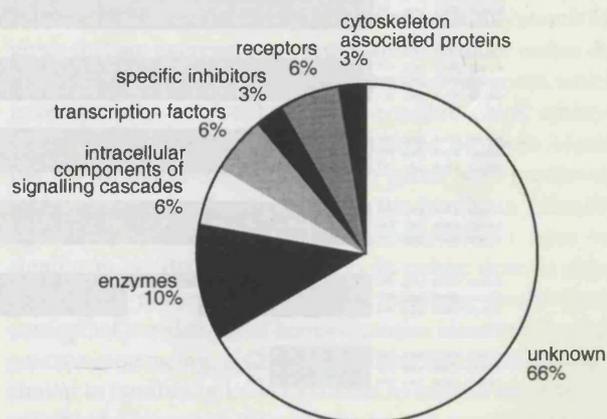


Fig. 2. Functional classification of clones identified during this screen with expression restricted to cranial neural tissues. ESTs corresponding to 66% of clones have no significant sequence similarity and therefore encode proteins of unknown function.

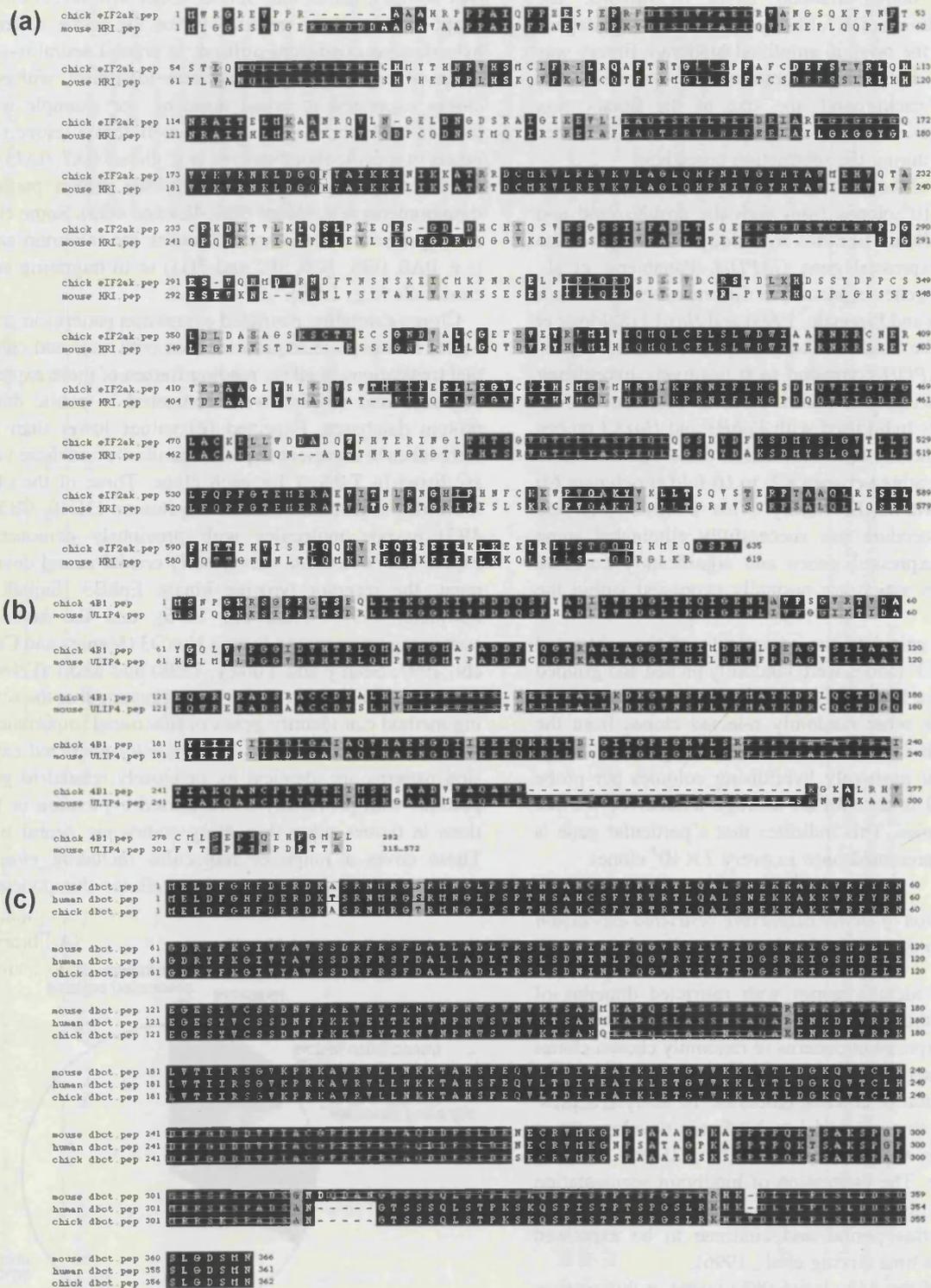


Fig. 3. (a) Amino acid alignment of the entire coding region of clone 0A11 (chick eIF2ak) with the mouse HRI protein. (b) Amino acid alignment of the coding region of clone 4B1 with the mouse ULIP4 protein. The ULIP4 protein contains 257 extra amino acids relative to 4B1 (positions 315–572 as indicated). (c) Amino acid sequence comparison of the entire coding region of clone 0A8 (chick dbct) with the mouse and human doublecortin proteins. All alignments were performed using the BLOSUM30 algorithm. Identical amino acids are indicated by the black boxes and conservative amino acid substitutions are indicated by the grey boxed residues.

signalling cascades and specific inhibitors of other proteins (see Table 1 and Fig. 2).

Four clones encode enzymes (0A2, 0A11, 3F10 and 4B1). Clone 0A2, expressed in all rhombomeres at varying levels except for r5, is identical to the chick *brain-type creatine kinase (B-CK)* gene which encodes the cytoplasmic form of CK and is involved in energy metabolism (Hossle et al., 1986; Prichard et al., 1983). Clone 0A11 is expressed strongly in r5 and sequencing of the entire coding region (Genbank accession: AF330008) reveals that it encodes a serine/threonine kinase highly related to the eukaryotic translational initiation factor subunit 2 $\alpha$  (eIF2 $\alpha$ ) kinase, HRI (haem-regulated inhibitor of translation: Berlanga et al., 1998; Chen et al., 1991) and with lower similarity to PKR (interferon-inducible double-stranded RNA dependent protein kinase: Meurs et al., 1990) and PEK (pancreatic eIF2 $\alpha$  kinase: Shi et al., 1998) (Fig. 3a). Phosphorylation of eIF2 $\alpha$  by HRI prevents binding of the initiator methionine-tRNA to the ribosome, thus inhibiting translational initiation (Kimball, 1999). Clone 4B1 is expressed in the midbrain and r4–r6 and at lower levels in neural crest emigrating from r4. Sequencing of the entire coding region of this clone (Genbank accession: AF330010) showed that it is very highly related to the N-terminal half of the dihydro-pyrimidinase-related protein ULIP-4 (Fig. 3b). Some members of the ULIP protein family are upregulated during neuronal differentiation and have roles in axonal guidance (Byk et al., 1996; Hedgecock et al., 1987; Holm and Sander, 1997; Minturn et al., 1995). Clone 3F10 is expressed throughout the midbrain and hindbrain and exhibits moderate amino acid similarity to spermidine synthase (SPDSY) (Korhonen et al., 1995).

Two clones encode intracellular components of signal transduction pathways (0A7 and 0A10). Clone 0A7 is expressed at high levels in the midbrain and r5, and within r5, expression is restricted to the ventral two-thirds (not shown). 0A7 encodes the chick cellular inhibitor of apoptosis-1 protein (cIAP1; You et al., 1997). cIAP1 binds to the tumour necrosis factor alpha (TNF $\alpha$ ) receptor (Shu et al., 1996) to suppress the transcriptional activation of initiator caspase genes (Wang et al., 1998) and also binds certain effector caspases (Roy et al., 1997), both leading to an inhibition of apoptosis (Deveraux and Reed, 1999).

Clone 0A10, expressed at low levels in r3 and r5, is the chicken homologue of Sin1. Sin1 interacts with Sty1/Spc1 (stress-activated, mitogen-activated protein kinase, SAPK) in yeast, is phosphorylated following stress in a Sty1/Spc1 independent manner and is required for stress-dependent transcription via the substrate of Sty1/Spc1, Atf1 (Wilkinson et al., 1999). We have previously shown that chicken and yeast Sin1 are biochemically equivalent as chimaeric fusions of the two proteins can rescue loss of Sin1 function in yeast (Wilkinson et al., 1999).

Clone 1D10, expressed in the fore-, mid- and hindbrain, shares moderate sequence similarity to human ribonuclease/angiogenin inhibitor protein (RAI/RI, Schneider et al.,

1988). Clone 4F11 is expressed in specific dorsal cells posterior to r6 and shares a high similarity to the mammalian transmembrane G-protein coupled central cannabinoid receptor CB1 which is widely expressed in the adult brain (Howlett, 1998).

Clone 0A8 is expressed strongly in r2, r4 and neural crest migrating from r4. Initial analysis of EST sequence data suggested that this clone encodes the chicken homologue of doublecortin and this has been confirmed by sequencing the 0A8 clone in its entirety (Genbank Accession: AF330009, see also Fig. 3c). Mutations in human *doublecortin* result in X-linked lissencephaly, in which abnormal cortical lamination occurs due to defects in neuronal migration (desPortes et al., 1998; Gleeson et al., 1998). The doublecortin protein binds to and stabilises microtubules and is expressed widely in migrating neurons (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999; Taylor et al., 2000).

Two clones: 0B3 (expressed in the midbrain and spinal cord) and 3E2 (expressed in r3 and r5) exhibit sequence similarities to the hypothetical proteins L15344 (Ambrus et al., 1993, 1996) and G2 (U10991-unpublished) respectively identified through human genome sequencing.

The remaining 22 clones exhibiting restricted expression in cranial neural tissues have no significant sequence similarity to any known nucleic acid or protein sequence and represent novel genes of unknown function (i.e. 66% of clones with restricted expression patterns are novel). These data are summarised in Fig. 2 and Table 1.

### 3. Discussion

#### 3.1. Use of a subtracted hindbrain cDNA library to identify transcripts with spatially restricted expression patterns

We constructed a cDNA library from the chick hindbrain, subtracted this with a pre-streak embryo cDNA library and then carried out an in situ hybridisation screen to search for genes that are expressed in a regionalised manner within the midbrain or hindbrain. This has identified 36 genes which have spatially restricted expression within chick embryo cranial neural tissue. Sequence analysis of these clones has shown that three correspond to genes with previously known expression/function within the hindbrain (*HoxD3*, *EphB3* and *Msx1*). Twenty-two clones (66%) have no significant similarity to sequences in public domain databases. Two clones show moderate sequence similarity to conceptual translations of human proteins identified through genome sequencing, six encode proteins that are sufficiently similar to families of known proteins to infer an identity and possible function and three clones encode peptides that are identical to previously characterised proteins that have no known role in embryonic development.

On the basis of these sequence similarities, of the 12 clones identified with known or inferred functions, four

encode enzymes, two encode transcription factors, two intracellular components of signalling cascades, two transmembrane receptors, one a cytoskeleton associated protein and one a specific antagonist. We expect that there will be a similar diversity of functions within the clones that as yet have no assigned identities. It is perhaps surprising to find restricted expression of genes encoding metabolic enzymes in different rhombomeres and the identification of such genes suggests that there are differential requirements for their products.

We routinely obtained at least 700 nucleotides of high-quality sequence data from either end of the clones that were sequenced. The lack of identification of 3' sequence similarity of many clones from the library is not surprising given that 3' untranslated region sequences are poorly conserved between species and few chick ESTs are present in the databases. At the 5' end however, approximately one-third of clones from the hindbrain library extend into the coding region which raises the possibility of obtaining a hit whilst performing cross-species searches using protein databases. Molecular identification of the clones with no current similarity may have to await more data being deposited on public domain databases in conjunction with obtaining further sequence.

### 3.2. Co-expression of apoptotic regulatory genes in rhombomere 5

Neural crest that migrates from the chick hindbrain is divided into three distinct streams: crest cells from r1 and r2 migrate into the first branchial arch, crest from r4 into the second branchial arch, crest from r6 migrates into the third branchial arch and neural crest free regions are present adjacent to r3 and r5 (Lumsden et al., 1991). Rhombomeres 3 and 5 produce some neural crest cells but significantly fewer than adjacent even-numbered rhombomeres (Lumsden et al., 1991; Kulesa and Fraser, 1998). One mechanism underlying the decrease of neural crest from r3 and r5 is the apoptotic elimination of their precursors from these rhombomeres. Apoptosis occurs throughout the dorsal neural tube with raised levels over r2/3 and r5 at the time of neural crest production (Graham et al., 1994), and these apoptotic foci correlate with sites of *Bmp4* and *Msx2* expression. *Msx2* expression is induced by BMP4 (Graham et al., 1994) and both of these molecules lead to an increase in the apoptotic elimination of neural crest cells when ectopically expressed in even-numbered rhombomeres (Graham et al., 1994; Takahashi et al., 1998). Clones OA7 (cIAP1) and OA11 (eIF2 $\alpha$  kinase-related) identified during this screen encode peptides that are identical to or related to proteins with roles in the control of apoptosis.

One effect of inhibiting translation via the phosphorylation of eIF2 $\alpha$  can be an induction of apoptosis (Lee and Esteban, 1994; Srivastava et al., 1998). The expression of OA11 throughout r5 may therefore underlie a predisposition of r5 to undergo apoptosis. On the other hand, the overlap-

ping expression of the apoptotic inhibitor cIAP1 in ventral r5 may protect these cells from apoptotic elimination, such that cell death is restricted to dorsal regions. It will therefore be interesting to analyse whether these molecules modulate BMP4/*Msx2* induced apoptosis in r5.

### 3.3. Expression of genes in neural crest migrating from r4

In view of the role of doublecortin in the migration of neuronal cell bodies in the cerebral cortex (Barkovitch et al., 1996) it is intriguing that clone OA8, the chick homologue of *doublecortin*, is expressed in neural crest migrating from r4. *doublecortin* encodes a cytoplasmic protein expressed widely in migrating neurons (Gleeson et al., 1999) that contains several potential phosphorylation sites (Gleeson et al., 1998) and a tubulin binding domain (Taylor et al., 2000). The binding of doublecortin protein to tubulin facilitates polymerisation into microtubules and may influence neuronal migration by enabling a dynamic regulation of the cytoskeleton. Like migrating neurons, the motility of the neural crest is governed by the cytoskeleton and our observations raise the possibility that doublecortin has a role in r4 neural crest cell migration. Since r4-derived craniofacial structures are not missing in X-linked lissencephaly, and *doublecortin* expression occurs only in a subset of neural crest, presumably other genes have similar roles and have overlapping expression with doublecortin. However, the segmental expression of *doublecortin* in r2 and r4 does not correlate specifically with cell migration, raising the possibility that it may regulate other microtubule-dependent processes within the hindbrain.

Another clone expressed in neural crest cells migrating from r4 is 4B1, which encodes a protein highly homologous to the ULIP family (Unc-33 Like Proteins; Byk et al., 1998). Sequence similarity is highest to ULIP4, but 4B1 encodes a protein which lacks a ~300 amino acid region present at the C-terminus of all other ULIP proteins (Fig. 3b) and therefore may represent a product of alternate splicing. ULIPs are intracellular proteins that are phosphorylated in response to various stimuli (Byk et al., 1996) and some have roles in regulating neuronal outgrowth; these include *C. elegans* unc-33 (Hedgecock et al., 1985, 1987), rat ULIP2 (Toad-64) (Minturn et al., 1995) and chick ULIP4 (Crmp-62) which is essential for collapsin-mediated neuronal growth cone collapse (Goshima et al., 1995). Overexpression of ULIP2 in neuroblastoma cell lines has demonstrated that this protein associates with microtubules and can effect cell shape changes via interactions with the cytoskeleton (Gu and Ihara, 2000). In addition to regulating axonal outgrowth, collapsins have been implicated in controlling neural crest cell migration, since collapsin-1 is expressed in territories adjacent to migrating neural crest cells (which express the collapsin-1 receptor, neuropilin-1), and collapsin-1 protein can act in an inhibitory manner to the migration of these cells (Eickholt et al., 1999). 4B1 is co-expressed with neuropilin-1 in neural crest cells that migrate

from r4 and raises the possibility that ULIPs may mediate collapsin-induced repulsion of migrating neural crest cells.

### 3.4. Perspectives

We have used an in situ hybridisation screen to identify genes with previously unknown expression in the embryonic midbrain and hindbrain. Until recently, in situ hybridisation has been little used as a technique for large scale projects to assess gene expression patterns and this is probably due to the large amount of 'hands-on' time required for this method. With the advent of automated robots to perform this task (Plickert et al., 1997) the feasibility of carrying out in situ hybridisation screens has greatly increased.

This screen has identified a number of novel candidate genes whose expression is spatially restricted to cranial neural tissue. For some of these molecules, potential roles can be proposed based on sequence similarities to known genes. For others, it may be possible to group these into synexpression groups (Niehrs and Pollet, 1999) based on their expression patterns. The usefulness of this latter approach has been recently demonstrated by the cloning of BAMBI, an inhibitor of TGF- $\beta$  signalling which was identified by virtue of its expression pattern which is coincident with that of the TGF- $\beta$  family member, BMP4 (Onichtchouk et al., 1999).

The development of in ovo electroporation (Itasaki et al., 1999) to easily and efficiently overexpress genes within the chick neural tube/neural crest, and the use of either conventional or 'morpholino' based anti-sense oligonucleotides (Cooke and Isaac, 1999; Heasman et al., 2000; Nasevicius and Ekker, 2000; Summerton and Weller, 1997) to inhibit gene function means it should be feasible to extend this screen to assess the function of those genes identified. Our preliminary results indicate that morpholino oligonucleotides can be introduced into the chick hindbrain by electroporation. These approaches will in particular enable functional analyses of the candidate genes that have no homologies with genes of known function, which precludes the design of dominant negative reagents.

## 4. Experimental procedures

### 4.1. Embryo dissection

Chicken embryonic hindbrains were collected from Hamburger-Hamilton (1992) stage 10–12 embryos from fertilised brown chicken eggs (Needles Egg Farm, Hertfordshire). Gross dissection to remove non-neural tissue was performed in L15 media (GIBCO-BRL) without serum at 4°C. Enzymatic digestion to aid in freeing of adjacent endoderm and mesoderm was performed using 6 U/ml dispase (Boehringer) diluted in L15 media for 3–4 min at room temperature. Inhibition of dispase was achieved by transferring the sample to L15 media containing 20% sheep serum at 4°C. StageX (Eyal-Giladi and Kochav, 1975) embryos

were removed from unincubated fertilised chicken eggs in a mixture of 50% PC saline: 50% PBSA at 4°C prior to sample collection in L15 media.

### 4.2. RNA isolation

Total RNA was extracted from either 83 isolated embryonic hindbrains or from 113 embryos prior to the formation of Koller's sickle. Embryonic material was disaggregated in TRIzol reagent (GIBCO-BRL) and total RNA isolated according to the method of the supplier. Total RNA was resuspended in H<sub>2</sub>O and frozen at -70°C until 100  $\mu$ g was obtained. Poly(A)<sup>+</sup> RNA was prepared by binding to poly(T) coated magnetic beads (Dyna) and subsequent elution. Efficiency of poly(A)<sup>+</sup> selection was determined by northern blotting (Sambrook et al., 1989).

### 4.3. cDNA library construction

Plasmid libraries were constructed using the SuperscriptII System for cDNA Synthesis and Plasmid Cloning (GIBCO-BRL). One microgram of poly(A)<sup>+</sup> RNA was used to construct each library and following cDNA synthesis 50 ng of the largest cDNA molecules were cloned into either *NotI*- and *SalI*- digested pSPORT1 (for the hindbrain library) or pSPORT2 (for the pre-streak library). Each ligation mixture was introduced into ElectroMAX DH12S *Escherichia coli* cells (GIBCO-BRL) using a BioRad Gene-pulser electroporator under standard conditions. The number of independent clones for each library was determined by plating a known fraction of each library prior to amplification. The resultant hindbrain and 'pre-streak' plasmid libraries contained  $7 \times 10^5$  and  $6 \times 10^5$  independent clones respectively. The range of insert sizes for the hindbrain library is 0.4–3.0 kb with an average insert size of 1.9 kb ( $n = 15$ ). Insert sizes range from 0.7–2.0 kb for the 'pre-streak' library with an average insert size of 1.3 kb ( $n = 12$ ). For both libraries,  $5 \times 10^5$  clones were amplified and these amplified libraries were used in all subsequent procedures. To assess whether these libraries contained full length cDNA clones, a fraction of each library representing  $2 \times 10^5$  clones was subjected to southern blotting with a probe for a gene of known transcript size, *GAPDH* (Panabieres et al., 1984). In both cases insert sizes of 1.2 kb were observed indicating that full-length clones were present (not shown).  $5 \times 10^5$  clones from each library were amplified according to the method of Hanahan et al. (1991) and the amplified libraries were aliquotted and stored in 20% glycerol at -70°C.

### 4.4. Subtractive hybridisation

Removal of clones common to both the hindbrain and pre-streak cDNA libraries was performed according to the method of Harrison et al. (1995). Single (sense) stranded tracer DNA was prepared from the hindbrain library using the method recommended by the supplier of the vector

(GIBCO-BRL) and any residual double-stranded contaminants were removed by digesting the sample with *PvuII*. Antisense biotinylated mRNA derived from the driver (pre-streak) library was prepared using biotinylated-UTP (Clontech) and a T7 MEGAscript kit (Ambion) according to the manufacturer's instructions. Following a single round of solution hybridisation of the single stranded tracer and biotinylated driver, removal of excess biotinylated driver and driver:tracer hybrids was achieved by binding to streptavidin (GIBCO-BRL) followed by their removal by repeated phenol:chloroform extraction. Remaining single stranded molecules were converted to their double stranded form using the M13 universal primer and Sequenase DNA polymerase (Amersham) prior to introduction into ElectroMAX DH12S cells by electroporation. Preliminary analysis suggested a large amplification of empty clones had occurred during the subtraction procedure. Removal of clones without inserts from the subtracted library was achieved by digesting 5 µg of plasmid DNA prepared from the library with *NotI* followed by size fractionation by agarose gel electrophoresis, excision and purification of DNA molecules greater than 4kb in size and subsequent self ligation. This purified subtracted library was introduced into ElectroMAX DH12S *E. coli* cells by electroporation.

#### 4.5. Characterisation of the subtracted library

The effectiveness of the subtraction procedure was determined by plating  $2 \times 10^4$  clones from both the unsubtracted and subtracted hindbrain libraries on LB agar plates followed by colony screening (Sambrook et al., 1989) using probes for the following genes: *GAPDH* (Panabieres et al., 1984); *EphB3* (Sajjadi and Pasquale, 1993) and *HoxA3* (Saldivar et al., 1996). Determination of the level of enrichment/purification of these genes was achieved by comparing the number of positive signals from filters representing the unsubtracted or subtracted libraries. The complexity of the subtracted library was determined by plating  $1.2 \times 10^3$  clones on LB agar followed by colony screening (Sambrook et al., 1989) using probes derived from nine randomly chosen clones from the library followed by the determination of the average number of colonies hybridising to each of these probes.

#### 4.6. Whole mount in situ hybridisation

The subtracted hindbrain library was plated at low density and individual clones picked into 96 well plates. Plasmid DNA was extracted from each clone on a small scale (Qiagen plasmid mini kit) and linearised using *SalI*. The insert size of each clone was checked by running a portion of the digested DNA on an agarose gel. The remainder of the linearised DNA was used as template in the synthesis of digoxigenin (DIG) labelled antisense RNA probes. Probe integrity and approximate concentration was assessed by agarose gel electrophoresis and approximately 1 µg DIG labelled RNA was hybridised to at least 3 HH 9–12 embryos

for each clone. In situ hybridisation was performed either by hand or automatically using an InsituPro (AbiMed) in situ hybridisation robot (Plickert et al., 1997) using 'Protocol Four', previously described by Xu and Wilkinson (1998). Stained embryos were visualised with the aid of a stereomicroscope (Leica) scored for expression in the forebrain, midbrain, hindbrain, neural crest and spinal cord and photographed using an accompanying camera. Images were digitised using a Polaroid SprintScan 35 scanner and images were manipulated using AdobePhotoshop.

#### 4.7. DNA sequencing and sequence analysis

Clones were sequenced from both the 5' and 3' ends using the M13 reverse and M13 universal primers (Pharmacia) in conjunction with a Big Dye terminator kit and a 377 automated DNA Sequencer (Applied Biosystems). Sequences were analysed using the MacVector 5.1 package (Oxford Molecular). Nucleic acid and protein sequence similarity searches were performed using the gapped BLAST algorithms (Altschul et al., 1997) accessed via the internet at <http://www.ncbi.nlm.nih.gov/BLAST/>. EST sequence data generated during this study has been submitted to the Genbank EST database dbEST.

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