Functional Analysis Of Receptor Tyrosine Kinases Sek1 And Elk In Neural Crest In The Xenopus Embryo.

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For My Family.
Abstract

Segmentation underlies patterning of the hindbrain and of the branchial neural crest which migrates from the hindbrain to form skeletal components in the visceral arches. Branchial neural crest cells migrate in three streams to specific visceral arches, and since their anteroposterior identity is to some extent prepatterned, it is important that they migrate to the correct arch. Based on their spatially restricted expression several Eph-related receptor tyrosine kinases (RTKs) may be involved in patterning of the branchial neural crest. Recent work has identified a family of polypeptide ligands for these RTKs.

I have analysed the expression and function of two Eph-related receptors, Sekl and Elk which are expressed in a subset of neural crest. Sekl mRNA is first detected in branchial neural crest adjacent to rhombomere 5 and persists as these cells migrate into the third arch. Prior to migration there is also a ventral domain of expression in the mesoderm which may be the presumptive future migration pathway of this neural crest. Elk mRNA is first detected throughout the presumptive branchial neural crest, and continues in crest cells migrating into the third and fourth arches. In addition there is ventral mesodermal Elk expression in the third and fourth arches. These expression patterns suggest that Sekl and Elk may mediate cell interactions during branchial neural crest migration.

Previous studies have suggested a role of Sekl in restricting cell movement between rhombomeres. I have taken a dominant negative approach and expressed truncated Sekl and Elk receptor in Xenopus embryos to investigate their roles in neural crest. Analysis of the resulting embryos with a variety of neural crest markers showed abnormal migration of third arch neural crest cells into adjacent territories. A ligand for Sekl and Elk, Htk-L, was found to be expressed in the second arch neural crest, which is complementary to the expression of Sekl and Elk. Overexpression of Htk-L lead to aberrant migration of third arch neural crest into the adjacent territories. These results together with the expression data indicate that the receptors and ligands may mediate a cell repulsion that restricts neural crest cell migration to the correct destination. Since recent work has implicated Eph RTKs and their ligands in axon guidance, this indicates that common mechanisms may be used for regulating the migration of neural crest and axons.
Acknowledgements

I would like to thank my external supervisor Dr David Wilkinson for his support, enthusiasm and interesting discussions throughout my PhD.

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## Abbreviations

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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>A-P</td>
<td>anteroposterior</td>
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<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
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<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>CTP</td>
<td>cytidine-5’-triphosphate</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>DiI</td>
<td>1,1,dioctadecyl3,3,3,3teramethylindocarbocyaninperchlorate</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dpc.</td>
<td>days post coitum</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EDTA</td>
<td>diaminooethanetetraacetic acid</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<td>GTP</td>
<td>guanosine-5’-triphosphate</td>
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<td>HH</td>
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<td>PBTw</td>
<td>phosphate buffered saline/Tween 20</td>
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ρg  picogram
poly A  polyadenylic acid
pre-r  presumptive rhombomere
r  rhombomere
ps  parasegment
rRNA  ribosomal RNA
RNA  ribonucleic acid
RNase  ribonuclease
rpm  revolutions per minute
RT  room temperature
RTK  receptor tyrosine kinase
SDS  sodium dodecyl sulphate
sec  second(s)
st.  stage
TAE  Tris-acetate-EDTA buffer
TBE  Tris-borate-EDTA buffer
TE  Tris-EDTA buffer
TEMED  N,N,N',N'-tetramethylethylenediamine
TGF  transforming growth factor
Tris  2-amino-2-(hydroxymethyl)-propane-1:3-diol
UV  ultraviolet
v/v  volume per volume
w/v  weight per volume
μg  microgram
μl  microlitre
μm  micromolar
CHAPTER ONE.

1.1 Introduction.

During embryonic development a single cell, the fertilised egg, gives rise to many cell types in highly specific, functional arrangements in tissues and organs. This pattern emerges progressively during early development, and involves a number of interlinked processes, including: cell proliferation and death; cell differentiation to generate many distinct cell types; pattern formation in which different cell types are organised in specific spatial arrangements; and morphogenesis in which the physical form of tissues arises.

During early vertebrate development a series of processes establishes the major tissues and their organisation to form the body plan. The first morphogenetic event is the process of gastrulation in which mesodermal cells are induced to form, migrate and initiate differentiation. This involves signals from endoderm which divert presumptive ectodermal cells to differentiate into mesoderm. Concurrent with this process, a major source of inductive signals, the organiser, together with signals from mesoderm, induce dorsal ectoderm to form the neural plate, seen morphologically as a thickened epithelium. This neural plate subsequently folds so that the lateral edges meet and fuse to form the dorsal wall of the neural tube, the progenitor of the central nervous system (CNS). Planar signals and vertical signals are implicated in patterning the anteroposterior axis of the neural plate.

Neural crest cells form at the interface between the dorsolateral neural plate and the presumptive epidermis, through inductive interactions between these tissues (Selleck and Bronner-Fraser, 1995). Neural crest cells then migrate along characteristic pathways from the dorsal neuroepithelium to form a variety of different derivatives (Bronner-Fraser and Fraser, 1988) (Le Douarin, 1982). In the trunk, neural crest cells give rise to sensory and sympathetic ganglion cells, components of the enteric nervous system and also to pigment cells and adrenomedullary cells. In the head, neural crest cells give rise to similar cell types as trunk neural crest but also additionally give rise to connective tissue, cartilage and bone.

An important aspect of animal development is patterning along the anteroposterior (A-P) axis which generates many of the specialised body parts that underlie the functional complexity of the adult organism. It is now well established that this involves conserved mechanisms of "positional information" that regulate the organisation of tissues according to position along the A-P axis (Hunt and Krumlauf, 1992). For example, as the neural plate is formed it is already morphologically different along the A-P axis presaging the formation of the different regions of the brain and spinal cord. Another widespread mechanism is
segmentation, in which a tissue is subdivided into a series of building blocks (segments) each of which later forms a more or less similar set of differentiated derivatives depending on its location along the body axis. These processes of A-P patterning and segmentation are understood in most detail in the fruit-fly, *Drosophila melanogaster*, in which like other arthropods, the segmented patterns is obvious in the adult as well as in the embryo.

1.2 *Drosophila* Segmentation.

Segmentation is visible in the *Drosophila* embryo both at a morphological and a molecular level. Initially the *Drosophila* embryo develops as a syncytium in which nuclear division is not accompanied by cytokinesis (reviewed in (St Johnston and Nusslein-Volhardt, 1992)). The nuclei of the syncytium divide several times until there are approximately 128 nuclei, at which point the nuclei migrate to the periphery of the egg to form the syncytial blastoderm and cellularisation begins soon afterwards. After cellularisation is complete, gastrulation begins with the invagination of the presumptive mesoderm through the ventral furrow. The first morphological signs of segmentation in the *Drosophila* embryo appear as a series of thickenings in the mesoderm followed by grooves appearing in the ectoderm (Turner and Mahowald, 1977). The grooves divide the embryo into segmental units referred to as parasegments (Martinez-Arias and Lawrence, 1985). The *Drosophila* embryo has 14 parasegments with each one functioning as a compartment which contains a precise set of lineage restricted cells. Proper segments form later during the development of the larva, each consisting of the posterior two thirds of one parasegment and the anterior third of the adjacent parasegment.

Segmentation of the *Drosophila* embryo is generated through a hierarchy of gene expression that acts to divide the embryo into progressively smaller and more specialised regions (reviewed by (St Johnston and Nusslein-Volhardt, 1992)). Extensive genetic screens have shown that the patterning of the embryo is established in the syncytial blastoderm by the localised transcription of zygotic patterning genes. These genes are controlled by maternally inherited gene products that are deposited in the egg at oogenesis and specify the anteroposterior and dorsoventral axes. The specification of the anteroposterior axis of the embryo is controlled by the anterior, posterior and terminal systems which act independently or additively to define discrete parts of the pattern. The anterior system determines the segmented region of the head and thorax, the posterior system determines the segmented abdominal region and the terminal system determines the formation of non-segmental structures at the anterior and posterior tips (Nusslein-Volhardt, 1991).
The maternal effect gene bicoid has been shown to be required to determine the pattern and polarity of anterior structures (Fronhoffer and Nusslein-Volhard, 1986). Females lacking the bicoid gene produce embryos which fail to develop a head and thorax (Fronhoffer and Nusslein-Volhard, 1986). bicoid mRNA is concentrated at the anterior end of the egg and is translated after fertilisation to produce an anterior to posterior concentration gradient of the protein that extends over the anterior two thirds of the syncytial blastoderm (Berleth et al., 1988) (Driever and Nusslein-Volhard, 1988b). This gradient of bicoid protein appears to determine anterior positional values in a concentration dependent manner (Driever and Nusslein-Volhard, 1988a) (Struhl et al., 1989). bicoid encodes a sequence-specific transcription factor that contains a homeodomain and directly regulates zygotic genes (Berleth et al., 1988). The anterior fate map depends on the shape of the bicoid protein gradient indicating that different thresholds of the protein elicit different responses through the specification of distinct anterior positional values. The way in which this is achieved has been demonstrated through the analysis of the gap gene hunchback, which is required for the development of the thorax and the head (Lehmann and Nusslein-Volhard, 1987). hunchback expression is completely dependent on bicoid as can be seen by the absence of its expression in bicoid/ mutants. hunchback is only transcribed above a certain threshold of bicoid concentration and if the bicoid gene dosage is increased then hunchback expression extends posteriorly (Schroder et al., 1988) (Struhl et al., 1989) (Tautz, 1988). Analysis of the hunchback regulatory sequences has identified six bicoid binding sites, which can be sub-divided into three weak and three strong binding sites (Driever et al., 1989) (Driever et al., 1989). The bicoid protein is seen in a broad concentration gradient and the existence of high and low affinity bicoid binding sites provides a means by which the bicoid protein gradient may be converted into discrete domains of zygotic gene expression (Driever et al., 1989).

bicoid, together with other maternal effect genes, establishes overlapping patterns of gap gene expression such as hunchback, giant, and kruppel, which act as transcriptional activators and repressors to regulate pair rule genes. This is exemplified by the regulation of the pair rule gene even-skipped, which is expressed in seven transverse stripes in the embryo (Frasch et al., 1987). even-skipped encodes a homeodomain protein which is required for segmentation. Mutant embryos lacking even-skipped protein fail to form even numbered segments (MacDonald et al., 1986). Distinct regulatory elements have been identified in the promoter of even-skipped which direct the expression of individual stripes (Goto et al., 1989). Genetic studies have indicated that regulation of the transcription of the even-skipped gene in stripe 2, positioned between the posterior head and anterior thorax of the embryo, is controlled by bicoid and three gap genes hunchback, giant, and kruppel (Frasch and Levine, 1987) (Goto et al., 1989). The product of the hunchback gene is a zinc
finger containing factor, which together with bicoid activates the expression of the pair rule
gene even-skipped in a broad anterior domain (Stanojevic et al., 1991) (Small et al., 1992)
(Ip et al., 1992). The expression of even-skipped, however, is restricted to a tight band by
the activity of two gap genes, giant and kruppel. High concentrations of bicoid activate the
expression of giant, which acts as a transcriptional repressor of even-skipped expression,
defining its anterior edge. Likewise, expression of kruppel defines the posterior boundary
resulting in a narrow band of even-skipped expression in stripe 2 (Stanojevic et al., 1991)
(Small et al., 1992) (Ip et al., 1992). All four of these DNA binding proteins bicoid,
hunchback, giant, and kruppel have been shown to bind to multiple sites in the even-
skipped stripe 2 promoter element (Stanojevic et al., 1989) (Small et al., 1991).

Pair rule genes in turn regulate the expression of segment polarity genes, which are
responsible for organising the epidermal pattern. Wingless and hedgehog are segment
polarity genes encoding signalling molecules, whose expression flanks the boundary
between adjacent parasegments (Mohler and Vani, 1992) (Tabata et al., 1992) (Lee et al.,
1992) (Kornberg et al., 1985) (Fjose et al., 1985). After an initial stage when their
expression is regulated by pair rule genes, wingless and hedgehog expressing cells signal
to one another across the parasegment boundary in a manner which positively reinforces
the expression of one another. These rows of wingless and hedgehog expressing cells act
as signalling centres that organise the pattern of the segment through other downstream
segment polarity genes.

The homeotic selector genes specify anteroposterior (A-P) parasegment identity and are
regulated by the products of the gap and the pair rule genes. There are seven homeotic
genes expressed in the Drosophila embryo that are organised in two clusters, four in the
Antennapedia complex, proboscipedia, Deformed, Sex combs reduced, Antennapedia, and
The products of the homeotic genes are homeodomain containing transcription factors
which can bind to and regulate other genes involved in conferring segment structure and
function in the Drosophila embryo (Scott et al., 1989) (Lewis, 1978) (Kaufman et al.,
1990). Homeotic gene expression boundaries exactly coincide with the morphological
limits of the parasegments and each parasegment is characterised by the combinatorial
expression of one or more homeotic genes. Mutations in homeotic genes cause
transformation of the A-P identity of a parasegment(s). For example, in loss of function
mutants of Ultrabithorax, parasegments 5 and 6 are transformed into two parasegment 4’s
(Struhl, 1983).
In summary, the patterning of the Drosophila embryo is achieved by a cascade of molecular interactions through which the maternal effect genes regulate a hierarchy of zygotic genes leading to its morphological segmentation and A-P specification.

1.3 Vertebrate Segmentation.

As in Drosophila, segmentation is also an important feature of vertebrate development. During vertebrate embryogenesis segmentation is evident in the formation of somites, vertebrae, spinal and cranial nerves and ganglia, the developing hindbrain and forebrain, and branchial and trunk neural crest.

1.3.1 Somitic mesoderm segmentation.

The most conspicuous segmental feature of the vertebrate embryo are the somites, which form in a rostrocaudal sequence one pair at a time. Somites are initially epithelial spheres of tightly apposed cells which bud off from the rostral end of the mesenchymal loose paraxial mesoderm (reviewed by (Christ and Ordahl, 1995)). The cells in a newly formed somite are radially arranged so that the apical surfaces of the cells face in towards a central lumen which contains mesenchyme-like cells. The luminal cells and the ventromedial cells of the somite form the sclerotome which along with the notochord gives rise to the vertebrae. The dorsolateral cells form the dermamyotome which further subdivides so that the cells immediately underneath the ectoderm, the dermatoine, give rise to the dermis of the trunk as well as muscles of the limb and other non-axial muscles (Christ et al., 1983) (Ordahl and Le Douarin, 1992). The remainder of the dermamyotome, termed the myotome, gives rise to axial skeletal muscle (Christ and Wilting, 1992).

The subdivision of the sclerotome into rostral and caudal halves patterns motor axon outgrowth and trunk neural crest migration from the spinal cord (Keynes and Stern, 1984) (Rickmann et al., 1985) (Bronner-Fraser, 1986a). Trunk neural crest migration ventrally is segmented, with cells moving through the rostral but not the caudal portion of the somites. These cells later give rise to sensory and sympathetic ganglia (Rickmann et al., 1985). Experiments have been performed to establish whether the segmental migration of trunk neural crest occurs because of intrinsic patterning within the neural tube or because of cues inherent to the somitic mesoderm and have involved the rostrocaudal rotation of the paraxial
mesoderm which give rise to the somites (Bronner-Fraser and Stern, 1991). After the rotation of the mesenchyme, neural crest cells only migrate through the caudal half of the somite suggesting that segmental migration of the trunk neural crest is due to cues inherent to the somitic mesoderm and that these cues are specified prior to the overt differentiation of the somites.

In the chick, motor axons grow from the neural tube laterally through the sclerotome towards the myotome, exclusively through the rostral half of each somite (Keynes and Stern, 1984). In order to determine whether this segmental outgrowth is an intrinsic property of the neural tube or due to cues in the somite, neural tube and somite rotation experiments have been performed. These experiments demonstrate that the caudal half of the somite is inhibitory to motor axon outgrowth (Keynes and Stern, 1984).

1.3.2 Hindbrain segmentation.

The vertebrate hindbrain is divided into seven segments termed rhombomeres which can be seen as transient bulges in the neuroepithelium. Boundaries form between the rhombomeres in a fixed order (Vaage, 1969). Segmentation of the hindbrain is important as it provides a means of patterning neurones and neural crest which arise from the rhombomeres. In the chick the branchial motor neurones arise from two adjacent rhombomeres: rhombomeres 2 and 3 give rise to the Vth nerve, rhombomeres 4 and 5 to the VIIth nerve and rhombomeres 6 and 7 to the IXth nerve (Figure 1.1). Axons of these motor neurones exit from the even numbered rhombomeres and enter the anterior part of the first, second and third branchial arches respectively. The formation of motor neurones from two rhombomeres gives rise to the idea of two segment periodicity (Lumsden and Keynes, 1989) suggesting parallels with pattern formation of the parasegments of the *Drosophila* (Garcia-Bellido et al., 1973).

Differentiation of other neurones also proceeds in a rhombomere specific fashion (Lumsden and Keynes, 1989) suggesting that each rhombomere may have evolved by the duplication of a primitive segmental unit (Metcalf et al., 1986). Studies in the zebrafish have suggested that the serially repeated pattern of reticulospinal neurones in the hindbrain reflect its segmental organisation (Metcalf et al., 1986) (Kimmel et al., 1985). The reticulospinal neurones of the hindbrain are found in seven bilateral clusters which are periodically spaced. Each cluster contains several different types of reticulospinal neurones with morphologically similar cells being found at rhombomeric intervals along the rostrocaudal axis (Metcalf et al., 1986). It has been argued that each rhombomere has a unique set of reticulospinal neurones through changes in the projections of individual axons as well as the addition or deletion of certain neurones (Metcalf et al., 1986). Similarly in the chick, it
Figure 1.1: Axonal pathways in the chick hindbrain.

A diagrammatic representation showing the positions of cranial nerve axons and cell bodies in the chick embryonic hindbrain. (Adapted from (Lumsden and Keynes, 1989)).

m2, midbrain; isth, isthmus; r1-8, rhombomeres 1-8; Nerves: III, oculomotor; IV, trochlear; V, trigeminal; VI, abducens; VII, facial; VIII, vestibuloacoustic; IX, glossopharyngeal; X, vagus; XI, accessory;
Chick

Nerve roots:
Motor
Sensory
has been shown that each rhombomere contains the same set of basic neuronal types but some contain more of one particular neuronal type than another (Clarke and Lumsden, 1993). This indicates that the initial neuronal development of each rhombomere is conserved and the differences arise from subtle variations of this repeated pattern.

1.3.3 Cell lineage restriction.

Parasegments in *Drosophila* are compartments of lineage restricted cells that do not intermingle with their neighbours, which allows each parasegment to maintain a distinct identity. Similarly, chick rhombomeres have also been demonstrated to be compartments of cell lineage restriction (Fraser *et al.*, 1990). This has been demonstrated by labelling individual cells prior to rhombomere boundary formation and identifying the progeny of these cells at later stages. In most cases the descendents of the marked cell were found in more than one rhombomere. In contrast, progeny of cells labelled after the morphological formation of the rhombomere boundaries, are restricted to a single rhombomere. If the initially marked cell was located next to a boundary its progeny were seen to abut the rhombomere boundary but not cross it. These results suggest that cells are able to move across presumptive rhombomere boundaries before overt boundary formation but become restricted to a single rhombomere after the morphological formation of the boundaries. It has, however, been demonstrated that the cell lineage restriction of rhombomeres is violated by approximately 5% of cells, indicating that they are not absolute (Birgbauer and Fraser, 1994).

1.3.4 Segmental migration of cranial neural crest.

Cranial neural crest migrates in a segmental manner and has been shown to play a central role in the patterning of the vertebrate head. Much of the cranial neural crest cells arising from the hindbrain and midbrain migrates to fill the branchial arches. Unlike trunk neural crest, cranial neural crest gives rise to skeletal as well as neurogenic derivatives. In both the chick and the mouse it has been demonstrated that cranial neural crest cells populate the arches in a ventral to dorsal order (Kontges and Lumsden, 1996)(Serbedzija *et al.*, 1992). In the mouse, neural crest cells delaminate away from the neuroepithelium before closure of the neural tube, whereas in the chick they remain in the dorsal neural tube and commence their migration shortly after its closure (Tosney, 1982). Studies in the chick show that there is rostrocaudal wave of neural crest migration with neural crest cells emanating from the hindbrain in a segmented manner. Cranial neural crest cells emerge from rhombomere 2
and more anterior regions to populate the mandibular (first) arch and the trigeminal ganglion. Neural crest from rhombomere 4 fills the hyoid (second) arch and geniculate and vestibulocochlear ganglia, and crest from rhombomere 6 and more posterior regions to fill the third and fourth branchial arches and the superior and petrosal ganglia of the glossopharyngeal nerve (Lumsden et al., 1991). In order to ascertain the fate of these cells in the arches, chick-quail grafts of rhombomeres have been carried out. This showed that the segmental migration of the cranial neural crest is maintained throughout craniofacial ontology with skeletal elements and their sites of muscle attachment originating from the same stream of neural crest (Kontges and Lumsden, 1996).

Grafting experiments performed in the chick have shown that transplanted cranial neural crest develop into structures that reflect their origin and not their eventual location (Noden, 1983) (Noden, 1988). When chick embryos have donor rostral hindbrain neural crest cells transplanted caudally, some embryos develop a duplicate set of first arch structures in the second branchial arch and in some cases extra first arch-type muscles are also formed (Noden, 1983) (Noden, 1988). This suggests that the rostral hindbrain neural crest cells are patterned before migration.

1.4 Molecular mechanisms of hindbrain segmentation and segmental identity.

1.4.1 Gene expression in the hindbrain.

Certain genes have been identified whose expression precedes the morphological segmentation of the hindbrain suggesting that these gene products may underlie patterning. These genes include transcription factors such as the Hox gene family, the zinc finger gene Krox20, and the leucine zipper gene Kreisler. Hox genes are implicated in segment identity whereas Krox20 and Kreisler are implicated in segmentation of the hindbrain.

1.4.2 Hox genes.

Hox genes are the vertebrate homologues of the Drosophila HOM-C genes and encode DNA binding proteins which have been conserved throughout animal evolution. In Drosophila there are two complexes, the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C), whereas in the red flour beetle Tribolium castaneum and the nematode Caenorhabditis elegans, these complexes are contiguous. It is therefore thought these are
derived from an ancestral cluster that arose from the serial duplication of a HOM gene (Beeman et al., 1989) (Kenyon and Wang, 1991) (Salser and Kenyon, 1994).

Vertebrate homologues of Drosophila HOM-C genes are thought to have arisen through duplication and divergence of a common ancestral complex (Graham et al., 1989) (Duboule and Dolle, 1989) (Kessel and Gruss, 1990) (Boncinelli et al., 1991). They are arranged in four separate clusters termed Hoxa, Hoxb, Hoxc and Hoxd (Scott, 1992). At equivalent positions within each cluster there are highly related, paralogous genes which tend to share similar expression domains with the exception of the group 1 Hox genes (Hunt et al., 1991a) (Figure 1.2). Hox genes are expressed from the posterior part of the CNS to anterior limits of expression correlating with their position in the cluster: the 5' genes having the most posterior limits of expression with each successive 3' gene having a more anterior limit of expression. This relationship is known as “colinearity” and is a conserved feature of Hox/HOM gene expression. Paralogous genes share the same anterior limits of expression which reflect their position in the cluster. For example, the paralogous group 3 has an expression domain up to the rhombomere 4/5 boundary and the more 5' set, paralogous group 4, are expressed up to the rhombomere 6/7 boundary. The exception to this rule are the group 1 and 2 genes. Hoxa1 is expressed early during hindbrain development up to the rhombomere 3/4 boundary, then downregulated. Hoxb1 is also expressed initially up to the rhombomere 3/4 boundary, then is restricted and upregulated in rhombomere 4 thus, it does not follow colinearity (Hunt et al., 1991a) (Murphy et al., 1989) (Murphy and Hill, 1991) (Frohman et al., 1990).

Patterning information is believed to be transferred from the rhombomeres to the branchial arches through the neural crest (Hunt et al., 1991d) (Hunt et al., 1991b) which migrate from specific rhombomeres to specific destinations (Figure 1.3). A possible mechanism by which the cranial neural crest cells are prespecified is by the overlapping expressions of the Hox genes resulting in what has been termed a Hox code (Kessel and Gruss, 1990) (Kessel and Gruss, 1991) (Hunt et al., 1991b). The migrating neural crest cells maintain their Hox code as they migrate (Hunt et al., 1991c) and the branchial arch ectoderm subsequently expresses an identical pattern of Hox gene expression to that of the rhombomeres (Hunt et al., 1991d) (Hunt et al., 1991b). Therefore, it would seem important that that the correct population of neural crest cells migrate to and populate the correct branchial arch.

The function of the Hox genes during embryogenesis has been investigated by making loss of function or gain of function mouse mutants. Generally, loss of function mutants show an anterior homeotic transformation whereas gain of function mutants show a posterior homeotic transformation (reviewed by (Krumlauf, 1994)). An example of a homeotic
Figure 1.2: Summary of *Hox*, *Krox20* and *Kreisler* gene expression in the hindbrain.

Representation of the segmentally restricted expression pattern of *Hox*, *Krox20* and *Kreisler* genes in the hindbrain of a 9.5 day mouse embryo. The shading depicts the relative levels of transcripts. The *Hox* paralogues have similar anterior boundaries of expression, with the exception of *Hoxa2* and *Hoxb2*, and show a two segment periodicity.

rl-8, rhombomeres.
Figure 1.3: The branchial *Hox* code.

Expression patterns of the paralogous *Hox* genes in the rhombomeres, the branchial neural crest and the branchial arches after neural crest migration is complete. The coloured arrows indicate the migration of the neural crest expressing a combinatorial *Hox* code into specific branchial arches. The pattern of *Hox* code expression is transferred to the cranial ganglia and branchial arches. The short red arrow indicates that *Hoxbl* expression is confined to the neurogenic crest. The open arrows indicate that first arch crest do not express *Hox* genes. The relationship between the paralogous genes and those of *Drosophila* are indicated below the *Hox* clusters (Adapted from (Hunt et al., 1991)).
transformation is seen in loss of function mutations of the \textit{Hoxa2} gene. \textit{Hoxa2} is normally expressed in second arch but not first arch neural crest (Hunt \textit{et al.}, 1991a). Mice homozygous for mutated copies of \textit{Hoxa2} have structures of the second branchial arch transformed into mirror image duplications of those in the first arch (Gendron-Maguire \textit{et al.}, 1993) (Rijli \textit{et al.}, 1993) indicating that a homeotic transformation of the neural crest-derived structures of the head has occurred. It is interesting to note that only proximal and not distal structures are duplicated in the light of fate mapping experiments which have suggested that proximal structures of the mandibular arch skeleton are formed by neural crest emanating from rhombomeres 1 and 2 and that midbrain neural crest gives rise to the distal skeletal elements of this arch (Kontges and Lumsden, 1996). Loss of function of the \textit{Hoxa2} gene does not seem to affect the patterning of the hindbrain as seen by the expression patterns of rhombomere-specific genes such as \textit{Krox20}.

1.4.3 \textit{Krox20}.

\textit{Krox20} encodes a transcription factor with three zinc fingers capable of binding to specific sequences of DNA. It was first identified as an immediate early response gene in serum stimulated fibroblasts (Chavrier \textit{et al.}, 1988). Homologues with conserved expression in rhombomeres 3 and 5 and the third arch neural crest have been identified in \textit{Xenopus} (Bradley \textit{et al.}, 1992), chick (Nieto \textit{et al.}, 1991), mouse (Wilkinson \textit{et al.}, 1989) and zebrafish (Oxtoby and Jowett, 1993) suggesting a conserved role. \textit{Krox20} expression is initiated before morphological segmentation of the hindbrain in two fuzzy stripes coinciding with presumptive rhombomeres 3 and 5. These stripes of expression become sharper as the rhombomere boundaries are formed (Irving \textit{et al.}, 1996). Disruption of the \textit{Krox20} gene leads to a loss of rhombomeres 3 and 5 indicating that \textit{Krox20} is vital for segmentation of the hindbrain (Swiatek and Gridley, 1993) (Schneider-Maunoury \textit{et al.}, 1993). \textit{Krox20} expression is also detected in the third arch neural crest which migrates posterior to the otic vesicle. Homozygous embryos which have a LacZ reporter gene inserted so as to disrupt the \textit{Krox20} gene, show LacZ expression in neural crest cells both rostral and caudal to the otic vesicle (Schneider-Maunoury \textit{et al.}, 1993).

Studies in mice have also shown that \textit{Krox20} plays a direct role in the regulation of \textit{Hox} gene expression during hindbrain segmentation (Sham \textit{et al.}, 1993) (Nonchev \textit{et al.}, 1996) (Schneider-Maunoury \textit{et al.}, 1993) (Swiatek and Gridley, 1993). In particular, analysis of the \textit{Hoxb2} gene has identified an enhancer that directs expression in rhombomere 3 and 5 and contains two \textit{Krox20} binding sites. These have been shown by mutational analysis and the effects of ectopic \textit{Krox20} expression to be necessary for \textit{Hoxb2} expression in rhombomeres 3 and 5 (Sham \textit{et al.}, 1993).
1.4.4 *Kreisler*.

*Kreisler* was initially described as an X-ray induced recessive mutation in mice in which the homozygous mutant adults suffer from deafness and abnormal circling behaviour, resulting from a deformation of the inner ears (Frohman *et al.*, 1993) (McKay *et al.*, 1994). This phenotype can be traced to abnormalities in the developing hindbrain, as seen in the abnormal positioning of the otic vesicle and a loss of hindbrain segmentation posterior to the rhombomere 3/4 boundary (Deol, 1964).

The *Kreisler* gene encodes a leucine zipper transcription factor of the MAF superfamily which is expressed during hindbrain segmentation (Cordes and Barsh, 1994). The rostral limit of *Kreisler* expression at the presumptive rhombomere 4/5 boundary and the caudal limit of expression decreases in the area of rhombomere 6.

Molecular analysis of homozygous *Kreisler* mutant mice indicate that there are altered patterns of gene expression in the hindbrain. For example *Krox20* is expressed in rhombomere 3 but is absent in rhombomere 5 (McKay *et al.*, 1994) (Frohman *et al.*, 1993). The expression domain of *Hoxb1* which is normally expressed within the boundaries of rhombomere 4 is expanded to cover a domain slightly broader than the width of one rhombomere. The anterior limit of expression of *Hoxd4* no longer abuts with the rhombomere 6/7 boundary but coincides with the posterior limit of *Hoxb1* expression. These patterns of disrupted gene expression suggest that there is a loss of rhombomeres 5 and 6 in homozygous *Kreisler* mutants (McKay *et al.*, 1994).

The zebrafish homologue of *Kreisler, Valentino*, has been identified in a genetic screen to identify genes involved in brain regionalisation (Moens *et al.*, 1996). Mutations in *Valentino* disrupt the expression of *Krox20* in rhombomere 5 and mutant embryos display an absence of visible rhombomere boundaries and boundary specific gene expression posterior to the rhombomere 3/4 boundary. The hindbrain of the mutant embryos is also shortened by the length of one rhombomere. It has, therefore, been suggested that the region between rhombomeres 4 and 7 fails to expand and become subdivided to form rhombomeres 5 and 6 but remains as a distinct but developmentally earlier “protosegment”. Phenotypic similarities between *Kreisler* and *Valentino* mutants indicate that the function of these genes may be conserved between the species. The differences in interpretation of the phenotypes between the two species could be explained due to a failure to analyse earlier phenotypes in *Kreisler* mutants. Nonetheless, the phenotypes of both *Kreisler* and *Valentino* mutants indicates that this gene is important for the segmentation of the hindbrain.
1.5 Mechanisms of restricting cell movement.

The restriction of cells to individual compartments allows each compartment to maintain a unique identity. This can be observed in the hindbrain during the formation of the rhombomeres and an important question is how this is established.

1.5.1 Compartments and boundaries.

The restriction of cell movement between rhombomeres could be due to intrinsic differences between adjacent segments, or the formation of a barrier at rhombomere boundaries. In support of the latter possibility, rhombomere boundaries contain cells which have distinct characteristics to cells in the centre of rhombomeres (Heyman et al., 1995). These boundary cells have different rates of cell division and unlike most cells of the neural epithelium and do not undergo interkinetic nuclear migration during cell division (Guthrie et al., 1991), in which the nuclei of dividing cells migrate basally as the cells go through S-phase in the cell cycle and subsequently return to the apical surface of the cell. They are therefore a relatively static population of cells. The extracellular space is increased between boundary cells with higher levels of chondroitin sulphate proteoglycan in the extracellular spaces (Heyman et al., 1995). The rhombomere boundary cells also show differences from the rhombomere cells themselves in patterns of gene expression. There is an increase in levels of the intermediate filament protein, vimentin, at the rhombomere boundaries (Heyman et al., 1995) along with increased levels of NgCAM (Lumsden and Keynes, 1989) and peanut lectin (Layer and Alber, 1990). This pattern of gene expression may indicate a radial glial phenotype for these cells (Guthrie, 1996). In mature rhombomere boundaries there is a decrease in expression of Krox20 and Hox-b1. Correspondingly there is an increase in expression of PLZF, Fgf-3, and Pax-6 only in the boundaries (Heyman et al., 1995). The existence of these distinctive boundary cells raises the possibility that they act as a barrier to prevent intermingling between rhombomeres.

Another possibility is that differences in the cell surface properties of cells from different rhombomeres prevent cell mixing. To test this hypothesis donor rhombomeres lacking their boundaries have been transplanted so that rhombomeres from different anteroposterior levels are juxtaposed (Guthrie and Lumsden, 1991). In this way it was demonstrated that when even numbered rhombomeres are placed next to odd numbered rhombomeres the boundaries reform, but when two even or odd numbered rhombomeres are juxtaposed boundaries do not re-establish themselves. In other experiments, rhombomere fragments grafted into various positions along the anteroposterior axis in the chick have been used to
show that when even numbered rhombomeres are combined with cells from other even numbered rhombomeres, extensive cell mixing occurs (Guthrie et al., 1993). Similarly, cell mixing also occurs when odd numbered cells are placed in odd numbered rhombomeres, but to a lesser degree. When even numbered tissue is placed in an odd numbered rhombomere, however, the transplanted cells do not intermingle with the cells of the recipient rhombomere. These phenomena suggest that there are differences in cell surface and/or adhesion properties in neighbouring rhombomeres that serves to restrict cell movement and points to a two segment periodicity model in hindbrain segmentation.

1.5.2 Segmentation of neural crest.

In the chick and the mouse, streams of cranial neural crest migrating to the first, second, and third branchial arches are each separated by neural crest free zones that lie opposite to rhombomeres 3 and 5. Experiments in the chick have demonstrated that there are increased levels of cell death in rhombomeres 3 and 5 and it has been suggested that this may account for the crest free zones (Lumsden et al., 1991) (Jeffs et al., 1992). Rhombomere explant experiments have shown that the increased levels of cell death in rhombomeres 3 and 5 are caused by the presence of the adjacent even numbered rhombomeres since rhombomeres 3 and 5 produce migrating neural crest cells when explanted alone (Graham et al., 1993). It has also been demonstrated that even numbered rhombomeres are responsible for the maintenance of expression of msx2 in rhombomeres 3 and 5. msx2 expression has been shown to correlate with increased levels of apoptosis (Graham et al., 1993). Moreover, it has been shown that BMP4 expression in rhombomeres 3 and 5 is also dependent on the presence of even numbered rhombomeres. Addition of recombinant BMP4 protein to isolated explant cultures of either rhombomere 3 or 5 upregulate the expression of msx2 and suggest that the levels of apoptosis are also increased (Graham et al., 1994). These experiments suggest that rhombomeres 3 and 5 do not produce neural crest cells because they are eliminated by apoptosis through the action of msx2 through BMP4 (Lumsden et al., 1991).

Lineage tracer injections into individual rhombomeres, however, have revealed that rhombomeres 3 and 5 do produce a small number of neural crest cells (Sechrist et al., 1993) which deviate either rostrally or caudally to join the streams of migrating crest emanating from the adjacent even numbered rhombomeres (Birgbauer et al., 1995). Analysis of the timing of cell death in rhombomeres 3 and 5 have shown that it occurs after the neural crest cells have migrated and is thus unlikely to account wholly for the neural
crest free zones opposite rhombomeres 3 and 5 (Sechrist et al., 1993). It has been shown that rhombomere 3 ceases to produce neural crest cells earlier than other rhombomeres (Sechrist et al., 1993) and that the neural crest cells also preferentially exit near the rhombomere 3/4 border when rhombomere 3 is transplanted to the position of rhombomere 4 indicating either an attractive exit point or inhibitory signals in rhombomere 3 (Sechrist et al., 1994). This may account for the crest free zones opposite rhombomere 3. When rhombomere 4 is transplanted to the position of rhombomere 3 it has been shown that neural crest cells enter the mesenchyme adjacent to rhombomere 3 (Sechrist et al., 1994) which is normally devoid of neural crest cells. This indicates that although the mesenchyme adjacent to rhombomere 3 is normally inhibitory to neural crest cells from rhombomere 3 it is not inhibitory to neural crest cells emerging from rhombomere 4. In experiments where otic placodes are transplanted neural crest cells are observed migrating towards the ectopic otic vesicle, suggesting the presence of a chemoattractant (Sechrist et al., 1994). Ablation or partial ablation of the otic vesicle alters the pattern of neural crest cell migration such that labelled rhombomere 4 neural crest cells contribute to the third branchial arch as well as the second branchial arch (Sechrist et al., 1994). Together, these suggest that in addition to the presence of the otic vesicle being a physical barrier that blocks neural crest cell migration opposite rhombomere 5 and rhombomere 6, it may also have an active role in guidance (Sechrist et al., 1994).

1.6 Adhesion molecules involved in neural crest migration.

A mechanism by which migrating neural crest cells could be segmented into streams is by the selective expression of adhesion molecules. In the chick, trunk neural crest cells arise from the neural tube adjacent to somites 8 to 28, and migrate along either of two routes, the dorsal pathway or the ventral pathway. In the dorsal pathway neural crest cells migrate uniformly beneath the ectoderm and eventually give rise to pigment cells. Neural crest migration along the ventral pathway is segmented with the neural crest cells migrating through the rostral and not the caudal portion of the somites eventually giving rise to sensory and sympathetic ganglia (Rickmann et al., 1985). Manipulations of somite halves in the chick have demonstrated that placing rostral halves of somites, which are permissive for neural crest migration, adjacent to one another result in abnormally large ganglia (Kalcheim and Teillet, 1989). When caudal somite halves, which are inhibitory for neural crest migration, are placed together, the resulting ganglia are smaller than normal and are located dorsally. These experiments demonstrate that the size and morphology of these ganglia is due to the somite imposed segmental migration of the neural crest. Experiments to establish whether this segmental migration of trunk neural crest occurs because of intrinsic patterning within the neural tube or because of cues inherent to the somitic
mesoderm have been performed (Bronner-Fraser and Stern, 1991). Rostrocaudal rotation of segmental plate mesenchyme gives rise to somites with reversed polarity and results in neural crest cells migrating only through the caudal half of the somite. This suggests that segmental migration of the neural crest is imposed by the somitic mesoderm and that either the somite contains an inhibitory factor or the rostral somite contains an attractive factor which functions to mediate migration of the trunk crest along the ventral pathway. The presence of an inhibitory molecule in the segmental plate before the initiation of trunk neural crest migration has also been suggested as neural crest cells do not migrate through unsegmented paraxial mesoderm.

Time lapse studies performed on explants of the trunk region of the chick embryo allow the behaviour of prelabelled trunk neural crest cells to be examined. It has been found that labelled individual neural crest cells migrate in an unpredictable manner but maintain the normal segmental migration pattern through the somites (Krull et al., 1995). However, in similar experiments when lectin peanut agglutinin (PNA) is ectopically applied, the trunk neural crest cells migrate through the whole somite (Krull et al., 1995). PNA-binding molecules have been shown to be strictly localised in the caudal sclerotome (Oakley and Tosney, 1991), therefore, it has been suggested that the segmental migration of trunk neural crest cells is due to the presence of PNA binding molecules in the caudal somite which prevents neural crest cell migration (Krull et al., 1995). This suggests that segmentation of neural crest migration in the trunk can occur due to the localisation of inhibitory molecules.

Other inhibitory molecules have been identified in the chick which may regulate trunk neural crest migration in the dorsolateral path (Oakley et al., 1994). These molecules initially inhibit the migration of trunk neural crest cells. Molecular studies have shown the transient expression of PNA-binding activity and C6S-immunoreactivity in the dorsolateral pathway but the expression levels of these markers significantly decrease as the neural crest cells enter the path. Deletion of the dermamyotome allows precocious migration of trunk neural crest cells along the dorsolateral path, possibly by removing the expression of both glycoconjugates (Oakley et al., 1994).
1.7 Eph receptor tyrosine kinases.

Based on their expression patterns Eph receptor tyrosine kinases have been implicated as a potential candidates involved in cell interactions in cranial neural crest emanating from the hindbrain.

Receptor tyrosine kinases (RTKs) play critical roles in signal transduction pathways in which they relay signals such as growth factors from the extracellular environment across the plasma membrane. All RTKs possess an extracellular domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. Upon ligand binding to the extracellular domain, receptor dimerisation occurs and subsequently there is a transient activation of the intracellular tyrosine kinase domain by autophosphorylation of specific tyrosine residues (Ullrich and Schlessinger, 1990) (van der Geer et al., 1994). The phosphorylated receptors are then free to interact with the SH2 domains on intracellular signalling proteins by means of short phosphotyrosyl-containing amino acid sequences (van der Geer et al., 1994).

RTKs can be subdivided into 14 subfamilies depending on the structure of the extracellular domain. The largest of these subfamilies is the Eph family which comprises at least 13 members. All members of the Eph family are characterised by a highly conserved extracellular domain consisting of a single immunoglobulin-like domain, two fibronectin type III repeats and 20 conserved cysteine residues (Lindberg and Hunter, 1990) (Lhotak et al., 1991) (Pasquale, 1991) (Gilardi-Hebenstreit et al., 1992) (Sajjadi and Pasquale, 1993). The cysteine residues are thought to hold the receptors in a conserved conformation. The Eph receptors have a highly conserved intracellular domain but little is known about the molecules which interact with the intracellular domain of the Eph receptors.

Members of the Eph family were initially identified as orphan receptors but recently a family of ligands have been characterised. All the ligands are membrane bound and the attachment to the cell membrane has been shown to be necessary for activation of the receptor (Davis et al., 1994). This makes them unique as RTK ligands in that they are inactive in a soluble form and this suggests that the Eph receptors and their ligands may mediate cell contact dependent signalling. Artificially clustering the soluble ligands results in receptor activation, indicating that attachment to the cell surface may facilitate ligand oligomerisation (Davis et al., 1994).

The ligands can be divided into two subclasses depending on their method of attachment to the cell surface. They can be anchored to the cell membrane either by a transmembrane
domain or a glycosylphosphatidylinositol (GPI) linkage. There are three transmembrane ligands, Elk-L, Htk-L and Elk-L3 and these have strongest homology in the C-terminal portion of their cytoplasmic regions (Bergemann et al., 1995) (Gale et al., 1996a). Based on this strong conservation it has been postulated that this region may have a role (Brambilla et al., 1995).

*B61, EHK1-L, RAGS, Elf-1, and LERK4* constitute a separate class of ligands, which are attached to the cell surface via a GPI anchor attached at the C-terminal tail. These ligands share greater homology with each other than with members of the transmembrane subclass of ligands (Gale et al., 1996b) and the region of greatest homology within this subclass is the putative receptor binding region which includes four conserved cysteine residues (Kozlosky et al., 1995).

Functional binding studies have shown that the all the ligands for the Eph receptors can be divided into two subclasses (Gale et al., 1996b) (Figure 1.4) that correspond to the two structural subclasses. Transmembrane ligands, Elk-L, Htk-L and Elk-L3, have been shown to interact exclusively with four of the Eph receptors, termed the Elk subgroup, consisting of Sek-3, Sek-4, Elk and Htk (Gale et al., 1996b) (Beckmann et al., 1994) (Brambilla et al., 1995). The GPI-anchored ligands, B61, EHK1-L, RAGS, Elf-1, and LERK4, bind to a different subset of the Eph receptors known as the Eck subgroup (Gale et al., 1996b). There is a level of promiscuity within each group with various receptors able to bind to distinct ligands with different affinities (Kozlosky et al., 1995) (Cheng and Flanagan, 1994) (Gale et al., 1996b). The receptor Sek1 is the only exception, with the ability to interact with all the GPI-anchored ligands and Htk-L and Elk-L3 of the transmembrane ligands (Gale et al., 1996a) (Gale et al., 1996b).

Fusing of the extracellular portion of the receptors or ligands to the Fc domain of IgG and has been used to analyse the distribution of the different classes of receptors and ligands in whole mouse embryos. Detection of transmembrane or GPI-anchored ligands using the IgG Fc fusion proteins shows complementary expression patterns to their corresponding subclass of receptor in embryos at embryonic day 10.5 (Gale et al., 1996b) and in embryos at embryonic day 9.5 (Flenniken et al., 1996) in a variety of tissues. In addition, whole mount in situ hybridisation has also shown this complimentary in the hindbrain where receptors and ligands are expressed in alternating rhombomeres (Figure 1.5). Sek1, Sek-3 and Sek-4, are expressed at high levels in rhombomeres 3 and 5 whereas the ligands Elk-L, Elf-2, and Elk-L3 are expressed at high levels in even numbered rhombomeres.

The Eph family of receptors and their ligands have been implicated in a number of different functions including establishing a topographic map from the retina to the tectum. Expression patterns and binding studies using receptor or ligand affinity probes show that
Figure 1.4: Binding specificity subclasses of the Eph RTKs and their ligands.

The Eph RTKs and their ligands are divided into two subfamilies depending on the binding specificities. This also correlates with their structural homologies. Members of one receptor subfamily can bind to all of the transmembrane ligands whereas members of the other subfamily bind to the GPI-anchored ligands. The exception is Sek1 which can bind to the GPI-anchored ligands and two of the transmembrane ligands. Based on (Gale et al., 1996).
GPI-Anchored Ligands

Transmembrane Ligands

Receptors

Ligands
Figure 1.5: Segmental expression patterns of Eph RTKs and their ligands.

The diagram shows the segment restricted expression pattern of the Eph RTKs and their ligands in the hindbrain of a 9.5 day mouse embryo. The shading depicts the relative levels of transcripts. Note the complimentary expression patterns of the transmembrane ligands and their receptors.
there are complementary gradients of the ligands Elf-1 and RAGS in the developing tectum with Mek4 in the developing retina (Drescher et al., 1995) (Cheng et al., 1995). RAGS and Elf-1 are expressed in decreasing posterior to anterior gradients in the tectum with the highest levels in the posterior tectum, whereas Mek4 has a complementary gradient of expression in the retina with the highest concentration in the temporal retinal ganglion axons (Cheng et al., 1995). These axons project to the anterior tectum where the ligands are expressed at low levels. Nasal retinal ganglion axons, which are expressing low levels of the receptor project to the posterior tectum where there is high levels of ligand expression. Whereas, temporal retinal ganglion axons, which are expressing high levels of the receptor project to the anterior tectum where there is low levels of ligand expression. The migration of retinal axons to the tectum has been analysed in vitro using a “membrane stripe assay” (Walter et al., 1987). In this assay, retinal axons are given the choice of growing along anterior or posterior tectal membranes. Although temporal axons can grow on membranes from the posterior tectum they grow preferentially along stripes of membranes from the anterior tectum. If posterior membranes are added to growing cultures of temporal axons their growth cones are immediately paralysed and retract but if anterior membranes are added, no effect is observed (Walter et al., 1987). These in vitro assays have shown that Elf-1 is repellent for temporal and not nasal axon guidance, whereas, RAGS is repellent for both types of axons, though to different degrees (Monschau et al., 1996) (Nakamoto et al., 1996). Additional experiments involving retroviral overexpression of Elf-1 in the chick tectum leads to retinal axons avoiding ectopic patches of Elf-1 expression and their subsequent mapping to abnormally anterior positions (Nakamoto et al., 1996). Based on these data a high level of Mek4 receptor expression in the temporal retinal ganglion cells appears to cause the axons to be repelled away from high levels of expression of the ligands RAGS and Elf-1 in the tectum.

There appears to be a similar role for RAGS (AL-1) in axon fasciculation (Winslow et al., 1995). It has been observed in vitro that rat cortical neurones bundle together as they grow across astrocytes (Winslow et al., 1995) and that RAGS is expressed on astrocytes during development and a putative receptor Ehkl (Rek7) is expressed on the cortical neurones. Receptor blocking experiments have been performed where the extracellular domain of Ehkl has been fused to the constant region of the IgG domain and added to cultures of cortical neurones growing across astrocytes. The chimeric protein inhibits binding of the ligand to the receptor and prevents the growing neurones from bundling together suggesting that RAGS is required for axon bundling. Taken together with studies in the retinotectal system, it has been proposed that this is due to repulsion by the astrocytes.
The receptor Sek1 has been shown to play a role in the developing hindbrain. Sek1 was originally identified by screening a mouse cDNA library in a search for tyrosine kinases which are segmentally expressed in the hindbrain (Nieto et al., 1992) and is expressed at high levels in rhombomeres 3 and 5. Homologues of Sek1 have been identified in zebrafish (Xu et al., 1994), chick (Sajjadi and Pasquale, 1993) (Patel et al., 1996) and Xenopus (Winning and Sargent, 1994) (Xu et al., 1995). In vivo functional studies on Sek1 have been performed in zebrafish and Xenopus by a dominant negative approach (Xu et al., 1995) (Xu et al., 1996). Cleavage stage zebrafish embryos, or one cell of a two cell Xenopus embryos were injected with mRNA encoding truncated dominant negative Sek1 lacking the intracellular kinase domain. In the injected embryos display disrupted hindbrain segmentation. This is observed by Krox20 expressing cells in even numbered rhombomeres in a domain contiguous with the Krox20 expression in rhombomeres 3 and 5. Since Krox20 is normally expressed in rhombomeres 3 and 5 this demonstrates that the expression of dominant negative Sek1 results in the presence of odd numbered rhombomere cells in even numbered rhombomere territory (Xu et al., 1995). Cell lineage labelling experiments of rhombomeric cells have shown that there is little crossing of rhombomere boundaries by cells once the boundaries have formed (Birgbauer and Fraser, 1994) (Fraser et al., 1990). Therefore, the phenotypes caused by expression of dominant negative Sek1 may indicate that the cells of rhombomeres 3 and 5 are now able to cross rhombomere boundaries and enter even numbered rhombomere territory. Alternatively, these phenotypes may indicate that Sek1 is required to switch rhombomere cell identity. During hindbrain development cells are able to cross between rhombomeres prior to the formation of boundaries, but analysis using molecular markers shows that ectopic odd or even numbered rhombomeric cells are never observed in inappropriate territories. This suggests that Sek1 could be involved in regulating cell fate changes (Xu et al., 1995). The hindbrain phenotypes can be rescued by coinjecting full length Sek1 with dominant negative Sek1, indicating a need for the intracellular kinase domain in the normal function of Sek1 (Xu et al., 1995). In the zebrafish embryo the expression of dominant negative Sek1 also causes a forebrain phenotype which involves an expansion of the retina and loss of diencephalic structures (Xu et al., 1996). As with the hindbrain phenotypes this suggests a role for Sek1 in regulating cell fate change or cell movement in the forebrain.
1.8 Aims of the project.

One strategy in studying cranial neural crest migration is to identify and characterise molecules that are expressed by migrating cranial neural crest cells and/or their pathway of migration. Two members of the Eph family of receptor tyrosine kinases Sekl and Elk are expressed in Xenopus cranial neural crest. In this study the expression pattern of both Sekl and Elk during Xenopus development has been analysed and a functional study of both of these genes in cranial neural crest migration in Xenopus has been undertaken using a dominant negative approach. During these studies, the Xenopus homologue of Htk-L was isolated. As Htk-L is able to interact with both Sekl and Elk it was of particular interest when it was found to be expressed in a complementary expression pattern to that of Sekl, therefore, I have collaborated with a colleague, Dr. Vicky Robinson, to investigate the effect of overexpression of Htk-L on cranial neural crest migration in Xenopus.
CHAPTER TWO

Materials And Methods

The laboratory chemicals are supplied by BDH, Boehringer Mannheim or Sigma. The restriction endonucleases and T4 DNA ligase including their buffers were supplied by Boehringer Mannheim. The oligonucleotides were produced at NIMR, Mill Hill. All molecular biology protocols were carried out as described in (Sambrook et al., 1989). Any deviations are described below.

2.1 Standard solutions

Acid alcohol: 70% ethanol, 0.1% concentrated HCl.

10 x nucleotide mix: 10mM A/C/UTP, 1mM GTP.

10x NAM: 110mM NaCl, 2mM KCl, 1mM Ca(NO₃)₂, 1mM MgSO₄, 0.1mM EDTA.

20x SSC: 3M NaCl, 0.3M Tri-sodium citrate pH7.0

DEPC H₂O: 0.05% (v/v) Diethyl pyrocarbonate added to deionised H₂O, shaken, left overnight and autoclaved.

Hybridisation Mix: 50% deionised formamide, 5xSSC, 2% Boehringer Blocking Powder, 0.1% Triton X-100, 0.5% CHAPS, 1mg/ml Torula yeast RNA (Sigma), 5mM EDTA, 50μg/ml heparin.

DIG Mix 10mM GTP, 10mM ATP, 10mM CTP, 6.5mM UTP, 3.5mM DIG-UTP.

KTBT: 50mM Tris.HCl pH7.5, 150mM NaCl, 10mM KCl, 1% Tween-20.

L-agar: As L-Broth but with the addition of 1.5% (w/v) bacto-agar.

Supplied by NIMR media.

L-Broth: 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto yeast extract, 0.5% NaCl. Supplied by NIMR media.
MEMFA: 0.1M MOPS, pH7.4, 2mM EDTA, 1mM MgSO$_4$, 3.7% formaldehyde.

NTM: 100mM NaCl, 100mM TrisHCl pH 9.5, 50mM MgCl$_2$.

NTMT: 100mM NaCl, 100mM TrisHCl pH 8.0, 50mM MgCl$_2$, 0.1 % Tween-20.

Orange loading buffer: 50% glycerol, 20mM Tris.HCl, 20mM EDTA (pH 8.2), 0.1% orange G.

PBS: Prepared by adding phosphate buffered saline tablets (Oxoid BR14a) to H$_2$O and autoclaving.

PBT: PBS, 0.1% Triton X-100

Solution One: 50mM glucose, 10mM EDTA, 25mM TrisHCl, 2mg/ml lysozyme.

TBE: 0.089M Tris Borate, 0.002M EDTA pH 8.

TfBI: 30mM Potassium Acetate, 100mM RbCl, 10mM CaCl$_2$, 50mM MnCl$_2$(4H$_2$O), 15% glycerol. Adjusted to pH 5.8 with 0.2M acetic acid and filter sterilised.

TfBII: 10mM MOPS, 75mM CaCl$_2$, 10mM RbCl, 15% glycerol. Adjusted to pH 6.5 with 1M KOH and filter sterilised.

Victoria blue: 1% solution in acid alcohol.

2.2 Cloning and DNA manipulation.

The bacterial strain used was *Escherichia coli* K12 DH5 alpha F'.

The plasmid vectors used were:

pSP64TK1 (Xu *et al.*, 1995).

pBluescript KS +/- or SK +/-
2.2.1 Production of competent cells for transformation.

K12 DH5 alpha F' were streaked on an L-agar plate and grown overnight at 37°C. A single colony was used to inoculate 10ml L-Broth for an overnight culture at 37°C with shaking. The following day 50ml of L-Broth was inoculated with 0.5ml of culture and grown until the OD was 0.45-0.55. The culture was then chilled on ice for 10 minutes and spun at 13,000rpm for 10 minutes to pellet the cells. The supernatant was discarded and the pellet was resuspended in 20ml TfBI and incubated on ice for 5 minutes. The suspension was spun as before and the resulting pellet was resuspended in 2ml TfBII and again incubated on ice but this time for 10 minutes. The cells were snap frozen in aliquots of 100μl on a mixture of dry ice and ethanol and stored at -70°C until required.

2.2.2 Subcloning and ligation.

The T4 DNA ligase enzyme and buffer were used according to the manufactures instructions with the ratio of vector to insert being 1:3. The reaction was either performed overnight at 16°C for blunt ended ligations or for a few hours at room temperature for cohesive ended ligations.

2.2.3 Bacterial transformation.

3μl of a ligation reaction was added to 50μl of competent cells which had been allowed to defrost slowly on ice. This mixture was incubated on ice for 15 minutes then given a brief heat shock by placing in a 42°C waterbath for 90 seconds. The cells were then incubated for a further 2 minutes on ice before adding 200μl of L Broth. The cells were allowed to recover at 37°C for 50 minutes and then plated out on L agar plates containing 100μg/ml ampicillin. The plates were incubated overnight at 37°C and single colonies were picked for further analysis.
2.2.4 Isolation of plasmid DNA by a mini-preparation method.

A single bacterial colony was used to inoculate a 5ml culture of L-Broth containing 100µg/ml ampicillin, which was incubated overnight at 37°C with shaking. 4ml of the culture were spun down at 13,000 rpm using sterile 2ml Eppendorf tubes, saving 1 ml for possible future analysis. The pellet obtained was resuspended in 180µl of Solution One and allowed to sit at room temp for 5 minutes and then on ice for 5 minutes. 400µl of 0.2M NaOH, 0.1% SDS was added to the mixture before being mixed gently by hand and allowed to remain on ice for a further 5 minutes. Next 300µl of 3M NaOAc was added and the tube was mixed gently. The tubes were put on ice for 10 minutes and subsequently spun for 15 minutes at 4°C at 13,000 rpm. 750µl of the supernatant was transferred to fresh 2ml Eppendorf tube, where 450µl of isopropanol was added and the resulting mixture was vortexed. The tubes were spun for 5 minutes at room temperature and the pellet was washed in 70% EtOH and air-dried before resuspension in 100µl of double distilled H₂O. At this stage diagnostic digests were carried out to analyse the DNA construct.

2.2.5 Isolation of plasmid DNA using the Wizard Maxiprep DNA purification system.

100-500ml of L-Broth containing 100µg/ml ampicillin were inoculated with 1ml of a desired culture and shaken overnight at 37°C. The plasmid DNA was subsequently purified using the wizard maxiprep DNA purification system (Promega A7270). Diagnostic digests were carried to authenticate the DNA.

2.2.6 Restriction endonuclease digests.

All restriction enzymes were used according to the manufacturers instructions, using the appropriate buffer supplied.
2.2.7 Electrophoretic separation of DNA and RNA.

DNA or RNA samples were analysed using agarose gel electrophoresis in TBE buffer. The concentration of agarose used depended on the size of the fragments that were to be separated but generally 1% gels were used. DNA or RNA samples were loaded after mixing with one tenth volume of orange loading buffer. Fragment sizes were determined by corunning a 1 Kb ladder and ethidium bromide stained DNA or RNA was visualised using a UV transilluminator and photographed.

2.2.8 Polymerase Chain Reaction analysis.

A 25 base pair oligonucleotide was designed to check the orientation of the Elk insert being subcloned into pBluescript. The conditions were 94°C for 1 minute, 57°C for 1 minute, then 72°C for 2 minutes for 25 cycles. A fraction of each reaction was then loaded on an agarose gel to determine which reaction yielded a product.

2.2.9 In vitro transcription and translation.

cDNA was transcribed and translated using the coupled transcription-translation rabbit reticulolysate system (Promega) labelling with 35S-methionine. The products were heated at 95°C for 2 minutes and separated on a 1% SDS PAGE gel using rainbow markers (Amersham) as size standards.

In vitro transcription of synthetic mRNA was achieved by mixing the following at room temperature: 35.5μl DEPC H2O, 20μl 5x transcription buffer, 12.5μl 100mM DTT, 10μl 10x nucleotide mix, 10μl 10mM GpppG, 4μl RNAsin, 4μl SP6 RNA polymerase, 4μl linearised plasmid (1μg/μl) and incubated at 37°C for 2.5 hours. 1μl aliquot was removed and frozen before adding 2μl of RNase-free DNAsase (Promega M6102) and the tube further incubating at 37°C for 20 minutes.

Synthetic mRNA was then purified by phenol/chloroform and chloroform extraction, followed by the use of a Quick Spin Column (Boehringer Mannheim 1274015) and subsequent precipitation at -20°C with 1/3 volume 10M ammonium acetate and 2.5
volumes of ethanol. The RNA was pelleted by centrifugation at 13,000rpm, 4°C for 20 minutes before being washed in 70% ethanol and air-dried. The RNA pellet was dissolved in ultrapure water obtained from Sigma (W4502) and a 1μl aliquot was run adjacent to 1μl aliquot kept prior to DNase treatment on a 1% TBE RNase-free gel to estimate the final concentration of RNA.

2.3 Embryo culture and manipulations.

*Xenopus laevis* embryos were obtained from adult females that had been injected 12 hours previously with 800 units of human chorionic gonadotrophin (HCG). The eggs were expelled by gentle peristalsis of the mother's ventrolateral surface and fertilised by rubbing them with macerated testis from a sacrificed male. Ten minutes later the eggs were flooded with 1/10 Normal Amphibian Medium (NAM). Approximately 30 minutes later the rotated embryos were dejellyed using 2% cysteine hydrochloride (pH 7.8). A volume of 10nl was injected into dejellyed embryos using an air driven injector at either the one, two or 32 cell stage depending on the experiment. All injections were performed in 3/4 NAM containing 4% Ficoll and the embryos allowed to recover for six hours before being gradually transferred to 1/10 NAM and allowed to develop to the required stages. Embryos which were to be injected at the 32 cell stage were placed in 4% Ficoll in order to maintain their spherical shape after de-jellying. The embryos were staged according to Nieuwkoop and Faber (1967). Refer to appendix for table of number of embryos with disruptions to neural crest migration.

2.3.1 Whole mount in situ hybridisation.

2.3.1.1 Synthesis of probe:

The following were mixed in an RNase free Eppendorf tube at room temperature; 10μl of DEPC H2O, 4.0μl of 5x transcription buffer, 2.0μl of 0.1 M DTT, 2.0μl of DIG mix, 1.5 μl of linearised plasmid (1μg/ml), 1.5μl of SP6, T7 or T3 RNA Polymerase, and incubated for 2 hours at 37°C. At this point a 1μl aliquot was removed and frozen. 2μl of RNase-free DNase (Promega M6102) was added and the tube was further incubated at 37°C for 20 minutes. To precipitate the RNA, 50μl DEPC H2O, 25μl 10M Ammonium acetate and 20μl ethanol were added before placing in dry ice for 30 minutes. The tube was spun in a
microfuge at 4°C for 20 minutes at 13,000 rpm. The resulting pellet was washed with 70 % ethanol and air-dried before being re-dissolved in 50µl DEPC H$_2$O. 2µl of this final riboprobe was run along with the initial 1µl aliquot on an RNAse-free 1% agarose TBE gel to estimate the amount of riboprobe synthesised. The riboprobe was stored at -70°C and 2µl were added for each ml of hybridisation mix.

2.3.1.2 Pre-treatments of embryos.

Once at the required stages the vitelline membranes surrounding the embryos were removed and they were fixed at 4°C in MEMFA overnight. The following day the embryos were rinsed twice in PBT for 5 minutes before being treated for 10 minutes with 10µg/ml proteinase K in PBT. Subsequently the embryos were carefully rinsed in PBT and re-fixed in 4% formaldehyde at room temperature for 20 minutes. They were then rinsed in PBT before adding 1ml of hybridisation solution for overnight prehybridisation at 55°C with gentle rocking. After prehybridisation embryos can be stored at -20°C.

2.3.1.3 Hybridisation.

1ml of fresh hybridisation solution containing 2µl of DIG labelled probe was added and the embryos were incubated overnight with gentle rocking in a heater block at 55°C.

2.3.1.4 Post hybridisation.

The embryos were washed with the following solutions for 5 minutes each at 55°C; 100 % Solution 1; 75% Solution 1: 25% 2x SSC; 50% Solution 1: 50% 2x SSC; 25% Solution 1: 75% 2x SSC. They were then washed twice in 2x SSC, 0.1% CHAPS for 30 minutes at 55°C and twice in 0.2x SSC + 0.1% CHAPS for 30 minutes at 55°C. Subsequently the embryos were rinsed in KTBT at room temperature before preblocking in 10% sheep serum and 2% BSA in KTBT at room temperature for 2-3 hours or longer. The blocking solution was then replaced with fresh blocking solution containing 1µl/1ml DIG-AP antibody and the embryos were rocked overnight at 4°C.
2.3.1.5 Post antibody washes.

The embryos were washed 5 times at room temperature for 1 hour in KTBT, then overnight at 4°C. After washing the embryos were transferred into glass embryo dishes and washed in NTM for 5 minutes at room temperature before incubating in NBT/BCIP (Boehringer Mannheim 65597921) staining solution at room temperature in the dark, whilst rocking gently. The reaction was stopped once the desired intensity was reached, by washing in KTBT. If necessary the reaction was restarted the following morning after incubating in the dark overnight at 4°C in NTM.

2.3.2 Detection of localisation of injected mRNA.

In order to determine whether the correct blastomere had been injected at the 32 cell stage the embryos were coinjected with dextran-fluorescein. This was assumed to co-localise with the injected synthetic RNA. In order to evaluate the efficiency of the blastomere injections and the localisation of the injected message an anti-fluorescein antibody was used. The embryos were kept in the dark as much as possible during the subsequent steps.

Embryos were fixed for one hour at room temperature in MEMFA after the in situ hybridisation colour reaction. The embryos were washed twice in PBT and blocked for 2-3 hours or longer in PBT containing 10% sheep serum, at room temperature. The blocking solution was replaced with fresh blocking solution containing 1μl/2ml anti-Fluorescein-AP antibody and the embryos were rocked overnight at 4°C.

The embryos were washed 5 times at room temperature for 1 hour in PBT, then overnight at 4°C. After washing the embryos were transferred into glass embryo dishes and washed twice in NTMT for 5 minutes at room temperature. Fast Red tablets (Boehringer Mannheim) were dissolved in 0.1 M Tris.HCl pH 8.2, 0.1% Tween-20 (2ml/tablet). The embryos were incubated in this Fast Red staining solution at 4°C in the dark for one hour, whilst rocking gently to allow full penetration of the staining solution into the embryos. After one hour the reaction was continued at room temperature and stopped by washing in PBT, once the desired intensity was reached.
2.3.3 Histological studies.

Embryos to be sectioned were additionally fixed in 4% formaldehyde, 1% gluteraldehyde in PBS at room temperature for one hour. The embryos were then washed with PBS and placed twice in methanol for 5 minutes. The methanol was replaced twice with propanol for 10 minutes which in turn was replaced with Cedarwood oil for 30 minutes, three times. The embryos were then placed in 1:1 Cedarwood oil: wax, at 60°C for 30 minutes which was replaced three times with paraffin wax at 60°C for 30 minutes each. Embryos were then transferred into a plastic mould and orientated as required. 6μm sections were cut on a microtome and the resulting ribbons were lowered onto a waterbath at 55°C to smooth the creases. Glass slides were used to pick up sections from the waterbath and these were placed on a hot plate at 45°C to dry for several hours. Once dry the slides were de-waxed in Histoclear before being mounted in DPX mountant (BDH 360294H).

2.3.4 Victoria blue staining.

This procedure was performed on Xenopus embryos stage 33 onwards when cartilage has formed.

The embryos were anaesthetised for up to one minute in 0.2% MS222 before fixing them in 4% paraformaldehyde at room temperature for a minimum of 24 hours. In order to bleach any natural pigmentation in the embryo they were incubated in a solution of 30% hydrogen peroxide for approximately four hours. The embryos were subsequently placed in 50% ethanol for a minimum of one hour and in acid alcohol for a minimum of two hours. The embryos were then stained in Victoria blue for 45 minutes. They were then serially dehydrated from 70% ethanol to 100% ethanol and cleared in methyl salicylate.
 sklearn

expression patterns in multiple tissues during the development of the vertebrate embryo. One of these receptors, Sekl, was initially identified in a screen for segmentally expressed kinases in the mouse hindbrain (Nieto et al., 1992). Expression studies performed in both the mouse and the chick have shown that Sekl is expressed in a broad domain in the presumptive hindbrain and later upregulated in presumptive rhombomeres 3 and 5 (Nieto et al., 1992) (Irving et al., 1996). The Xenopus homologue of Sekl has also been found to be expressed in rhombomeres 3 and 5 as well as third arch neural crest (Winning and Sargent, 1994) (Xu et al., 1995). Similarly the zebrafish homologue, rtkl is expressed in rhombomeres 3 and 5 (Xu et al., 1994). Additional sites of Sekl expression include forebrain, the otic vesicle, the pronephros, the developing somites, the notochord and newly formed mesoderm (Winning and Sargent, 1994) (Xu et al., 1995) (Nieto et al., 1992) (Irving et al., 1996) (Xu et al., 1994) (Xu et al., 1996).

By screening a Xenopus embryo cDNA library for novel Eph family members Dr Ketan Patel in my laboratory identified Xenopus Elk, a gene which is homologous to rat Elk and chick Cek5 (Scales et al., 1995). Initial investigations in my laboratory revealed that Elk is expressed in migrating cranial neural crest. As Sekl and Elk are both expressed in cranial neural crest, I set out to characterise the organisation of Xenopus cranial neural crest and the expression patterns of Sekl and Elk in more detail.

Cranial neural crest cells emerge from the hindbrain and migrate to populate specific branchial arches. Studies in the chick have shown that neural crest migrates from rhombomere 2 to the first branchial arch, from rhombomere 4 to the second arch and from rhombomere 6 and more posterior regions to the third and fourth branchial arches (Lumsden et al., 1991). Since the cranial neural crest cells appear to be patterned it would seem important that the streams of neural crest do not mix before or during their migration in addition to migrating to their correct destination. In the chick, the three streams of neural crest are separated by areas of crest free mesenchyme and it has been suggested that these prevent the intermingling of neighbouring streams of neural crest (Lumsden et al., 1991). The presence of crest-free mesenchyme may however, be required for this function as
there are a number of situations where juxtaposed populations of cranial neural crest remain segregated and migrate to distinct destinations. One example is seen in *Xenopus* where the third and fourth arch streams of cranial neural crest segregate only during migration. In *Xenopus*, the prospective neural crest is derived from both deep and superficial layers of the ectoderm (Schroeder, 1970). Morphological and fate mapping analysis using electron microscopy and interspecies grafting has been used to show that in *Xenopus* cranial neural crest cells delaminate away from the neural plate at stage 15 and form a discrete population adjacent to the neural plate (Sadaghiani and Thiebaud, 1987). By stage 16, three distinct masses of premigratory crest are located next to the neural plate. These do not start to migrate until between stages 19-22, in the space between the ectoderm and the dorsal mesoderm. The most anterior stream, migrates first and populates the first arch, with the second arch neural crest migrating soon after to fill the second arch. The most caudal mass of cranial neural crest divides into an anterior stream, which migrates to fill the third branchial arch and a posterior stream, which contributes to the fourth branchial arch (Figure 3.1A) (Sadaghiani and Thiebaud, 1987). In *Xenopus*, it has been suggested that the streams of migrating neural crest are separated by placodal thickenings in the surface ectoderm (Sadaghiani and Thiebaud, 1987).

In contrast to the delayed onset of migration in *Xenopus*, chick neural crest cells emerge from the dorsal midline of the neural tube shortly after its closure and immediately commence their migration into the branchial arches. Unlike *Xenopus*, in the chick there is crest free mesenchyme opposite rhombomeres 3 and 5 that are thought to arise by the apoptotic elimination of neural crest cells originating from rhombomeres 3 and 5 and the targeted migration of the surviving neural crest cells into neighbouring streams of migrating neural crest (Sechrist *et al.*, 1993) (Lumsden *et al.*, 1991) (Graham *et al.*, 1993).

### 3.2 Results

#### 3.2.1 Analysis of neural crest organisation in *Xenopus* embryos.

A number of molecular markers have been identified which are expressed in specific cranial neural crest streams. For example *Krox20* is expressed in rhombomeres 3 and 5 and neural crest migrating from the region adjacent to rhombomere 5 into the third branchial arch (Bradley *et al.*, 1992). The transcription factor *AP2* is expressed by all four streams of cranial neural crest throughout their migration into each arch (Bradley *et al.*, 1992). In order to examine the migration of cranial neural crest embryos were hybridised with riboprobes to detect *AP2* plus *Krox20* expression. This method of analysis allows the third
Figure 3.1: Restricted migration of branchial neural crest cells in *Xenopus*.

(A) The diagram shows the streams of cranial neural crest (1-4) prior to (upper part) and during their migration (middle and lower part) into specific branchial arches. The third and fourth arch neural crest streams do not segregate until during migration. Adapted from Sadaghiani and Theibaud (1987). (B) Expression pattern of *AP2* and *Krox20* in a stage 25 *Xenopus* embryo as revealed by whole mount *in situ* hybridisation. *AP2* is detected in all four streams of migrating neural crest (n1-n4), whereas *Krox20* is only detected in the third arch neural crest (n3), which therefore is stained more strongly. (C) Section through the dorsal region of migrating neural crest which has been previously analysed for the expression of *AP2* and *Krox20*. The streams of neural crest (n2-n4) can be seen to be juxtaposed, with no physical barrier to prevent intermingling. (D) A more ventral section of the embryo shown in (C). As the streams of neural crest migrate deep into the branchial arches they become separated by the pharyngeal pouches.
arch neural crest to be distinguished from the other populations of cranial neural crest as it expresses both molecular markers and is detected as a darker colour. Serial coronal sections were then prepared from embryos previously analysed for the simultaneous expression of both Krox20 and AP2. Analysis of Xenopus embryos during the early stages of cranial neural crest migration show the presumptive second, third and fourth arch crest are juxtaposed and there are no obvious physical barriers between them (Figure 3.1B,C). Coronal sections of older embryos show that as the cranial neural crest migrates more ventrally into the branchial arches the streams of neural crest become separated by the pharyngeal pouches (Figure 3.1D). Thus, unlike the situation described in the chick, the streams of neural crest only become separated from each other after having migrated deep into the branchial arches.

3.2.2 Expression of Sekl.

In Xenopus, Sekl is first expressed in presumptive rhombomere 3 at stage 13 and then in presumptive rhombomere 5 at stage 14 with the expression of Sekl in these rhombomeres remaining at a high level throughout cranial neural crest migration (Figure 3.2A-C). Expression of Sekl is also detected in the premigratory cranial neural crest adjacent to rhombomere 5. This expression slightly precedes that in rhombomere 5 and corresponds to the third arch neural crest. Sekl is expressed in this neural crest throughout its migration into the third branchial arch (Figure 3.2A-C). Sections taken from embryos analysed by whole mount *in situ* hybridisation demonstrate expression of Sekl is detected in the neural crest beneath the arch ectoderm (Figure 3.2D). In addition to expression of Sekl in migrating third arch neural crest, Sekl transcripts are also detected in a domain that extends ventrally around the embryo which may correspond to the future migration route of the third arch neural crest cells (Figure 3.2A-C). At stage 20 this expression is both contiguous with, and as broad as the Sekl expression domain in the third arch neural crest (Figure 3.2A) but then becomes narrower and confined to the anterior limit of the migrating third arch neural crest cells (Figure 3.2B). Section analysis of stage 18 embryos shows that this ventral band of expression is initially located in the mesoderm (Figure 3.2D). By stage 24 expression is downregulated in the deep layers of the ectoderm and mesoderm yet upregulated in a broader domain in the underlying endoderm (Figure 3.3A,B). More ventrally in the branchial arches, the broad domain of Sekl expression is divided into two smaller domains which appear to correspond to specific regions of the forming pharyngeal pouches (Figure 3.3C).
Figure 3.2: Expression of Sek1 in the *Xenopus* embryo.

Whole mount *in situ* hybridisation was performed to detect the expression of Sek1 during neural crest migration. (A) Stage 20 embryo prior to third arch neural crest migration. The ventral line of Sek1 expression is as wide as and contiguous with the third arch neural crest expression. The expression of Sek1 can also be detected in rhombomeres 3 and 5, the forebrain and the cement gland. (B) Stage 23 embryo. The third arch neural crest has started migrating towards the third branchial arch. The ventral line of expression has become confined to the anterior limit of the migrating third arch neural crest cells. Expression of Sek1 is now also detected in the pronephros. (C) Stage 27 embryo. The third arch neural crest has now migrated into the third branchial arch. (D) Transverse section of a tailbud stage embryo slightly posterior to r5, revealing Sek1 expression in the neural tube, neural crest, endoderm, and mesoderm.

n3, third arch neural crest; v, ventral line of expression; r, rhombomere; cg, cement gland; f, forebrain; pn, pronephros; nt, neural tube.
Figure 3.3: Analysis of *Sekl* expression in the ventral domain of expression.

Whole mount *in situ* hybridisation was performed to detect the expression of *Sekl* and then coronal sections prepared through the ventral domain of *Sekl* expression. (A) Stage 20 embryo. *Sekl* expression is observed in the mesoderm. (B) Stage 24 embryo. *Sekl* expression is observed to be downregulated and in a narrower domain in the ectoderm and mesoderm, and upregulated in a broad domain in the underlying ectoderm. (C) A more ventral section of the embryo shown in (B). The broad domain of *Sekl* expression in the endoderm becomes divided into two domains which appear to correspond to the future pharyngeal pouches.

ec, ectoderm; en, endoderm; m, mesoderm.
3.2.3 Expression of Elk.

Following the isolation of Xenopus Elk by my colleague Ketan Patel, I analysed the expression pattern of Elk in detail by whole mount in situ hybridisation. Transcripts of Elk are first detected around stage 15, lateral to the neural plate in the presumptive first, third and fourth arch neural crest masses prior to their migration (Figure 3.4A). Expression of Elk persists in the third and fourth arch neural crest populations during their migration into the branchial arches and continues to be expressed until at least stage 33 (Figure 3.4A-E). The expression of Elk in the third and fourth arch neural crest has been confirmed by serial sectioning of embryos (Figure 3.4E). By stage 21 there is also a band of Elk expression extending ventrally around the embryo, which is contiguous with and of similar width to the Elk expression domain observed in the migrating third and fourth arch neural crest (Figure 3.4C). This band of Elk expression remains as a broad domain of expression throughout third and fourth arch neural crest migration and appears to correspond to their future migration route. Transverse sections show that this band of expression is found within both the ectoderm and in the underlying mesoderm (Figure 3.4E).

Elk is also expressed in two stripes in the forebrain. The first stripe of Elk expression appears at stage 15 and the second at stage 20 with both domains persisting until at least stage 32. Transverse sections through tailbud stage embryos show expression of Elk in the ventral portion of the neural tube excluding the floorplate (Figure 3.4E).

3.2.4 Simultaneous detection of Sek1 and Elk.

In order to confirm the relative domains of expression of Elk and Sek1 in the neural crest (Figures 3.2,3.4), whole mount in situ hybridisation was carried out to detect Elk and Sek1 expression simultaneously. Elk and Sek1 show overlapping domains of expression in the premigratory and the migrating third arch neural crest (Figure 3.5). The expression of Elk (lower level signal) extends posteriorly to that of Sek1 (high level signal), confirming that Elk is expressed in a broader domain than Sek1 and that it is expressed in fourth arch mesoderm and neural crest.
Figure 3.4: Expression of *Elk* in the *Xenopus* embryo.

Whole mount *in situ* hybridisation was carried out to detect the expression of *Elk* during neural crest migration. (A) At stage 15 *Elk* expression is detected in the forebrain, and the presumptive first, third and fourth arch neural crest. (B) At Stage 20 expression of *Elk* occurs in the migrating third and fourth arch neural crest. A band of *Elk* expression extending ventrally around the embryo is detected that is contiguous with and as wide as the expression in the neural crest. The border of the neural crest is difficult to see as expression occurs at similar levels in the ventral region. (C,D) Stage 23 embryo and stage 27 embryo respectively. *Elk* expression is seen in the migrating third and fourth arch neural crest and in a domain extending ventrally around the embryo. *Elk* expression is also detected in the forebrain. (E) Transverse section through a tailbud stage embryo previously analysed for *Elk* expression. Expression can be detected in the neural crest, the ventral part of the neural tube excluding the floorplate, the mesoderm and the endoderm.

n1, first arch neural crest; n3, third arch neural crest; n4, fourth arch neural crest; v, ventral line of expression; fb, forebrain; pn, pronephros; nt, neural tube; nc, neural crest.
Figure 3.5: Simultaneous detection of Sekl and Elk by whole mount in situ hybridisation.

Whole mount in situ hybridisation was carried out to detect the expression of Sekl and Elk concurrently. Sekl expression is seen as the strong staining in the neural crest and Elk as the weaker. The expression of Elk can be seen to extend more caudally than that of Sekl in the migrating neural crest to include fourth arch neural crest, and also in the ventral domain of expression. This pattern of expression remains throughout neural crest migration as seen in (A) stage 23, (B) stage 24, (C) stage 26, and (D) stage 28 embryos.
3.3 Summary.

In *Xenopus* the populations of cranial neural crest are juxtaposed prior to and during the early stages of their migration. There are no obvious physical barriers seen by analysis of serial coronal sections which raises the question of what mechanisms exist to prevent individual neural crest cells from neighbouring streams from intermixing to ensure that the neural crest cells migrate to their correct location.

I have found that the Eph RTK *Sekl* is expressed in the third arch neural crest both prior to its migration and during its migration into the third branchial arch. Preceding migration of third arch neural crest *Sekl* is also expressed in a domain in branchial arch mesoderm which is as broad as and contiguous with *Sekl* expression in the third arch neural crest. This mesodermal expression is eventually confined to the anterior limit of the migrating neural crest cells. The ventral domain of expression becomes downregulated in the deep layers of the ectoderm and mesoderm but becomes upregulated in the underlying endoderm.

In contrast, *Elk* is expressed in the third and fourth arch neural crest prior to and during their migration into the respective arches. Expression is also detected in a broad band which extends ventrally around the embryo which is contiguous with *Elk* expression in the third and fourth arch neural crest.

*Sekl* and *Elk* are thus expressed in specific streams of cranial neural crest prior to and during their migration into their prespecified arches. They show overlapping expression in the third arch neural crest and may also be co-expressed in the mesoderm of the presumptive third arch. Their expression in the mesoderm appears to correspond to the future migration route of the third arch neural crest and represent the first markers which are known to distinguish the mesoderm of different arches.
Chapter Four.

Functional Analysis Of Sek1 And Elk In The Migration Of Xenopus Neural Crest.

4.1 Introduction.

The expression pattern of Sek1 and Elk in the Xenopus embryo suggests that they may mediate cell interactions in specific streams of cranial neural crest. To test their function the effects of expressing dominant negative forms of the receptors that lack the intracellular tyrosine kinase domain were analysed. This approach has been used to study the role of signalling through the FGF, activin and BMP4 receptors in Xenopus embryos (Amaya et al., 1991) (Hemmati-Brivanlou and Melton, 1992) (Graff et al., 1994). As receptor kinases are activated by ligand-induced dimerisation of the receptors leading to transphosphorylation and activation of the intracellular catalytic domains (Ullrich and Schlessinger, 1990), the activation can, be blocked by the overexpression of a truncated receptor comprising the extracellular and transmembrane domains, but lacking in kinase function (Amaya et al., 1991) (Ueno et al., 1991) (Ueno et al., 1992). In addition the truncated receptors could also disrupt the function of the endogenous receptors by sequestering their ligands. Using the dominant negative approach to block the endogenous function of Sek1 in zebrafish and Xenopus it has been shown that Sek1 function is required to restrict the segmental domains of rhombomeres 3 and 5 gene expression (Xu et al., 1995).

4.2 Results.

4.2.1 Subcloning a dominant negative construct of Elk.

A dominant negative Sek1 construct lacking the intracellular kinase domain was already available that had been subcloned into the vector pSP64T (Xu et al., 1995). This vector provides 5’ untranslated sequences of β globin, stop codons to terminate the truncated coding region, followed by the 3’ untranslated regions of the Xenopus β globin gene. It
has been shown that the untranslated regions enhance the translational efficiency and stability of mRNA (Krieg and Melton, 1984).

In order to make a dominant negative Elk construct a 2.2 Kb cDNA fragment encoding the extracellular and transmembrane domains of Elk was subcloned into the BgII and HindIII restriction sites of the vector pSP64T. From the published sequence of Elk it was predicted that the truncated receptor protein lacking the intracellular kinase domain would have a relative molecular weight of 70 kDa. In vitro transcription and translation from the truncated Elk cDNA clone using the rabbit reticulocyte lysate system in conjunction and subsequent SDS-PAGE gel analysis shows that the size of the resultant protein is approximately 66 kDa (Figure 4.1).

4.2.2 Microinjection of mRNA encoding truncated Sek1.

RNA encoding either truncated Elk or Sek1 was microinjected together with a lineage tracer into one cell of two cell stage Xenopus embryos. This technique allows for an internal control for the injections as only one side of the embryo expresses the truncated receptors whereas the other side acts as an uninjected control. β globin mRNA which has previously been reported to have no specific effects on development in Xenopus embryos, was injected into 20-30 embryos of every batch and used as a control for non-specific effects of RNA injection. Tritrating the amount of RNA injected indicated that it was necessary to inject 3ng of RNA in order to disrupt the function of Sek1 or Elk during mid-neurula stages of development, however, these levels of injected RNA are toxic to embryonic development and result in approximately 50% of embryos injected with either control RNA or RNA encoding truncated receptor showing disrupted gastrulation phenotypes. These embryos were discounted from subsequent analysis. Embryos were usually allowed to develop to stages 20-23 and were subsequently analysed by whole mount in situ hybridisation using Krox20, a marker for third arch neural crest.

After injection of RNA encoding truncated Sek1, 28% of embryos had normal Krox20 expression and 72% showed a disrupted pattern. Embryos analysed at the onset of third arch neural crest migration around stage 20, show that although the neural crest cells on the uninjected side are contiguous with rhombomere 5, third arch neural crest cells are found
Figure 4.1: Verification of the molecular sizes of the proteins for the truncated receptors \textit{Sek1} (S) and \textit{Elk} (E).

Autoradiograph of radiolabelled \textit{in vitro} translation products of truncated \textit{Sek1} (Δ Sek1 66 kDa) and \textit{Elk} (Δ Elk 66 kDa) cDNA electrophoresed against rainbow markers on an SDS-polyacrylamide gel. Control indicates a positive control cDNA used for this experiment.
adjacent to rhombomere 6 and sometimes adjacent to rhombomere 4 on the injected side (Figure 4.2A,B). Analysis of later stage embryos (stages 22-25) show that a range of phenotypes occur during third arch neural crest migration. The least severe phenotype observed was characterised by most of the third arch neural crest cells appearing to migrate towards their appropriate destination but with a few ectopic groups of Krox20 expressing cells anterior and/or posterior to the normal stream (Figure 4.2E). Other phenotypes are observed: embryos show third arch neural crest cells appear to originate from an incorrect anteroposterior location but then migrate towards the correct destination of the third branchial arch (Figure 4.2E,F). This phenotype could indicate that the location of some third arch neural crest is initially disrupted (as in Figure 4.2A), but the stream of neural crest “recovers” and then migrates towards the correct branchial arch. Other embryos show Krox20 expression occurred in populations of third arch neural crest cells which appeared to be migrating posteriorly along the spinal cord (Figure 4.2D).

4.2.3 Microinjection of mRNA encoding truncated Elk.

Embryos injected with RNA encoding truncated Elk show similar phenotypes to those expressing truncated Sek1. In these cases 34% of the injected embryos which appeared morphologically normal showed no phenotype, and 66% showed disrupted neural crest migration. Whole mount in situ analysis of embryos expressing the truncated receptor at early stages of third arch neural crest migration show the presence of Krox20 expressing cells posterior to the normal stream of third arch neural crest (Figure 4.3A,B). As in the case of truncated Sek1 injected embryos, Krox20 expressing cells were also observed leaving the main bulk of third arch neural crest to migrate posteriorly (Figure 4.3B). Other phenotypes seen in truncated Elk injected embryos are characterised by some of the third arch neural crest originating from the wrong anteroposterior location but apparently migrating towards its correct destination of the third arch (Figure 4.3C). In some embryos this posterior population of Krox20 expressing cells completely detaches from the main stream of neural crest (Figure 4.3D).

4.2.4 Microinjection of mRNA encoding both truncated receptors.

As Sek1 and Elk have overlapping mRNA expression patterns and Sek1 can interact with two of the three ligands for Elk it is possible that Sek1 and Elk may have overlapping functions or show cooperativity. In order to address this possibility I coinjected equal
RNA encoding truncated Sekl was microinjected into one cell of a two cell stage Xenopus embryo. The embryos were allowed to develop until stage 21 (A,B) or stages 23-25 (D-F), then fixed and Krox20 expression was detected as a marker of third arch neural crest. (A,B) show dorsal views of flat mounted embryos, with the uninjected side to the left and the injected side to the right. (C) shows the uninjected side of an injected embryo and (D-F) show the injected side of the embryos. The white arrows indicate a normal restricted stream of third arch neural crest (n3) and the black arrows indicate cells that are in abnormal locations.
Figure 4.3: Effects of expressing truncated *Elk* on third arch neural crest.

RNA encoding truncated *Elk* was microinjected into one cell of a two cell stage *Xenopus* embryo. The embryos were allowed to develop until stage 21 (A,B) or stages 23-25 (C,D), then fixed and *Krox20* expression was detected as a marker of third arch neural crest. (A) shows a dorsal view of a flat mounted embryo, with the uninjected side to the left and the injected side to the right. (B) shows a higher power magnification of the injected side of a flat mounted embryo. (C,D) show the injected side of the embryos. The white arrows indicate a normal restricted stream of third arch neural crest (n3) and the black arrows indicate cells that are in abnormal locations.
quantities of RNA encoding both truncated receptors into one cell of two cell stage *Xenopus* embryos. The phenotypes observed were similar in both nature and proportion to those obtained by injection of either truncated Sek1 or truncated Elk alone. At early stages of cranial neural crest migration, third arch neural crest cells were observed to aberrantly migrate anteriorly (Figure 4.4A) and during later stages of development (stages 22-26) these third arch neural crest cells continued to migrate into inappropriate territories (Figure 4.4C). In other embryos, third arch neural crest cells originate from the wrong anteroposterior location but then migrate towards the appropriate arch. An additional phenotype was characterised by third arch neural crest cells migrating in a straight line from rhombomere 5 initially in the right direction, but then later deviating to migrate at right angles to their original path (Figure 4.4B). In some embryos small clumps of posterior ectopic Krox20 expressing neural crest cells could be seen adjacent to the normal third arch stream (Figure 4.4D).

In order to determine whether the truncated receptors had a selective effect on individual neural crest streams, embryos which had been injected with RNA encoding both truncated receptors were also analysed with the pan neural crest marker AP2. The uninjected side of the embryos showed the first, second, third and fourth arch neural crest streams migrating to their correct destination (Figure 4.5A). The injected side of these embryos, however, whilst showing unaffected first and second arch neural crest streams displayed aberrant caudal migration of the third and fourth arch neural crest cells, though it is not possible to distinguish which of these streams gave rise to the ectopic neural crest cells (Figure 4.5B). To determine the position of these ectopic neural crest cells, coronal sections were cut of embryos expressing both truncated receptors which had been analysed by whole mount *in situ* hybridisation to detect Krox20 and AP2 expression. This indicated the presence of Krox20 expressing cells in fourth arch crest territory (Figure 4.5C).

To ascertain whether defects were occurring in second arch neural crest and to confirm the location of the ectopic Krox20 expressing neural crest cells, the second arch neural crest marker Eck (G. Alldus, unpublished) was used. The injected embryos were analysed by whole mount *in situ* hybridisation simultaneously to detect both Krox20 and Eck. As Eck is not expressed as strongly as Krox20 it was possible to distinguish between the expression patterns of the two genes. The uninjected side of the embryos expressing the truncated receptors shows the normal migration of the second and third arch neural crest streams into their appropriate branchial arches (Figure 4.5D). The injected side of these embryos did not show a disruption to second arch neural crest migration, but revealed that third arch neural crest occasionally invaded second arch neural crest territory (Figure 4.5E).
Figure 4.4: Effects of expressing truncated Sekl and truncated Elk on third arch neural crest.

A mixture of RNAs encoding truncated Sekl and Elk were microinjected into one cell of a two cell stage Xenopus embryo. The embryos were allowed to develop until stage 21 (A,) or stages 23-25 (B-D), then fixed and Kroxl0 expression was detected as a marker of third arch neural crest. (A) shows a dorsal view of a flat mounted embryo, with the uninjected side to the left and the injected side to the right. (B-D) show the injected side of stage 23-25 embryos. The white arrows indicate a normal restricted stream of third arch neural crest (n3) and the black arrows indicate cells that are in abnormal locations.
Figure 4.5: Effects of expressing truncated Sekl and truncated Elk on cranial neural crest.

A mixture of RNAs encoding truncated Sekl and Elk were microinjected into one cell of a two cell stage Xenopus embryo. The embryos were allowed to develop to stages 23-25 and \textit{in situ} hybridisation was carried out to detect either AP2 or Krox20 and Eck. (A,D) show the uninjected sides of the injected embryos. (A,B) AP2 expression. (B) shows the first and second arch neural crest streams have migrated normally, but the third and fourth arch neural crest have migrated less ventrally and there are ectopic caudal cells. (C) Section of a stage 22 embryo which has been analysed for both Krox20 (stronger staining) expression and AP2 expression (weaker staining) demonstrating the presence of ectopic Krox20 expressing third arch neural crest cells in fourth arch territory. (D,E) Krox20 expression (stronger staining) is detected in the third arch neural crest and Eck staining (weaker staining) is detected in the second arch neural crest. (E) Krox20 expressing third arch neural crest cells are detected in second arch territory, whereas, the stream of second arch neural crest appears unaffected.
Analysis of neural crest development in *Xenopus* has shown that the third and fourth branchial arches give rise to the cartilages of the gills (Sadaghiani and Thiebaud, 1987). Since expression of the truncated receptors disrupts third arch neural crest migration I analysed effects on the structures derived from the third arch neural crest. Embryos expressing both truncated receptors were allowed to develop until stage 43 when the gill arches have formed and cartilage has been deposited. The cartilaginous skeleton of the embryos was then visualised using alcian blue. A large proportion (greater than 75%) of injected embryos died before reaching stage 43, but 5-10% of the surviving embryos were found to lack cartilage elements of the gill arches (Figure 4.6B).

### 4.2.5 Targeted blastomere injections of *Sek1* and *Elk*.

Since *Sek1* and *Elk* are expressed in both neural crest and mesoderm of the presumptive arches it was important to establish whether the aberrant third arch neural crest migration observed is due to disruptions in the underlying mesoderm or in the neural crest cells themselves. To address this problem targeted blastomere injections of the truncated receptors were performed. Fate maps of the 32 cell stage *Xenopus* embryo have shown that A1 and A2 blastomeres have a high probability of giving rise to head neural tissue and not to mesoderm (Dale and Slack, 1987) (Moody, 1987). mRNAs encoding the truncated receptors were coinjected into the A1 or A2 blastomere and the phenotypes were analysed (Figure 4.7A-D). In order to confirm the correct targeting of the mRNAs, in some experiments a fluorescein-dextran lineage tracer was coinjected with the mRNAs (Figure 4.8). The location of the lineage tracer was detected using an alkaline phosphatase conjugated anti-fluorescein antibody and then visualised using a red substrate (Figure 4.7C,D). The injected embryos were analysed with *Krox20* and showed aberrantly migrating neural crest cells similar to those seen with injections at the two cell stage. Some embryos displayed aberrant caudal migration of the third arch neural crest cells (Figure 4.7A), and some showed third arch neural crest cells originating from the incorrect rostrocaudal location (Figure 4.7B) and others displayed ectopic *Krox20* expressing neural crest cells both anterior and posterior to the stream of third arch neural crest (Figure 4.7C-F).
Figure 4.6: Effects of expressing truncated *Sek1* and *Elk* on cartilage.

A mixture of RNAs encoding truncated *Sek1* and *Elk* were microinjected into one cell of a two cell stage *Xenopus* embryo. (A,B) Cartilage staining of stage 43 embryos. (A) shows an uninjected embryo. (B) shows an injected embryo with the uninjected side to the top of the page. Anterior is to the left. The embryo is missing two gill arches. The arrows are pointing to the gill arches.
Figure 4.7: A1 and A2 blastomere injections with truncated Sek1 and Elk.

A mixture of Sek1 and Elk RNAs were injected into A1 or A2 blastomere of a 32 cell stage Xenopus embryo. Some embryos were also coinjected with the lineage tracer fluorescein-dextran. The embryos were allowed to develop until stage 23-25 before being fixed and analysed for the expression of Krox20 (blue) (A-F) and fluorescein (red) (E,F). The black arrows indicate the presence of ectopic posterior and anterior Krox20 expressing cells (A-D). (F) A section through the embryo in (E) indicating the co-localisation of the ectopic Krox20 third arch neural crest cell in fourth arch territory and the injected fluorescein. The white arrow indicates the staining for fluorescein.
Figure 4.8: Representation of targeted blastomere injections.

Upper panel. The 32 cell *Xenopus* embryo. Adapted from (Dale and Slack, 1987). Lower panel. By coinjecting RNA and dextran-fluorescein into specific blastomeres it is possible to target distinct regions of the embryo. The embryo has been injected into either the A1 or A2 blastomere with fluorescein and allowed to develop before subsequent analysis for *Krox20* expression (blue) and the localisation of the injected fluorescein-dextran (red) detected using an alkaline phosphatase antibody against fluorescein. In this embryo, the lineage tracer has been targeted to the branchial neural crest.
4.2.6 Summary.

The inhibition of Sek1 and/or Elk function by a dominant negative approach causes third arch neural crest to migrate to inappropriate adjacent territories whereas second arch neural crest migration remains unaffected. This defect is observed both prior to and during the early stages of third arch neural crest migration suggesting that both Sek1 and Elk function are required to prevent third arch neural crest mixing with adjacent populations of second or fourth arch neural crest. Increased early mixing could lead to isolation of small clusters of third arch neural crest cells which are later detected in inappropriate territories. These ectopic posterior cells either continue to migrate into abnormal territories or re-join the main stream of normally migrating cells. In some instances, however, the initially disrupted third arch neural crest cells appear to recover and migrate to their correct destination. Sek1 and Elk appear to be specifically required for targeted migration of third arch neural crest since targeted migration of second arch neural crest is unaffected by expression of the truncated receptors.

As Elk is expressed in both third and fourth arch neural crest it seems probable that the expression of the truncated receptors would also affect the migration of fourth arch neural crest. The embryos analysed using AP2 as a molecular marker for all cranial neural crest suggest that expression of the truncated receptors does disrupt the migration of the fourth arch neural crest as well as the third arch neural crest. However, the lack of a molecular marker expressed only in fourth arch neural crest means that it is not possible to unequivocally address this possibility. It is also not possible to determine whether any effects seen on fourth arch neural crest migration are secondary effects due to disruption of the third arch neural crest.

Given that Sek1 and Elk are both expressed in the neural crest as well as the mesoderm of the presumptive second and third arches, it was necessary to determine whether the aberrant migration of the third arch neural crest was due to disruption of a mesodermal function of these receptors. To test this, expression of truncated Sek1 and truncated Elk was targeted to the cranial neural crest. This resulted in defective third arch neural crest migration and indicates that Sek1 and Elk function in third arch neural crest is required for its correct migration. The function of Sek1 and Elk in the mesoderm remains unknown.
There are two potential explanations of the defects in third arch neural crest migration. Firstly, the neural crest cells may no longer be able to perceive a positive signal expressed along their migration pathway, and secondly, they may not be able to detect a negative cue expressed in adjacent territories that restricts their migration. In light of experiments in other systems that implicate Eph receptors and ligand interactions in cell migration, a critical issue that must be addressed in order to understand third arch neural crest migration is determining the expression patterns and functions of ligands for both Sek1 and Elk.
Chapter Five.

Htk-L, A Ligand For Sekl And Elk.

5.1 Introduction.

The expression of dominant negative forms of Sekl and Elk in the Xenopus embryo disrupts the migration of the third arch neural crest, a process characterised by cells entering inappropriate territories. In order to understand these effects and the function of endogenous Sekl and Elk, it is important to know the distribution of the ligands they can interact. In vitro binding assays have shown that Elk can bind to all of the transmembrane class of ligands while Sekl can bind to Htk-L and Elk-L3 in this subclass (Gale et al., 1996b) (Gale et al., 1996a). As both of the receptors are able to interact with transmembrane ligands, a Xenopus cDNA library was screened for members of the transmembrane ligand subclass. The Xenopus homologue of Htk-L was previously isolated in my laboratory (Graham Alldus, unpublished) and in collaboration with Dr Vicky Robinson we set out to analyse the function of this ligand.

5.2 Results.

5.2.1 Expression pattern of Htk-L.

Dr Vicky Robinson showed by whole mount in situ hybridisation analysis shows that expression of Htk-L occurs in rhombomeres 2, 4 and 6, the eye, the second arch neural crest and in the mesoderm of the presumptive second arch (Figure 5.1). As described in chapter three, analysis with AP2 had demonstrated that during early migration of the cranial neural crest the populations of neural crest cells are juxtaposed (Figure 3.1), so it was important to confirm whether the domains of Sekl and Htk-L expression were also juxtaposed such that receptors and ligands might interact with one another. Whole mount in situ hybridisation was performed to simultaneously detect Sekl and Htk-L and revealed that the third arch Sekl expressing cells and the second arch Htk-L expressing cells are initially in contact with each other prior to and during the initial stages of migration (Figure 5.1). Thus, interactions could initially occur at the interface between the two domains of
Figure 5.1: Expression of Htk-L.

(A-C) Expression of Htk-L in stage 21 (A,B) and stage 26 (C) Xenopus embryos. Transcripts are detected in the eye, rhombomeres 2, 4 and 6, in the neural crest migrating to the second branchial arch and in a ventral domain contiguous with this neural crest. (B) A coronal section through this ventral domain reveals expression in the mesoderm. The expression of Htk-L on the left of the section is in the eye and the expression on the right is in the second arch neural crest.

(D-G) Double detection of Htk-L plus Sekl expression. Expression of Sekl (stronger staining) in the third arch neural crest (n3) and Htk-L (weaker staining) in the second arch neural crest (n2). Expression of Sekl and Htk-L are juxtaposed during neural crest migration. (D,E) show the neural crest streams as they commence their migration, stage 21, and (F,G) during their migration stage 24. (D,F) are lower power magnifications of (E,G) respectively.
expression. As the streams migrate further, however, the Sek1 expressing third arch stream and the Htk-L expressing second arch stream separate so that the streams are no longer in contact with one another. Since Elk has an overlapping domain of expression with Sek1 it can be assumed that it too could interact with Htk-L at the interface between second and third arch neural crest.

5.2.2 Overexpression of Htk-L.

Overexpression of truncated Sek1 and truncated Elk leads to a disruption in the migration of third arch neural crest with cells entering inappropriate anterior and posterior territories. Since Htk-L is expressed in second arch neural crest adjacent to third arch neural crest expressing Sek1 and Elk, it is possible that the function of Htk-L is to restrict the anterior movement of third arch neural crest. To test this idea the effects on neural crest migration by ectopic activation of Sek1 and Elk by widespread overexpression of Htk-L were observed. In such a case, overexpression of Htk-L would allow the receptors expressed in third arch neural crest to interact with Htk-L from all directions rather than unidirectionally at the border between the second and third arch neural crest.

As full length Xenopus Htk-L had not been isolated, mouse Htk-L cDNA was subcloned into pSP64T vector so that the insert was flanked by the untranslated regions of Xenopus β globin. 1-3 ng of synthetic mRNA derived from this construct was microinjected into one cell of two cell Xenopus embryos. The embryos were allowed to develop until stages 22-25 and the expression of Krox20 was subsequently analysed by whole mount in situ hybridisation. Phenotypes observed following injection of mRNA encoding Htk-L were characterised by disrupted migration of the third arch neural crest. During all stages of third arch neural crest migration, ectopic Krox20 expressing cells could be seen both anterior and posterior to the main stream of third arch neural crest (Figure 5.2A-D). Whilst the numbers of ectopic posterior Krox20 expressing cells in embryos overexpressing Htk-L was similar to those seen in embryos expressing the truncated receptors the numbers of anterior ectopic Krox20 expressing cells was greater. Some embryos showed discontinuous streams of third arch neural crest or abnormally shaped streams of third arch neural crest (Figure 5.2C).
Figure 5.2: Overexpression of Htk-L on cranial neural crest.

RNA encoding Htk-L was injected into one cell of a two cell stage Xenopus embryo. The embryos were fixed at stages 21-25 and in situ hybridisation was carried out to detect Krox20 (A-F) and Eck (E,F). (A,B) show dorsal views of flat mounted embryos. (B) The uninjected side is to the left and the injected side to the right. (C,D) Stage 24 embryos. (E,F) Show the injected side of embryos which have been simultaneously analysed for the expression of Krox20 (darker staining) and Eck (weaker staining). (E) Note the lack of neural crest cells adjacent to the second arch stream.

The black arrows indicate ectopic Krox20 expressing third arch neural crest cells which are detected either rostrally or caudally to the third arch stream. The white arrows indicate the normal stream of neural crest cells.
5.3 Analysis of the second arch neural crest.

To investigate whether widespread expression of Htk-L causes effects specific to third arch neural crest embryos overexpressing Htk-L were analysed with Eck, a molecular marker for second arch neural crest. Under these circumstances second arch neural crest appeared to be unaffected by the widespread expression of Htk-L, in contrast to the stream of third arch neural crest which showed ectopic Krox20 expressing cells (figure 5.2E, F). In some cases a crest free region could be seen between the second arch neural crest and the third arch neural crest suggestive of a precocious separation (Figure 5.2F).

5.4 Targeted microinjection of Htk-L.

As Htk-L is expressed in the mesoderm of the presumptive second arch as well as in neural crest it was important to ascertain whether the disruption to third arch neural crest migration caused by widespread expression of Htk-L is either due to specific effects on the third arch neural crest or was secondary to effects on the mesoderm. A1 and A2 blastomere injections were performed as described in section 4.2.5, and Krox20 expression was subsequently analysed. As with some of the injections performed with the truncated receptors, fluorescein-dextran lineage tracer was coinjected with Htk-L and was subsequently detected as a red colour reaction product. When ligand overexpression was targeted to the neural crest ectopic Krox20 neural crest cells were seen posterior to the third arch neural crest stream (Figure 5.3A). Sectioning of embryos injected with lineage tracer was carried out to confirm the correct targeting of the injected mRNA to the neural crest (Figure 5.3B) and the location of the ectopic Krox20 expressing cells in the territory of fourth arch neural crest. The lineage tracer was seen to overlap with the Krox20 expressing cells.
Figure 5.3: A1/A2 targeted blastomere injections with truncated \textit{Htk-L} coinjected with fluorescein.

\textit{Htk-L} RNA was injected into the A1/A2 blastomere of a 32 cell stage \textit{Xenopus} embryo. The embryos were allowed to develop until stage 23-25 before being fixed and analysed for the expression of \textit{Krox20} (blue) and fluorescein (red). (A,B) the black arrows indicate the presence of ectopic \textit{Krox20} cells. (B) The section through the embryo in (A) indicates the co-localisation of the ectopic \textit{Krox20} third arch neural crest cell in fourth arch territory and the injected fluorescein. The white arrow indicates the staining for fluorescein.
5.5 Summary.

_Htk-L_, a ligand for both _Sek1_ and _Elk_ is expressed in the second arch neural crest and mesoderm. This expression pattern is complementary to that of _Sek1_ and _Elk_ in the third arch neural crest and mesoderm. Since the expression of the truncated receptors sometimes leads to third arch neural crest entering second arch territory, the hypothesis that _Htk-L_ may play a role in the targeted migration of third arch neural crest was addressed. Widespread overexpression of _Htk-L_ leads to a disruption of third arch neural crest, with cells entering inappropriate adjacent territories. Second arch neural crest cell migration is however, unaffected. Finally, disruption of normal receptor and ligand interactions in the cranial neural crest by targeted blastomere injections was found to result in defects in the migration of the third arch neural crest.
Chapter Six.

Discussion.

6.1 Mechanisms underlying formation of neural crest streams.

Cranial neural crest cells migrate along stereotyped routes from specific rhombomeres of the hindbrain to specific branchial arches. It has been demonstrated in the chick that cranial neural crest cells migrate from rhombomere 2 to the first branchial arch, from rhombomere 4 to the second arch and from rhombomere 6 and posteriorly to the third and fourth branchial arches (Lumsden et al., 1991). Transplantation experiments performed in the chick demonstrate that when second arch neural crest cells are replaced with presumptive first arch crest cells, structures normally associated with the first arch form in the second arch (Noden, 1983) (Noden, 1988). This suggests the rostrocaudal identity of the cranial neural crest cells is established before they start their migration. Studies of the expression (Hunt and Krumlauf, 1991) and function (Rijli et al., 1993) (Gendron-Maguire et al., 1993) of the *Hox* genes suggests that they confer rostrocaudal identity to neural crest. In view of this early specification it would seem important that neural crest cells are targeted to the correct destination.

Several mechanisms have been proposed to account for the formation of the segmented streams and their correct targeted migration. In the chick and the rat it has been shown that areas of neural crest free mesenchyme exist which appear to separate individual streams of neural crest (Anderson and Meier, 1981) (Tan and Morriss-Kay, 1985). Studies performed in the chick have suggested that the crest free zones adjacent to rhombomeres 3 and 5 may arise through the selective apoptotic elimination of neural crest cells from these rhombomeres (Lumsden et al., 1991). It has been further demonstrated that the presence of the even numbered rhombomeres is necessary for the elimination of neural crest cells from rhombomeres 3 and 5 by maintaining the expression of the signalling molecule *BMP4* in rhombomeres 3 and 5 (Graham et al., 1993). Expression of *BMP4* mediates the upregulation of *msx2* and apoptosis in these rhombomeres (Graham et al., 1994).

Lineage tracing experiments performed in chick rhombomeres have demonstrated that individual cells and their progeny are mostly confined to a particular rhombomere once their boundaries have formed (Fraser et al., 1990). However, tracer injections into rhombomeres 3 and 5 has revealed that some neural crest cells originating from these rhombomeres do not die but instead migrate along rostral or caudal routes from these
rhombomeres to join cells arising from adjacent even numbered rhombomeres. This indicates that there is a restricted migration and guidance of the cranial neural crest cells that contributes to the formation of crest free zones (Birgbauer et al., 1995) (Sechrist et al., 1993).

In addition, it appears that the presence of the crest free zones are not essential to prevent adjacent streams of neural crest from mixing. In the chick this can be seen in the neural crest migrating from the midbrain and rhombomere 1, and from rhombomere 2 contributing to the anterior and posterior halves of the first arch respectively (Lumsden et al., 1991) (Kontges and Lumsden, 1996). A lack of intermingling between the streams of migrating neural crest can also be seen in rodent embryos which have been treated with retinoids. Treatment at late stages of embryogenesis induces the fusion of the first branchial arch with the second arch and of the trigeminal ganglia with the acousticofacial ganglia, however, the segmental pattern of migration of the first and second arch streams remains unaffected (Lee et al., 1995). The absence of mixing between juxtaposed migrating streams of neural crest can also be seen in *Xenopus*. Morphological studies have demonstrated the lack of neural crest free mesenchyme in the premigratory masses of cranial neural crest adjacent to the neural plate (Sadaghiani and Thiebaud, 1987). The neural crest masses segregate into separate streams as they migrate deep into the appropriate arches, with the streams of third and fourth arch neural crest only separating part way through their migration to their respective arches. By section analysis this study has confirmed the lack of crest free mesenchyme between the streams of neural crest cells prior to early during migration and the lack of any obvious physical barrier preventing the neural crest streams from mixing. Therefore, the areas of neural crest free mesenchyme may contribute to the segregation of streams in chick and in rodents but this is not the case in *Xenopus*. These observations suggest that normal segmental migration of cranial neural crest is dependent on the ability of the neural crest cells to interpret migratory cues and/or intrinsic differences between the streams which prevent their mixing.

### 6.2 Role of Eph receptors and their ligands in branchial neural crest.

I have found that the Eph RTK Elk is expressed in the premigratory masses of third and fourth arch neural crest in *Xenopus* and during their migration to the third and fourth branchial arches. Sek1 is expressed in the premigratory third arch neural crest mass adjacent to the neural plate and during its migration into the third branchial arch. There is, therefore, an overlapping expression of Sek1 and Elk in the third arch neural crest. The *Xenopus* homologue of Htk-L, a transmembrane ligand able to interact with both Sek1 and Elk, has been shown to have a complementary expression pattern to both receptors.
*Htk*-L is expressed in the second arch neural crest prior to and during its migration into the second branchial arch. These domains of expression allow the neighbouring streams of ligand-expressing second arch neural crest and receptor-expressing third arch neural crest to interact at the boundary (Figure 6.1).

As the *Sek1* expressing third arch neural crest begins to migrate there is also a band of *Sek1* expression in the mesoderm of the prospective third arch. Similarly, there is expression of *Elk* in the mesoderm of the third and fourth branchial arches which is maintained throughout neural crest migration. In addition, expression of *Htk*-L is also detected in a ventral line in the mesoderm of the presumptive second arch. The initial expression of *Elk*, *Sek1* and *Htk*-L in the mesoderm may correspond to the future migration route of the second, third and fourth arch neural crest cells to their respective branchial arches.

The expression patterns of *Sek1* and *Elk* in specific streams raises the question of whether they may mediate cell interactions during migration. To address this issue, embryos expressing *Sek1* or *Elk* lacking the intracellular kinase domain, were examined. As *Sek1* and *Elk* can both bind to and activate the transmembrane ligands, their overlapping expression domains in the third arch neural crest raises the possibility that they may have co-operative or overlapping functions. Therefore, embryos were injected with truncated *Sek1* or *Elk* alone or with both truncated receptors together. Embryos which expressed either truncated *Sek1* or truncated *Elk* alone showed phenotypes which were similar both in nature and proportion to embryos which co-expressed both truncated receptors. This is consistent with each of the truncated receptors being able to interfere with the function of both endogenous receptors, possibly by sequestering a common ligand.

Expression of *Sek1* and *Elk* truncated receptors in *Xenopus* embryos results in aberrant migration, anteriorly or posteriorly into neighbouring territories. During the initial stages of neural crest migration aberrant third arch neural crest cells can be detected in either second or fourth arch territory. In some embryos analysed for *Krox20* expressing third arch neural crest, cells were present in more caudal locations than during normal development and appeared to be migrating along the spinal cord. In other cases, ectopic cells were observed in fourth arch and/or second arch territory, either isolated from or contiguous with the third arch stream. In some embryos, after the initial abnormal caudal migration a stream of ectopic cells were seen to follow the unaffected cells towards the third arch. The presence of ectopic third arch neural crest cells in inappropriate territories could be due to these cells being unable to perceive a positive signal attracting them along their correct migration pathway or a negative cue that prevents them from entering adjacent territories. The phenotype seen in later stages of development, where third arch neural crest cells appear to change their direction of migration and migrate towards the correct branchial arch
Figure 6.1: Cell repulsion model of restricted cranial neural crest migration to the third arch.

The diagram depicts the expression domains of Sek1, Elk and Htk-L in the rhombomeres of the hindbrain, the cranial neural crest and the mesoderm. Htk-L is expressed in the second arch neural crest and mesoderm, adjacent to Sek1 and Elk expression in the third arch neural crest and mesoderm. Since the receptors and their ligand are expressed in adjacent territories and expression of the truncated receptors and widespread expression of Htk-L lead to defects in the migration of third arch neural crest, it suggest that the receptors and Htk-L mediate repulsion. Receptor-ligand interactions occur only on the rostral side of the receptor expressing cells at the rostral border of the third arch neural crest that are in contact with Htk-L expressing second arch neural crest or mesoderm. This would lead to a repulsion restricting third arch neural crest from migrating into second arch territory. The early expression of Sek1 Elk and Htk-L in the mesoderm of the presumptive second and third branchial arches may also be mediating repulsion between the mesodermal cells of the second and third branchial arches thus stabilising their domains.
rhombomeres

2 3 4 5 6

branchial arches

neural crest

mesoderm

Htk-L
Sek-1
Elk
Sek-1 + Elk
could arise because of other mechanisms also guiding the neural crest cells to their correct
destination. Based on the expression of *Htk-L* in adjacent second arch neural crest it is
possible that *Htk-L* may normally act to prevent the anterior migration of third arch neural
crest. To test this the effects of ectopic activation of *Sek1* and *Elk* by widespread
overexpression of *Htk-L* have been investigated. In embryos overexpressing *Htk-L*, third
arch neural crest cells contact cells expressing *Htk-L* from all directions and not just at the
border of the second and third arch. This results in a disruption to third arch neural crest
migration with cells entering inappropriate anterior and posterior territories and indicates
that *Htk-L* is required for the correct targeting of third arch neural crest. In particular,
embryos expressing the truncated receptors are unable to perceive a cue mediated by *Htk-L.*

As widespread expression of *Htk-L* also results in the presence of ectopic third arch neural
crest cells in inappropriate territories, *Htk-L* may mediate a repulsive cue that prevents third
arch neural crest from entering second arch territory. The expression patterns of *Sek1, Elk*
and *Htk-L* in the neural crest masses prior to and during early migration may thus reflect a
role in preventing the second and third arch neural crest streams from mixing. Indeed,
disruptions to the neural crest occurred after targeting of truncated receptors or of ligands to
the neural crest. Therefore, by inhibiting the activation of *Sek1* and *Elk,* the third arch
neural crest would no longer be able to perceive this repulsive signal and consequently
enter incorrect territory. Similarly, overexpression of *Htk-L* would mask the directionality
of the repulsion. The disruption to migration of third arch neural crest by embryos
overexpressing *Htk-L* may also be compounded by the fact that the third arch neural crest
cells are now expressing both *Htk-L* and its receptors and thus these neural crest cells
would repel each other. This mutual repulsion between individual neural crest cells may be
greater than the directional repulsion perceived from either the second or the fourth arch
neural crest thus, the third arch neural crest would abnormally migrate into second and/or
fourth arch territory. The caudal migration by third arch neural crest cells in embryos
expressing the truncated receptors may be due to masking of another as yet unidentified
ligand for *Sek1* expressed in the fourth arch neural crest. For example, a ligand which
repels *Sek1*-expressing cells more than *Elk*-expressing cells could be expressed in the
fourth arch mesoderm. This would prevent the third arch neural crest from migrating into
fourth arch territory. In order to ascertain whether this model is correct, and a whether an
equivalent mechanism is occurring in the first and second arch neural crest it is important to
identify more Eph receptors and their ligands which are expressed in these tissues.

Since receptors and ligands are expressed in both neural crest and mesoderm, targeted
blastomere injections were carried out to determine whether the abnormal migration of the
third arch neural crest cells in embryos expressing either the truncated receptors or *Htk-L,*
was due to disruption in their function in the neural crest cells or in the mesodermal cells.
The results of the targeted blastomere injections showed that expression of the truncated
receptors, or of their ligand, in the neural crest cells disrupt third arch neural crest migration. This suggests that the aberrant migration of third arch neural crest under these circumstances is due to an interference of receptor-ligand interaction in the neural crest itself. Blastomere injections of either the receptors or Htk-L targeting the mesodermal cells did not show a phenotype, however, this does not rule out a function for the mesodermal expression of Sek1, Elk and Htk-L as, this absence of a phenotype could be to technical reasons. One of these could be degradation of the injected mRNA and encoded protein by the time that the neural crest cells come into contact with mesodermal cells expressing either the truncated receptor or Htk-L after 21 hours of development. In addition, it could be that repulsion of the third arch neural crest by second arch neural crest is often sufficient to prevent the former from entering second arch territory.

6.3 Potential role in branchial arch mesoderm.

The function of Htk-L expression in the presumptive second arch mesoderm may be to prevent the receptor expressing third arch neural crest cells from migrating rostrally and entering second arch territory. Together with the repulsion by second arch neural crest the neural crest cells would be unable to migrate rostrally into the inappropriate branchial arch. However, this does not account for why expression of Sek1 and Elk occurs in the third arch mesoderm. One possibility is that the early expression of Sek1, Elk and Htk-L in the mesoderm of the presumptive second and third branchial arches may be mediating repulsion between the mesodermal cells of the second and third branchial arches, thus stabilising their domains.

6.4 Role of Eph receptors and their ligands in trunk neural crest.

This study has shown that Eph receptors Sek1 and Elk and their ligand Htk-L, are required for the correct targeted migration of third arch neural crest. It is proposed that this is mediated by repulsive mechanisms so that third arch neural crest does not mix with second arch neural crest. Interestingly, members of this RTK family and their ligands have been shown to have a central role in regulating movement of axons and neural crest in the trunk. Trunk neural crest cells, like cranial neural crest migrate in a segmental manner and move through the rostral and not the caudal half of each somitic sclerotome. It has been shown that cues inherent to the somite impose this pattern of migration (Bronner-Fraser and Stern, 1991). The molecular mechanisms responsible for this segmentation were previously unknown and it has been suggested that the caudal half of each somite may contain repulsive molecules which inhibit the migration of the trunk neural crest and later
the motor axons. A protein recognised by peanut agglutinin which inhibits both axon out
growth and neural crest migration has been found in the caudal somite (Krull et al., 1995)
(Davies et al., 1990). Recently, Htk-L and Elk-L have also been identified as inhibitory
molecules (Wang and Anderson, 1997) (Krull et al., 1987). Htk-L and Elk-L are
expressed in the caudal half of the somite, the area avoided by trunk neural crest.
Moreover, the trunk neural crest cells and motor axons have been shown to express Nuk, a
receptor which can interact with both of these ligands (Wang and Anderson, 1997). In
order to test the function of these ligands and their receptor, soluble Elk-L was added to
trunk explant cultures to inhibit receptor function. This resulted in the entry of trunk neural
crest into the caudal half of the somite (Krull et al., 1987). Furthermore, clustered soluble
Htk-L and Elk-L restrict the migration of trunk neural crest cells on a fibronectin substrate
in explant cultures (Wang and Anderson, 1997). Similarly, Htk-L and Elk-L cause the
collapse of the growth cones of motor axons although a guidance function has not yet been
shown. It is interesting to compare the situation in the trunk and the branchial region. In the
trunk, the crest cells express receptor (Nuk and Sek4) and the caudal half of the somites
express a ligand for them. Similarly, in the branchial region, third and fourth arch crest
cells express receptors (Sek1 and/or Elk) and second arch mesoderm expresses Htk-L.
Thus, this ligand could restrict the migration of trunk and cranial neural crest, albeit
through receptors. In contrast, Htk-L is expressed in second arch neural crest but not in
trunk neural crest. This may be due to the need to segregate the neighbouring populations
of cranial neural crest which have distinct rostrocaudal identities and prevent their
intermingling prior to and during their migration into their correct branchial arches.

6.5 Other mechanisms of neural crest guidance.

Previous studies have implicated a number of other mechanisms in neural crest migration,
and it seems likely that, as for axonal pathfinding (Tessier-Lavigne and Goodman, 1996),
multiple cues are utilised for guidance. It is useful to categorise these according to the range
of action and response to these cues. Firstly, these could be long-range diffusible cues that
either attract or repel specific neural crest cells. Secondly, these could be short-range or
contact-mediated attractive or repulsive cues and thirdly, there could be interactions
between neural crest cells that co-ordinate their migration. There is evidence for several of
these possibilities.

Several experiments have implicated long range diffusible factors in the migration of
cranial neural crest. In rhombomere rotation experiments performed in the chick, most of
the transplanted neural crest cells follow pathways which are appropriate for their new
location but some migrate towards their original destination (Sechrist et al., 1994) (Nieto et al., 1995). This would suggest the presence of long range attractive signals. Ectopically transplanted otic placodes lead to neural crest cells migrating towards the ectopic otic vesicle and ablation or partial ablation of the otic vesicle alters the pattern of neural crest cell migration which suggests the presence of a chemoattractant emitted from the otic vesicle (Sechrist et al., 1994). Additionally, it has been observed in rat embryos treated with retinoic acid at early stages of development, a process which transforms first arch neural crest cells into second arch identity, that some of these transformed cells aberrantly migrate into the second arch (Lee et al., 1995), perhaps indicating long range attractive signals. One alternate explanation for this experiment could be that neural crest cells of the first arch normally express an Eph receptor and neural crest cells of the second arch normally express its ligand, and thus repel first arch neural crest. When first arch neural crest is transformed into second arch neural crest with retinoic acid, the cells would now express the ligand and thus be able to migrate into inappropriate areas.

Short range or contact mediated signals could be mediated in part by extracellular matrix molecules. Laminin and fibronectin are extracellular matrix molecules which have been isolated and implicated in promoting cell migration. Laminin and fibronectin expression has been detected in the chick in regions of the cranial mesenchyme through which neural crest cells migrate (Krotoski et al., 1986). In the trunk, fibronectin and laminin immunoreactivity levels are uniform throughout the somite. An antibody recognising a 140-kD cell surface receptor which binds to both fibronectin and laminin has been detected on both trunk and cranial neural crest cells. Application of function blocking antibodies raised against a receptor for laminin and fibronectin lead to an effect on the initial migration of cranial neural crest cells. After application of the antibody the number of migrating neural crest cells was greatly reduced accompanied by ectopic neural crest cells building up within the neural tube and/or external to it (Bronner-Fraser, 1986b). Migration of the trunk neural crest is however, unaffected by the application of the function blocking antibody (Krotoski et al., 1986), indicating different roles for fibronectin and laminin in trunk and cranial neural crest migration.

There have been a number of adhesion molecules which have also been shown to affect neural crest cell migration. Injection of function-blocking antibodies raised against N-CAM or N-cadherin in vivo show effects on avian cranial neural crest migration with clusters of neural crest cells accumulating in the neural tube or at its periphery (Bronner-Fraser et al., 1992). Likewise, antibodies against tenascin cause similar defects to neural crest migration (Bronner-Fraser, 1988). This implicates cell adhesion and extracellular matrix molecules in providing a permissive mechanism for cranial neural crest migration, but it is not known whether they are involved in guidance.
Short range inhibitory molecules have also been identified in the chick. These may regulate migration of neural crest cells in the dorsolateral pathway. Molecular studies have shown the transient expression of PNA-binding activity and C6S-immunoreactivity in the dermamotyotome of dorsolateral pathway (Oakley and Tosney, 1991) It has been postulated that these molecules may delay the entry of neural crest cells into the dorsolateral pathway, since entry of neural crest cells into the dorsolateral pathway is accompanied by a decrease in the level of expression of these molecules (Oakley and Tosney, 1991). Deletion of the dermamyotome allows precocious migration of trunk neural crest cells along the dorsolateral pathway possibly by removing such inhibitory factors that normally prevent migration (Oakley and Tosney, 1991).

Interactions occurring between migrating neural crest cells which possibly co-ordinate their movement have been observed in time lapse studies. These have been performed in the chick and show that trunk neural crest cells travel in cohorts of 2-4 cells that stay closely associated as they migrate (Krull et al., 1995). This co-ordinated movement may be mediated by small processes that are visible between cells as they migrate and suggests that these cells may be mutually attracted such that they form tightly clustered streams.

6.6 General roles of Eph receptors and ligands.

The Eph receptors and their ligands have been implicated in the topographic organisation of the retinotectal system. The receptor Mek4 was found to be expressed in a gradient in the developing retina, with its concentration being highest in temporal retinal ganglion cells (Cheng et al., 1995); these project to the anterior tectum. Two GPI-anchored ligands, RAGS (Drescher et al., 1995) and Elf-1 (Cheng et al., 1995), which are able to interact with Mek4, are distributed in a posterior to anterior gradient with the highest levels in the posterior tectum. Thus, Mek4, and Elf-1 plus RAGS have complementary expression patterns in the retina and the tectum. In vitro studies have demonstrated that both Elf-1 and RAGS cause growth cone collapse and the repulsion of temporal and not nasal retinal ganglion axons (Drescher et al., 1995) (Nakamoto et al., 1996).

Repulsion mediated by an Eph receptor and its ligand has also been demonstrated to play a role in the establishment of other axons tracts in the developing brain. Mice with null mutations in the receptor Nuk have defects in the lateral projection of the anterior commissure. However, in mutants in which the intracellular tyrosine kinase is replaced by βgalactosidase the lateral projection of the anterior commissure is unaffected. Nuk mRNA is not expressed in the axons of the developing anterior commissural tracts but ventral to the axons in the preoptic area and the hypothalamus, that is, the area from which the axons
are excluded (Henkemeyer et al., 1996). In the Nuk null mutants, the axons of the anterior commissural tract invade a territory they do not normally enter. In wild type embryos this region corresponds to the domain of Nuk expression, suggesting that Nuk normally functions to repel axons from entering this territory. Htk-L and Elk-L, ligands for the Nuk receptor have been shown to be expressed in the axons of the anterior commissure (Henkemeyer et al., 1996). This implies that these ligands may themselves transduce signals.

Similarly repulsion may play a role in the patterning of the hindbrain (Xu et al., 1995). Expression of the truncated Sek1 receptor in Xenopus and zebrafish embryos leads to the presence of cells with rhombomere 3 and 5 identity in even numbered rhombomeres. This suggests that Sek1 may mediate the restriction of movement of cells from rhombomeres 3 and 5. Alternatively these phenotypes may indicate that Sek1 is required to maintain rhombomere cell identity. During hindbrain development cells are able to cross between rhombomeres prior to the formation of boundaries, however, using molecular markers ectopic odd or even numbered rhombomeric cells are never observed in a misappropriate territories. Therefore, this suggests that Sek1 may be involved in regulating cell fate changes. (Xu et al., 1995) (Xu et al., 1996).

The Eph receptors and their ligands may have other roles. For example, Eck receptor and its ligand B61. Eck and B61 are co-expressed in endothelial cells and their interaction has been shown to stimulate endothelial cell migration during TNF-α-induced angiogenesis (Pandey et al., 1995b). Consistent with this, anti B61 antibodies are able to block TNF-α-induced angiogenesis in a corneal neovascularization assay (Pandey et al., 1995b). Thus, an emerging theme is that the Eph receptors and their ligands may regulate cell and axon migration, in some situations by mediating repulsion and in others by stimulating migration.

6.7 Overlapping roles of receptors and ligands.

Whole mount in situ hybridisation analysis has shown that overlapping expression patterns tend to exist between ligands and receptors of the same subfamily (Gale et al., 1996b). To address the possibility of functional redundancy or co-operativity occurring between members of the Eph RTK family, null mutations of the Sek4 and Nuk genes have been generated in mice. In Sek4 null mutant mice the corpus callosum, the main axon tract which links the two side of the cerebellum, fails to form (Orioli et al., 1996). In double null mutant mice for Sek4 and Nuk the disruption to the corpus callosum is more severe than that seen in the single Sek4 or Nuk null mutant mice (Orioli et al., 1996). These results
indicate not only that Sek4 and Nuk are required for the pathfinding of the axons of the corpus callosum but that they have partially overlapping functions (Orioli et al., 1996). By analogy it seems likely that this may also occur in other Eph RTKs which have overlapping expression patterns. In the retinotectal system RAGS and Elf-1 have overlapping expression patterns in the tectum implying an overlap of function since both interact with Mek4 which is expressed on retinal axons. RAGS has however been shown to have a more posteriorly restricted distribution than Elf-1. Moreover, RAGS, has been shown to have a higher binding affinity to Mek4 and stronger repulsion affinity than Elf-1, thus, their distinct gradients of expression may together enable the correct projection of the retinal axons along the anteroposterior axis (Monschau et al., 1996). In this manner overlapping expression patterns may help to achieve the correct axonal targeting.

The overlapping expression domains of Sek1 and Elk in Xenopus third arch neural crest raises the possibility that they may have co-operative or overlapping functions in the third arch neural crest. As the percentage of phenotypes is similar in embryos expressing either truncated Sek1 or Elk alone or both truncated receptors together, they may have overlapping functions. Perhaps analogous to the situation occurring in the tectum, a ligand, able to interact both receptors, may be expressed in the fourth arch neural crest. If the ligand had a higher affinity for Sek1 than Elk it would ensure differential migration of the third arch neural crest in the same manner as that which guides nasal axons to their correct destination in the posterior tectum.

6.8 Roles of different receptor and ligand classes.

The Eph receptors and their ligands can be subdivided into two families based on their binding specificities (Gale et al., 1996b). The ligand subclasses also correspond to the two different methods of anchorage to the membrane, either with a transmembrane domain or a GPI linkage. This raises the question as to whether there are different functions for each subclass. For example in the retinotectal system, the GPI-anchored ligands, Elf-1 and RAGS and their receptor Mek4 are expressed in gradients along the anteroposterior axis in the tectum and the retina respectively (Drescher et al., 1995) (Nakamoto et al., 1996) (Cheng et al., 1995). In contrast, expression of a transmembrane ligand, Elk-L (Cek5-L), and its receptor, Nuk (Cek5), display a dorsoventrally restricted expression pattern which is restricted to the retina (Holash and Pasquale, 1995) (Kenny et al., 1995) (Henkemeyer et al., 1996) (Holash et al., 1997). In addition in the early chick embryo, it has been shown that the expression of Nuk is restricted to the ventral retina and its ligand(s) to the dorsal retina (Holash et al., 1997). Experiments performed on dissociated dorsal or ventral retinal cells and receptor or ligand Ig chimeras demonstrated that cell adhesion and neurite
outgrowth occur between the ventral retinal cells and the ligand chimeras and vice versa. In addition, *in vitro* experiments suggest that interactions between receptor and ligand expressing cells result in intracellular signalling as well as cell adhesion. However, the role of Nuk is unclear, but these experiments suggest that it is distinct from the other subclass of receptors.

Detection with Fc fusion proteins of the GPI-anchored ligand class and corresponding receptor class reveals complementary expression domains in many tissues (Gale *et al.*, 1996b) (Flenniken *et al.*, 1996). In a number of tissues this is corroborated by whole mount *in situ* hybridisation to detect the location of mRNAs of individual receptors and ligands. However, there is also an overlap of expression of some receptors and ligands as can be seen in the branchial arches and the somites. LERK4 and RAGS RNA are expressed in all branchial arches whereas receptor-Fc detects ligands only in the first, second and fourth branchial arches. Similarly, LERK4 and RAGS are expressed throughout the somites whereas receptor-Fc only detects ligands in the ventral part. The places where binding to ligands is not detected are precisely coincident with the expression of corresponding receptors. This anomaly could occur if the majority of the ligand is sequestered by an excess of receptor, so the receptor-Fc would be unable to compete with the endogenous receptor to bind to the ligand. The complementary and overlapping of receptor and ligand expression may reflect their different roles. The co-expression of Eck and B61 in endothelial cells results in the stimulation of their migration (Pandey *et al.*, 1995b). In contrast, the complementary expression of Mek4 and RAGS with Elf-1 in the retinotectal system underlies a repulsive mechanism required for the correct targeting of the retinal axons (Drescher *et al.*, 1995) (Cheng *et al.*, 1995). It will be interesting to see whether this relationship between expression patterns and role emerge as a general theme.

### 6.9 Potential role of signalling through ligands.

The C-terminal end of intracellular domain, including several tyrosine residues, of the transmembrane ligands, are highly conserved (Holland *et al.*, 1996) raising questions about their biological function. Studies on the Eph receptor Nuk, have shown that bi-directional signalling may occur through both the receptor and its transmembrane ligands (Holland *et al.*, 1996) (Henkemeyer *et al.*, 1996). Whereas mice homozygous for a null Nuk allele show aberrant migration of the posterior tract of the anterior commissure. However, mice in which the intracellular tyrosine kinase domain of the Nuk receptor has been replaced by βgalactosidase, do not show this phenotype (Henkemeyer *et al.*, 1996). Furthermore, *in vitro* experiments in which both Nuk receptor and either Htk-L or Elk-L were co-expressed in cells show that both receptor and ligand were phosphorylated (Holland *et al.*, 1996).
The ligand could be phosphorylated both in vivo and in vitro by an activated Src tyrosine kinase, implying that this may occur in vivo upon binding to receptor and that the transmembrane ligands themselves transduce signals (Holland et al., 1996). Signalling through the ligand may be mediating the repulsion of Htk-L expressing axons by the adjacent Nuk expressing cells (Henkemeyer et al., 1996). It is interesting to consider whether Htk-L could transduce a repulsion of second arch crest by third arch crest. To test this I have looked at the effects of widespread expression of the extracellular domain of Sek1 and Elk on second arch neural crest migration. As second arch neural crest migration remains unaffected by expression of truncated Sek1 and Elk it is unlikely that Htk-L is mediating repulsive cues to third arch neural crest. Although there is no evidence to imply that Htk-L is itself transducing a repulsive signal, these experiments do not rule out the possibility that this has a role in some other aspect of neural crest development.

6.10 Biochemistry of Eph receptors and ligands.

Little is known about the molecules which physically interact with the intracellular domain of the Eph receptors. However, as neuronal growth cones collapses upon interaction with Elf-1, RAGS, Htk-L and Elk-L, one possibility is that they may be involved in the regulation of the actin cytoskeleton. Rho, Rac, and Cdc42, members of the Rho GTPase family, have been demonstrated to be important in the control of the actin cytoskeleton (Tapon and Hall, 1997). In particular these molecules have been shown to be important in the formation of stress fibres, lamelipodia and filopodia. Interestingly studies in Drosophila have shown that expression of mutated forms of Rac or Cdc42 leads to defects in axonal and dendritic outgrowth (Luo et al., 1994). Rac or Cdc42 may be targets of biochemical pathway involving the Eph receptors. Time-lapse microscopy has shown that migrating neural crest extend filopodia which collapse upon contact with cells which are inhibitory for neural crest migration (Jesuthasan, 1996). If members of the Eph RTKs and their ligands are involved in regulating the migration of these cells, it is possible that members of the Rho GTPase family may mediate the collapse of the filopodia.

In vitro studies have shown that activated Eck receptor associates with the p85 subunit of the PI-3 kinase resulting in increased activation of PI-3 kinase (Pandey et al., 1994). Interestingly, PI-3 kinase has been implicated in membrane ruffling and chemotactic responses involving the actin cytoskeleton (Wennstrom et al., 1994). The activated Eck receptor has also been shown to specifically interact with SLAP, a Src-like adapter protein which contains a C-terminal Src homology SH3 or SH2 domain but lacks its own catalytic kinase domain (Pandey et al., 1995a). The exact phosphorylated tyrosine residues on the
Eck receptor which mediate these interactions are not known. However, the residues which mediate intracellular interactions of Sek1 have been established (Ellis et al., 1996a). Sek1 has two in vitro sites of tyrosine autophosphorylation located in the non-catalytic juxtamembrane region at positions Y596 and Y602 (Ellis et al., 1996b). In vitro binding assays indicate that the cytoplasmic tyrosine kinase p59fyn binds through interaction of its SH2 domain with the conserved motif around the Y602 location of Sek1. The Y596 site is thought to play a role in stabilising the interaction of the p59fyn SH2 binding site and Y602 site in Sek1 (Ellis et al., 1996b). The Y596 and Y602 autophosphorylation sites lie within the consensus sequences YV/EDP and TYEDP respectively and are conserved in many of the Eph family members (Ellis et al., 1996b). This raises the possibility that the Src family of kinase have general roles in transducing signals for the Eph receptors.

6.11 Future directions.

There are a number of approaches that can be taken to extend the studies described in this thesis, and further understand the role of Eph receptors and ligands in neural crest.

This includes the determination of whether other Eph RTKs and their ligands are involved in the targeted migration of cranial neural crest and requires the expression patterns of these genes to be examined to determine if any are expressed in cranial neural crest. The expression profiles of these receptors and ligands which are expressed in cranial neural crest need to be closely examined to determine whether they have reciprocal or overlapping expression patterns. These data may provide insights into the function of these genes. For example, it may uncover other receptors able to interact with Htk-L and would provide evidence that the first arch neural crest may be targeted in a similar manner to third arch neural crest. To ascertain the role of other Eph RTKs expressed in cranial neural crest, a dominant negative approach could be taken. Similarly overexpression studies on ligands expressed in the cranial neural crest could be used to determine their roles. In order to determine whether the GPI-anchored ligands and receptors play a role in cranial neural crest cell migration, a dominant negative approach should taken with Eck, which can bind to GPI-anchored ligands. The receptor Eck is normally expressed in second arch neural crest and therefore it will be important to analyse effects on the migration of this neural crest stream. A GPI-anchored ligand related to EhklL has recently been isolated (T. Khan, J. Smith, and D. G. Wilkinson unpublished observations) and found to be expressed in neural crest in Xenopus. It will be important to analyse effects of overexpressing this ligand. This also relates to the question of whether Sekl mediates effects of GPI-anchored ligands as well as transmembrane ligands in the neural crest. It will therefore be important to ascertain the binding affinity for Sekl of all the ligands that are expressed in neural crest.
To determine the different mechanisms employed by GPI-anchored or transmembrane ligands, a stripe assay performed using migrating neural crest could be informative. Stripes of GPI-anchored or transmembrane ligands could be used to determine whether both ligands mediate repulsive responses to cranial neural crest. This assay would be performed using cranial neural crest cells obtained from chick embryos as this species allows neural crest to be easily and cleanly isolated. By isolating distinct rhombomeres it is possible to obtain discrete streams and in this manner the effects of individual receptors or ligands on the different streams.

To enable the cellular behaviour of migrating neural crest cells to be followed, the stripe assay and time lapse analysis could be performed. In this way the behaviour of the filopodia of the neural crest cells at boundaries between receptor and ligand expressing cells could be determined. If a repulsive mechanism was indeed being utilised the filopodia would retract on encountering a boundary. These methods of analysis would also provide evidence for the distinct roles of the transmembrane and the GPI-anchored ligands.

*In vivo* time lapse cinematography of migrating neural crest cells would provide a more clear idea of the cellular responses involved in guiding neural crest migration, for example, that repulsion occurs between neural crest streams. The effects of expressing dominant negative receptor and ligand could then be analysed in time lapse to reveal the effects on cell behaviour.
Bibliography.


Goto, T., MacDonald, P., and Maniatis, T. (1989). Early and late periodic patterns of even-skipped expression are controlled by distinct regulatory elements that respond to different spatial clues. Cell 57, 413-422.


### Appendix

Table Of Number Of Embryos With Disruptions To Neural Crest Migration.

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<th>Date</th>
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<th>ΔSek-1 + ΔElk</th>
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The table indicates the number of embryos in which abnormal migration of third arch neural crest was observed (number of abnormal/total) after injection of RNA encoding truncated Sek-1, truncated Elk or coinjection of both. Each line corresponds to a different batch of *Xenopus* embryos. There was a variability in the number of affected embryos between different batches of embryos and RNA preparations, so comparisons can be made within each batch, but not between batches or the net total.