Cell Signalling in
Limb Development
and Hindbrain Segmentation

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ABSTRACT

Receptor protein tyrosine kinases are important for the regulation of cell growth, differentiation and pattern formation. To date 14 subfamilies are known, of which the Eph subfamily is the largest. In this thesis I examine the regulation of expression of the Eph-related Cek-8 gene during limb development and hindbrain segmentation.

Cek-8 was found to be expressed in the mesenchyme at the tip of chick limb buds with high levels of transcripts posteriorly and apically but fading out anteriorly. Expression of Cek-8 in distal mesenchyme was found to be regulated by signals from the apical ridge, which could be substituted by FGF, and also by signals from the polarising region and by retinoic acid. Cek-8 expression was uniform across the antero-posterior axis of limb buds in talpid^2, a chick mutant with polydactylous limbs with up to 7-8 morphologically similar digits. These findings indicate that Cek-8 expression responds to regulatory signals during limb patterning and suggest that this receptor tyrosine kinase may have a role in coordinating responses to signals in the progress zone of early buds. Later on in limb development, Cek-8 expression was found to be associated with cell condensations that form tendons and their attachments to cartilage rudiments and then in developing feather buds.

Cek-8 is also expressed in the developing hindbrain in rhombomeres 3 and 5. Local treatment with retinoic acid led to a partly unsegmented hindbrain and this loss of segmentation (or boundaries) was found to be preceded by changes in segmental gene expression of Cek-8. Loss of boundaries was always correlated with changes in Cek-8
expression. Changes in \textit{Hox} (homeobox containing genes) gene expression were only apparent after morphological changes occurred and did not seem to impose a new identity on neural precursor cells. Normally, cells from adjacent rhombomeres do not mix and no substantial cell mixing could be detected even in the absence of a physical boundary. Possible roles of \textit{Cek-8} during hindbrain segmentation are discussed.
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CHAPTER ONE: General Introduction

The work presented in thesis describes factors which, directly or indirectly, regulate the expression of Cek-8, a member of the Eph subfamily of receptor protein tyrosine kinases, during chick limb and hindbrain development. In this chapter I will therefore first introduce the Eph receptor tyrosine kinases and their ligands, and then summarise hindbrain segmentation and limb development.

1 Receptor Protein Tyrosine Kinases

Cells in a multicellular organism need to communicate with one another in order to regulate their organisation into tissues, to control their growth and to coordinate their functions. There are three possible ways of cellular signalling in a multicellular organism: (1) A stimuli is either secreted to signal over a long distance; (2) a signalling molecule is bound to a cell’s membrane and signals only to neighbouring cells; and (3) gap junctions, directly joining the cytoplasms of neighbouring cells, allow the exchange of small molecules. For the purpose of this thesis, I will briefly focus on one of the mechanisms used by multicellular organisms to integrate long range (1) and contact dependant (2) signals: the use of signalling molecules and their receptor protein tyrosine kinases (RPTK).

These receptors were originally identified as cellular homologues of viral oncogenes that confer tumorigenicity (Schlessinger & Ullrich 1992). RPTKs are type I transmembrane proteins and they possess an extracellular, a transmembrane and a
catalytic intracellular protein tyrosine kinase domain. In general, receptors homo- or heterodimerize upon ligand binding, which in turn activates the catalytic activity of the intracellular tyrosine kinase and leads to trans-autophosphorylation of tyrosine residues in the intracellular region. Src like kinases, or adapter proteins (both intracellular), relay signals from activated RPTKs by binding to phosphorylated tyrosine residues usually via a SH2 (src homology 2) domain. This is the starting point for an intracellular signalling cascade which in most occasions will modulate gene expression.

A large number of RPTKs have been described and these are currently grouped in 14 subfamilies and I will limit my attention to the Eph subfamily.

2 Eph Receptor Protein Tyrosine Kinases

2.1 Brief History

The Eph subfamily is the largest known subfamily of RPTKs. The first member of this subfamily was isolated by Hirai et al (1987). The tyrosine kinase domain encoding the viral Fps protein (membrane associated protein tyrosine kinase) was used to screen a human genomic DNA library under low stringency. With the newly isolated probe they screened a erythropoetin-producing human hepato-cellular carcinoma cell line (ETL-1) cDNA library and isolated a novel putative RPTK: Eph. Since 1987 many different approaches led to isolation of new members of the Eph subfamily in several vertebrate species (Figure 1). Letwin et al (1988), for example, isolated protein tyrosine kinases involved in neural development by screening a rat brain cDNA expression library (λGT11) with a monoclonal antibody raised against phospho-tyrosine. One of the two cDNAs isolated was related to Eph (Elk) and the
other one to Fps (Flk). Another approach was to screen a rat brain cDNA library with a probe encoding for the kinase region of the insulin receptor under relaxed conditions and this led to the identification of a new human (EEK, ERK) as well as a new rat member of the Eph family (Chan & Watt, 1991). Other cloning strategies, including use of degenerate oligo-nucleotides from conserved regions within the kinase domain of many protein tyrosine kinases to amplify sequences from human epithelial cell line (HeLa) or rat brain cDNA libraries has also led to isolation of new members of the Eph family (Eck, Lindberg & Hunter 1990; Ehk1, Ehk2 Maisonpierre et al 1993). Screening of embryonic chick cDNA libraries with an Eph-related probe (Cek-4 or Cek-5) proved equally successful and led to identification of 5 new members, named Cek-6 to Cek-10 (Sajjadi & Pasquale, 1993). Only some of the various strategies which led to isolation of new members of the Eph subfamily are listed here to illustrate the importance and the abundant recruitment of these molecules during proliferation and also differentiation.

So far at least 13 members of the Eph subfamily have been isolated (see also figure 1) in humans (Hirai et al 1987 (EPH), Lindberg & Hunter 1990 (ECK); Chan & V Watt 1991(ERK); Wicks et al 1992 (HEK); Boehme et al 1993 (HEK2); Bennett et al 1994 (HTK); Kiyokawa et al 1994 (ERK); Fox et al 1995 (HEK4/HEK, HEK5/ERK, HEK7, HEK8, HEK11). mouse (Sajjadi et al 1991(Mek4); Gilardi Hebenstreit et al 1992 and Nieto et al 1992 (Sek1); Andres et al 1994 (myk1 and myk2); Zhou et al 1994 (Bsk); Ganju et al 1994 (Eck); Becker et al 1994 (Sek2-4); Henkemeyer et al 1994 (Nuk /Sek3); Ciossek et al 1995 (Mdk1); Ciossek et al 1995 (Mdk2 (myk1), Mdk5); Ellis et al 1995 (Ebk/Mdk1)), rat (Letwin et al 1988, (Elk (entire cDNA cloned by Lhotak et al 1991)); Chan & Watt (eek); Lai & Lemke 1991 (Tyro4-Tyro6, Tyro11); Maisonpierre et al 1993 (Ehkl, Ehk2); Valenzuela et al 1995 (Ehk3); Winslow et al 1995 (REK 7)), chick (Sajjadi et al 1991 (Cek4); Pasquale 1991
Eph Receptors

(Cek5); Sajjadi & E Pasquale 1993 (Cek6-Cek10), xenopus (Winning & Sargent 1994 (Pagliaccio); Jones et al 1995 (Xek); Xu et al 1995 (X Sek l); Weinstein et al 1996 (Xe10) and zebrafish (Xu et al 1994 (Rtk1-3)).

2.2 Structure

The Eph subfamily of RPTKs share some characteristic structural features as deduced from predicted amino-acid sequences and biochemical analysis. The extracellular portion comprises an immunoglobulin-like domain similar to those found in the fibroblast growth factor receptors (FGFR) and platelet derived growth factor receptor (PDGFR), a conserved region with 20 cysteine residues, and two fibronectin type III repeats (Friedman and O'Leary 1996). Cysteine residues may form disulphide bonds and link two parts of a single polypeptide chain (in the case of the Eph subfamily) thereby helping the protein to attain a particular shape, possibly an important feature for ligand binding or dimerisation. Fibronectin type III repeats are thought to be important for cell to cell interactions and have also been found in many neural adhesion molecules (Friedmann & O’Leary, 1996). The extracellular domain of the Eph subfamily also contains potential glycosylation sites. Glycosylation has been demonstrated for only one member so far (Soans et al 1994; Ellis et al 1996). Cek-8 has 3 glycosylation sites on the carboxy (C)-terminal half of extracellular domain and not more than 10 % of its mass is accounted for by carbohydrates. Eph RPTKs have only one membrane spanning region which can be flanked by a basic sequence on the intracellular side (Hirai et al 1987). This is in fact a common feature for the junction between the membrane and the cytoplasmic domain of many cell surface receptor (van der Geer et al 1994). Furthermore the intracellular domain comprises a single tyrosine kinase unit,
several tyrosine residues (potential phosphorylation sites), and a non-catalytic tail of about 100 amino acids (van der Geer et al 1994).

2.3 Ligands

Ligands for the Eph subfamily have only recently been identified. Bartley et al (1994) screened concentrated supernatants from several cell lines with the recombinant, truncated extracellular domain of the ECK receptor covalently linked to the surface of a BIAcore sensorchip. Several supernatants contained Eck binding material, and conditioned medium of two of those, SK-BR3 and HCT-8 (the origin of these cells is not revealed in the original work), were concentrated and loaded onto columns of immobilised extracellular domain of the ECK receptor. Eluates of the columns were analysed by electrophoresis and were found to be enriched in one wide band (Mr 21000-28000). N-terminal sequencing revealed that this band contained a single protein: B61. This protein had previously been isolated as a product of a novel immediate-early response gene induced by tumor necrosis factor (TNF) or interleukin 1 in human umbilical vein endothelial cells (HUVEC, Holzman et al 1990). B61 is a secreted protein and is attached to the cell membrane via a glycosylphosphatidyl inositol (GPI) anchorage (Holzman et al 1990, Shao et al 1995b). In parallel, Beckmann et al (1994) screened a human placental cDNA expression library for the ELK ligand by transfecting cells (CV-1/EBNA) on slides with pools of cDNA clones and testing them for the ability to bind ELK-Fc, a fusion protein consisting of the extracellular domain of the human ELK receptor and the Fc portion of the human immunoglobulin IgG. The Fc portion was used to identify which of the transfected cells bound to the ELK-Fc fusion protein by means of an anti-human IgG1 F(ab)2 antibody. A pool of cDNA clones was thereby identified and further screened until a single clone binding to ELK-Fc was
isolated. The largest open reading frame appeared to encode for a potential ligand: a unique type I transmembrane protein with a leader sequence, an extracellular domain containing 4 cysteine residues and a potential glycosylation site, a transmembrane and a cytoplasmic domain. Interestingly the sequence identified shared limited sequence identity with the B61 protein.

Several novel ligands were isolated in different vertebrate species (see also figure 1, humans (Bartley et al 1994 (B61), Beckmann et al 1994 (B61/LERK1, LERK2); Davis et al 1994 (B61/EF1/LERK1, EHKL1/ EFL2/ LERK3, ELK-L/EFL3/LERK 2); Bennett et al 1995 (Htk-ligand/LERK5); Kozlosky et al 1995 (LERK3, LERK4); Winslow et al 1995 (AL-1)), Lackmann et al 1996 (HEK-ligand/AL-1), mouse (Cheng & Flanagan 1994 (Elf1)); Shao et al 1994 (Cek-5 ligand), Bergemann et al 1995 (ELF-2); Takahashi & Ikeda 1995 (B61); Bennett et al 1995 (Htk-ligand/ ELF-2 ); Shao et al 1995 (Cek7 Ligand/Elf1)), rat (Takahashi & Ikeda 1995 (B61)), chick (Drescher et al 1995 (RAGS)), Xenopus (Weinstein et al 1996 (XELF-a)) and subdivided into two groups according to their membrane attachment. Eph ligands are attached to the cell membrane either via a GPI link (B61, LERK3, AL1, LERK4, ELF1) or via a transmembrane domain (LERK2, LERK5).

Membrane anchorage seems to be crucial for receptor activation (Davis et al 1994, Winslow et al 1995). Davis et al (1994) layered different forms of the ligands - soluble, membrane bound, or clustered- on top of reporter cell lines expressing either ELK (NIH3T3 cells) or EHK1 (C2C12 cells) and used receptor phosphorylation as an indicator for its activation. Tyrosine phosphorylation of ELK or EHK1 was only stimulated when ELK-L or B61 was available in a clustered or membrane bound form. In another experiment they analysed the growth response of reporter cells expressing a
receptor chimera with the extracellular domain of the ECK receptor fused to the cytoplasmic domain of the FGFR upon stimulation with either soluble or clustered ligand. Consistent with their initial observation EHK1-L and B61 were only able to induce proliferation of the reporter cells when they were presented to the receptor in a clustered form. These results suggest that ligands for the Eph-related receptors require cell to cell contact for receptor activation.

Furthermore these results also suggest that the same ligand can interact with several receptors and this promiscuity of the ligands has also been reported in other studies (Cheng & Flanagan 1994, Beckmann et al 1994, Bergemann et al 1995, Shao et al 1995, Brambilla et al 1995). Indeed, Gale et al (1996) recently grouped the Eph receptors and ligands into two major specificity subclasses based on their in vitro binding specificities. One subclass of ligands (GPI-linked: B61, Ehk1-L, LERK4, AL-1, and ELF-1 ) binds and activates one class of receptors (ECK, EHK1, EHK2, EHK3 and SEK1) whilst the other subclass of ligands (transmembrane : LERK2, LERK5 and Elk-L3) binds and activates the second class of receptors (ELK and NUK, see figure 1).

The precise signal transduction pathway following ligand binding to the Eph receptor has yet to be defined. B61 for example binds ECK and induces its autophosphorylation on a tyrosine residue (Davis et al 1994). Using the yeast two-hybrid screen with the cytoplasmic domain of ECK as bait, Pandey et al (1994, 1995) showed that the p85 subunit of phosphatidylinositol 3-kinase and SLAP (a new Src-like adapter protein lacking a tyrosine kinase domain) bound to the activated receptor via their SH2 domains. Similar tyrosine residues on the juxtamembrane (intracellular)
domain of SEK are autophosphorylated (Ellis et al 1996) and this mediates the association with FYN, another SH2-domain containing protein.

2.4 Expression of Eph receptors and Their Ligands


Recently Gale et al (1996) have classed the receptors and ligands into two major specificity subgroups and analysed their respective binding profiles in whole embryos. Embryos were either stained with soluble receptor bodies to visualise the distribution of their ligands or soluble ligand bodies (soluble ligand portion fused to antibody Fc domain) to define the distribution of their receptors. Receptors and also ligands of a particular subclass all identified similar ligand and receptor patterns respectively. Furthermore this study revealed that receptors and ligands (within a subclass) are expressed in a complementary fashion in many embryonic tissues and the authors suggest that this reciprocal expression compartmentalises the developing body plan, where ligands and receptors interact at their mutual expression boundary.
Figure 1: EPH receptors and their ligands

This figure is adapted from Gale et al 1996. Receptors are shown on the right and ligands on the left side. Receptors are divided into two groups: Elk- and Eck related receptors. Ligands are also divided into two groups: Trans-membrane and GPI-anchored ligands. Elk-related receptors bind the trans-membrane ligands whereas Eck-related receptors bind the GPI-anchored ligands. The Eck-related receptor Sek-1 is unique in that it binds to both type of ligands.

c, chicken; x, xenopus; h, human; m, mouse; r, rat; z, zebrafish.
2.5 Function of *Eph* Receptors and Their Ligands

2.5.1 Axon Guidance by Repulsion

The identification of ligands has shed light on the possible functions of *Eph*-PTKs and their ligands primarily in neural development. The biological activity of these molecules has been recently implicated in axon repulsion and the development of topographic maps (Cheng et al 1995 and Drescher et al 1995). The organisation of most axonal connections in the brain is topographical, where one neuronal population maps precisely onto another. In the visual system, axons from the retina project to the optic tectum (superior colliculus in mammals). For example, anterior (nasal) dorsal (superior) retinal ganglion cells project to the posterior ventral tectum, so that the map of the tectum is inverted with respect to that of the retina (Tessier-Lavigne 1995; figure 2).

These observations led to the design of two *in vitro* assays to analyse the molecular control of the development of a topographical map. In the membrane stripe assay, nasal (anterior) and temporal (posterior) retinal axons are grown on alternating lanes of anterior or posterior tectum. Nasal axons do not display any substrate preference whereas temporal retinal axons prefer to grow on anterior tectum derived stripes. Brief heat or protease treatment (Walter et al 1987) and also incubation with phosphatidylinositol-specific phospholipase C (PI-PLC, Walter et al 1990) converts posterior membranes to a substrate for temporal axons. The growth preference of temporal axons on anterior tectum has therefore been assigned to a repellent activity of the posterior tectum which is caused by GPI-linked molecules. This activity is present when the retinal axons arrive at the tectum (E6) and vanishes after E12, when all but a
few of axons have already connected to their targets (Walter et al 1987). In another assay, the growth cone collapse assay, temporal growth cones collapse once exposed to posterior tectal membranes (Stahl et al 1990, Cox et al 1990) reflecting the repellent activity of posterior tectum described by Walter et al (1987). A repellent factor therefore has to satisfy three criteria: It has to be GPI-anchored and expressed at higher levels in the posterior tectum between E6 and E12.

In order to isolate new candidate molecules for this repulsive activity found in the posterior tectum, Drescher et al (1995) used two dimensional gel electrophoresis to compare GPI anchored proteins from anterior and posterior chicken tectal membranes. They isolated a protein termed RAGS (repulsive axon guidance signal), which fulfils all three postulated criteria of a putative repellent protein in the optic tectum.

RAGS is a GPI-linked ligand for the Eph class of RPTKs, and is detected at higher levels in deep layers of the posterior tectum during retinotectal innervation (E6-E13). Although retinal axons might never traverse those deep layers it is conceivable that the RAGS expressing cells comprise radial glia that extend processes into more superficial (i.e. retinorecipient) ones.

COS cells transfected with RAGS cDNA mimic the repelling and growth cone collapsing activities of posterior tectum on temporal growth cones but interestingly has the same effect on nasal retinal axons. Since the biological activity of RAGS in vitro differs from the activity of posterior tectal membranes it may be that, in vivo, RAGS-associated molecules may modulate the repellent activity.
In another study, Winslow et al (1995) identified a receptor-ligand pair, REK-7/AL-1, which is involved in axon bundle formation. They tested the function of this pair of molecules in cultures of cortical, REK-7 expressing, neurons grown on monolayers of AL-1 expressing astrocytes. Cortical neurons normally fasciculate (bundle) in such cultures but the addition of soluble form of either receptor or ligand inhibited that process. Winslow et al (1995) suggest that this receptor-ligand pair may activate bundling by regulating fasciculation-protein expression but it could well be that AL-1 in astrocytes has a repellent activity and this encourages axons to grow on each other's surface (Keynes & Cook 1995, Tessier-Lavigne 1995).

In parallel Cheng et al (1995) described complementary gradients in expression and binding of ELF-1 (GPI linked ligand) and Mek4 (Eph-receptor) in development of the topographic retinotectal projection map in the mouse. Elf1 transcripts were found at highest levels in the posterior tectum, whilst Mek4 (but not Sek) mRNA was least abundant on the nasal retina. Neurons with high levels of Mek4 receptor expression (temporal retina) connect to areas in the tectum expressing low levels of Mek4-ligands (anterior tectum). Using a novel technique termed RAP (receptor affinity probe or receptor alkaline phosphatase) ligand binding activity can be localised with a soluble chimeric receptor-alkaline phosphatase fusion protein. By confronting tectal tissue from different antero-posterior levels with a chimeric MEK4 protein (the extracellular domain from MEK4 fused to alkaline phosphatase) they demonstrated that MEK4-ligand binding activity is highest in posterior tectal tissue. This activity and the complementary expression gradients of Mek4 and Elf-1 fit well with the activity of RAGS (Drescher et al 1995). High levels of ligand in the posterior tectum repel posterior (temporal, receptor expressing) retinal axons and they preferentially connect to the anterior tectum. Similarly, axons lacking the receptor (anterior/nasal) exhibit no
difficulties connecting to a region with high levels of ligands (posterior), although the mechanism used for guidance in this particular case remains unknown.

*Nuk* has also been implicated in axon guidance by controlling pathfinding of commissural axons in the mammalian central nervous system (Henkemeyer et al 1996). In mice lacking NUK protein the majority of the axons forming the posterior tract (acP) of the anterior commissure deviate from their normal path in to the ventral floor of the forebrain. *Nuk* expression analysis demonstrated that posterior tract axons of the anterior commissure did not express *Nuk*, and that these axons seemed to migrate along a path lacking *Nuk* expression. Furthermore they found *Lerk2* (a NUK-ligand) to be expressed in these and also other axons of the anterior commissure. The authors suggest therefore that NUK has a repulsive effect on acP axon migration thus ‘forcing’ ligand expressing acP axons to migrate in a receptor free area.

### 2.5.2 Forebrain Patterning

Using the dominant negative approach, Xu et al (1996) showed that the inhibition of RTK1 signal transduction in zebrafish led to an expansion of eye tissue into the diencephalic region. In the forebrain, *Rtk1* is expressed at high level in the dorsal diencephalon and at a lower level ventrally at 12 hours of development. When overexpressing a truncated *Rtk1* receptor, alterations to the *Rtk1* expression pattern were observed. Non-*Rtk1* expressing cells were now detected within the dorsal expression domain and reduced levels of transcripts were noticed ventrally. Furthermore, at 24 h of development *Pax6*, an eye marker normally expressed in the dorsal diencephalon, is now ectopically expressed in the ventral diencephalon. By labelling cells of presumptive ventral diencephalon they elegantly demonstrated that
those cells undergo a fate switch and are now incorporated into the expanded retina in embryos with blocked RTK1 activity. This implies a role for RTK1 in patterning the diencephalon.

2.5.3 Angiogenesis

Endothelial cells form the lining of blood vessels. They are responsible for blood supply and have a capacity to adjust their numbers and arrangement to suit local requirements. New vessels originate as capillaries sprouting from existing small vessels. This process is called angiogenesis and occurs in response to specific signals (Alberts et al 1992). B61 was originally identified as a secreted product of a novel immediate-early response gene induced by tumor necrosis factor -α (TNF-α) or interleukin 1 in human umbilical vein endothelial cells (Holzman et al 1990). Pandey et al (1995) demonstrated that the ligand-receptor pair B61/Eck mediates TNF-α- but not FGF-induced angiogenesis. Furthermore, they assigned the activity of B61, in vitro, to endothelial cell migration rather than proliferation.

Interestingly Eph is overexpressed in many tumors (Hirai et al, 1987, Wicks et al 1992) and overexpression of Eph has been implicated in transforming NIH3T3 cells (Maru et al 1990). Cells overexpressing Eph grow on soft agar (i.e. are anchorage independent) and induce tumors in nude mice. The growth of a tumor depends on the generation of new vessels (angiogenesis) to assure sufficient blood supply. It would be interesting to test whether the transforming activity of Eph is in any way linked to the angiogenic activity of B61/Eck. Antagonists to these receptors could prove a powerful tool against tumors.
Figure 2: Topographic projection and distribution of Eph tyrosine kinase receptors and their ligands in the retina and the optic tectum.

This figure is adapted from Tessier Lavigne (1995). (A) The letters "Read me" are projected upside down onto the retina. This projection is then inverted and projected upside down onto the optic tectum. This topographic projection is achieved because (Cek-5 expressing) axons from the ventral retina project to the dorsal tectum (B). Note there is a gradient of Cek-5 expression in the retina with highest levels of expression at ventral level. (C) Similar posterior retinal (Mek-4 expressing) neurons project onto the anterior tectum, where RAGS and Elf-1 are not expressed. Note the gradient of Mek-4 expression in the retina where transcripts are most abundant posteriorly. Elf-1 expression and RAGS activity is highest in the posterior and lowest in the anterior tectum. Any retinal neuron expressing the eph receptor is repulsed if it connects to an area with high levels of ligand activity.
2.5.4 Hindbrain Segmentation

*Eph* receptors have been implicated in vertebrate hindbrain segmentation (Xu et al, 1995—see later). Since the results described in chapter four deal with some aspects of molecular and cellular segmentation in the chick hindbrain I devote a substantial part of my introduction on hindbrain segmentation (see below, 3. Hindbrain Segmentation).

2.6 Future Prospects

The biological function of the numerous isolated receptor and ligands remain to be tested and this can be done either *in vitro* as elegantly demonstrated by Drescher et al (1995) or *in vivo* (Xu et al 1995, 1996). Mice lacking ECK activity do not display any obvious abnormal phenotype but it will be interesting to test them under certain pathological conditions (Chen et al 1996). Since B61 (ECK-ligand) induces angiogenesis, analysing the wound healing process (accompanied by neovascularisation) in these mice could prove a useful biological assay. Since the receptors and also ligands are broadly expressed in neural tissues, neurological defects, which are more difficult to detect, might also be expected in mice lacking either receptor or ligand.
3 Hindbrain Segmentation

Segmentation is fundamental to the organisation of the body plan in arthropods, annelids, chordates and possibly even in vertebrates. In vertebrates mainly three regions are thought to be "segmented", at least at morphological level: The somitic mesoderm, the forebrain and the developing hindbrain. The original description of segments in the hindbrain (termed rhombomeres) led to two different views of their importance (reviewed in Lumsden 1990): On the one hand, rhombomeres may be artefacts of fixation or 'transient embryonic structures resulting either from longitudinal compression of the neural tube or to the local strain of related nerves'. On the other hand, rhombomeres may be remnants of a primitive segmentation of the vertebrate nervous system. Evidence of cellular and also molecular segmentation support this latter interpretation, that rhombomeres may represent segmental developmental units.

3.1 Segmentation on a Cellular Basis

3.1.1 Neurogenesis

Rhombomeres are transient bulges in the vertebrate hindbrain neuroepithelium. The chick hindbrain consists of eight distinct rhombomeres (Lumsden 1990), whose formation begins at Hamburger & Hamilton (1951) stage 9- and is completed by stage 12. The rhombomere 5/6 boundary is the first to emerge, and three stages later all rhombomeres have formed. At stage 24 rhombomeres have "disappeared", at least at morphological level. Many neurons and most of the basic structures of the hindbrain
are formed during the period where rhombomeres are visible (Lumsden 1990).

In the chick hindbrain, the first signs of neurogenesis are apparent within alternate rhombomeres. Immunoreactivity with an anti-68K neurofilament antibody revealed that reticular neurons first appear at stages 13 and 14 in even rhombomeres (r2, r4 and r6; Lumsden & Keynes 1989). Later this alternate pattern of neurogenesis is diminished and by stage 17 reticular neurons can be found in both odd and even rhombomeres. Thus the timing of neuronal differentiation in the hindbrain occurs in a segmental manner which reflects its rhombomeric organisation (Lumsden & Keynes 1989).

The formation and disposition of cranial nerve roots and of branchio-motor neurons also reflects a segmental organisation. Cranial nerve roots are located at precise positions with respect to individual rhombomeres. For example, the Vth (trigeminal) nerve root is positioned at r2- whilst the VIIth (facial) nerve root is found at the level of r4. Furthermore, retrograde labelling of the Vth, VIIth and IXth nerve roots reveals a close relationship between the position of each of the branchio-motor nuclei (V, VII and IX) and their respective roots. More importantly, each motor nucleus recruits its motor neurons from two adjacent rhombomeres. For example, r2 and r3 branchio- motor neurons form the trigeminal nucleus, while branchio-motor neurons from r4 and r5 form the facial motor nucleus. The first, second and third branchial arches are innervated by branchial nerves V, VII and IX, respectively. Thus the position and innervation of each of the three principal branchial arches equally reflects the segmental organisation of the hindbrain during early development (figure 3, Lumsden & Keynes 1989).
Figure 3: Hindbrain segmentation

From Lumsden and Keynes 1989. (A) In the chick hindbrain rhombomere boundaries arise sequentially, beginning at stage 9- (Hamburger and Hamilton 1951, HH). By stage 12 (16 somites (s)) all rhombomere boundaries are formed and at least seven rhombomeres are visible.

(B) Diagram of a 3 day chick embryo hindbrain displaying the relationships between the pairs of rhombomeres, the branchio-motor nuclei, the cranial sensory ganglia and the branchial arches.

\begin{tabular}{ll}
r1-r7 & rhombomeres \\
gV-gIX & cranial sensory ganglia \\
b1-b3 & branchial arches \\
gV-gIX & branchiomotor nerves \\
ov & otic vesicle \\
fp & floor plate \\
\end{tabular}
3.1.2 Boundaries: Description and Formation.

By stage 12, seven distinct boundaries, from the r1/r2 boundary to the r6/r7 boundary, are present. From stage 10 onwards, at the basal part of boundaries, beneath the ventricular ridges, the intercellular space is increased compared to the space between cells at the centre of a rhombomere, as revealed in semi-thin sections and by electron-microscopy. By stage 16, this space is filled by enhanced levels of the extracellular matrix component chondroitin sulphate proteoglycan (CSPG, Heyman et al 1993). Furthermore, the positions of the nuclei of boundary cells during mitosis differ from those of the rest of hindbrain: Within the neural tube, the position of the nucleus of a neuroepithelial cell varies with the cell cycle. This is visualised by pulse labelling embryos with bromodeoxyuridine (BrdU) and then using an antibody directed against BrdU to detect which cells undergo DNA synthesis (S-phase). In the hindbrain, a cell within a rhombomere typically enters and leaves the S-phase when its nucleus is quite close to the pial (outer) surface of the neural tube (Guthrie et al 1991). In contrast, at boundaries, nuclei of S-phase cells lie close to the ventricular (inner) surface of the neural tube. Nuclei of boundary cells do not undergo interkinetic migration, thereby suggesting that boundaries are a tight and coherent group of cells with minimal cell mobility (Lumsden 1990, Guthrie et al 1991).

Later in development, from stage 14 onwards, transversely oriented axons are found to accumulate periodically in boundary regions (Lumsden & Keynes 1989). Furthermore increased levels of laminin, the adult and more adhesive form of N-CAM (Lumsden & Keynes 1989) and peanut-agglutinin binding proteins (Layer & Alber 1990) are found in rhombomere boundaries after stage 17. By stage 19, the
intermediate filament vimentin, a marker for radial glia, is located in mature boundaries with cells that are distinct from axon bundles (Heyman et al 1995).

The distinct morphology (Heyman et al 1995) and arrangement of boundary cells may provide an accessible route for axons. Alternatively, boundaries are regions where guidance cues operate to attract axon accumulation. The view that axon growth or fasciculation is promoted at the boundary is however disputed since boundaries are first populated by axons and all other cell surface or extracellular matrix characteristics are apparent much later (Guthrie 1996).

Interestingly, cells at boundaries display reduced junctional permeability of dyes with a low molecular weight when compared to other rhombomeric cells (Martinez et al 1992) and this may be an indication that boundaries are a barrier to short range signals.

Having established that boundaries have distinctive characteristics, it was possible to analyse the mechanisms underlying their formation. The cellular mechanisms underlying rhombomere formation have recently been dissected (Guthrie & Lumsden 1991). Boundaries between rhombomeres were removed by aspiration and within a day boundary regeneration occurred. This suggests that boundaries could be a third cell state, resulting from the interaction of two cell populations with different cell surface properties. To further investigate this idea Guthrie and Lumsden (1991) performed grafting studies where a part of r4, for example, was grafted in the absence of boundary regions, into a region normally occupied by r3 cells, so that in the host a “hybrid” rhombomere was created where one half contained r3 (host) and the other one r4 (donor) cells. The juxtaposition of two different cell populations again led to the
formation of a boundary. More interestingly, when an even rhombomere was juxtaposed to another even rhombomere no boundary was generated thus indicating that all even rhombomeres (r2, r4 and r6) share similar surface properties. Similarly r3 and r5 but not r7 share the same surface properties. Based on these observation it seems that rhombomeres 1 to 6 have alternating cell surface properties and that the confrontation of two different cell types induces boundary formation, mirroring the segmental organisation of the chick hindbrain.

In another experiment small pieces of neuroepithelium were dissected from a specific rhombomere, labelled with Dil and then transplanted at different levels into a chick host. A whole series of grafts was performed where the donor was either grafted at the same antero-posterior level (self with self) in a rhombomere with similar (odd to odd or even to even) or diverging (odd to even) surface properties. In summary, cells intermingled most freely when placed within their “original” position (self with self) and least dispersal was seen almost every time when odd and even rhombomere tissues were juxtaposed (Guthrie et al 1993). Results obtained in this study further confirmed the existence of alternating cell surface properties within the chick hindbrain but also suggest that each rhombomere may have its unique cell surface properties.

3.1.3 Boundaries Define Compartments

Rhombomeres have been described as being polyclonal lineage restriction units (Fraser et al 1990): When a precursor cell in the hindbrain was labelled before appearance of any morphological boundary, its progeny were found occasionally in two adjacent rhombomeres. However, when a clone was marked after morphological segmentation, the resulting clone intermingled freely within this rhombomere but
almost never crossed a boundary. This finding suggests that rhombomeres are analogous to the compartments described in Drosophila, and that early assignments of cell fates are maintained by prevention of cell mixing (Fraser 1990, Birgbauer et al 1994). The appearance of boundaries is thus coincident with lineage restriction, but it is not clear whether the boundaries themselves or different cell surface properties of adjacent rhombomeres prevent the intermingling of neighbouring cells. To address that question Guthrie et al (1993) repeated the juxtaposition experiments described above (Guthrie & Lumsden 1991) using quail rhombomeres as donors for chick hosts. Cell dispersal was analysed 36h- 48 h after grafting and donor tissue was distinguished from the host by Feulgen staining of the nucleoli. Similar to previous observations, a boundary was generated when large pieces of odd and even rhombomeric tissues were juxtaposed and additionally there was almost a complete absence of cell mixing at the interface. When odd rhombomere were juxtaposed no boundaries were generated and considerable cell mixing could be seen to occur preferentially at the pial surface.

3.1.4 Segmental Neural Crest Migration

Cells at the most dorsal level of the neuroepithelium are destined to become neural crest cells. These cells will migrate through the embryo and give rise to many different cell types depending on their position along the antero-posterior axis of the embryo. At the cranial level, neural crest cell derivatives comprise the sensory nervous system, the autonomic nervous system, skeletal and connective tissue and a small population of pigment cells (reviewed in Gilbert 1991). Grafting experiments (Noden 1973, 1975, LeLievre 1975) and the use of the fluorescent label Dil established the pattern of cranial crest generation and migration during chick hindbrain development (Lumsden et al 1991, Sechrist et al 1993). Three streams of neural crest are segregated
from the r1/r2, r4 and r6 levels to populate the first branchial arch and the trigeminal ganglion, the second arch and the geniculate ganglion and the third arch and the superior ganglion respectively. The lack of r3- and r5-derived streams of neural crest may be attributed to r3- and r5-derived crest also contributing to the three major migrating streams of neural crest cells (Birgbauer et al 1995, Sechrist et al 1993). This however is in conflict with the findings of Lumsden et al (1991) where the lack of r3 and r5 derived crest is correlated with selective apoptotic cell death at these precise levels (Graham et al 1993, 1994). This discrepancy could be a result of different scoring practices. Whilst Lumsden et al (1991) inferred the original site of Dil application to as yet unsegmented hindbrains (stage 9-) mainly from the position of the labelled cells after fixation (and only those in which the labelled zone remained confined to a single rhombomere were scored as successful), Birgbauer et al (1995) used intravital microscopy to directly visualise the rostrocaudal rearrangements of neural crest cells in vivo. Whilst the issue of segmental crest generation is probably not completely resolved, all studies describe a segmental neural crest migration intrinsic to the hindbrain. Grafting experiments have demonstrated that the neural crest is patterned before migration (Noden, 1988) and indicate that the segmental organisation of the hindbrain may not only be important for the development of the nervous system but also for the morphogenesis of the branchial regions of the head.

3.2 Molecular Basis of Segmentation

3.2.1 Hox Genes

3.2.1.1 Structure and Expression of Hox Genes

The evidence of the segmental cellular organisation of the vertebrate hindbrain led to the search for genes imposing that organisation. One approach was to clone
vertebrate homologues of genes known to be involved in segmentation in *Drosophila*. This strategy led to the identification of *Hox* (homeodomain containing) genes. *Hox* genes are vertebrate counterparts of homeotic (HOM) genes in *Drosophila*. Mutations in a homeotic gene cause the transformation of the structures of a specific segment into structures normally present at a different antero-posterior position. Homeotic genes map to the homeotic complex (HOM-C) comprising the Antennapedia (ANT-C, 3') and Bithorax (BX-C, 5') complexes both located on *Drosophila* chromosome 3 (Keynes & Krumlauf 1994). They encode for a family of transcription factors, all containing a conserved DNA-binding domain: the Antp like homeo-domain. Genes of that family include, from 3' to 5', *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *abdominal-B* (*abd-B*), (reviewed in McGinnis & Krumlauf 1992; figure 4). X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) revealed the similarity between the helix turn helix motif of certain yeast and prokaryotic DNA binding proteins with the homeodomain (Otting et al 1988, Qian et al 1989; Kissinger et al 1990).

There are striking similarities in structure and expression between HOM and *Hox* genes. Murine *Hox* genes for example are clustered on four different chromosomes. Sequence comparison of homedomains and flanking regions revealed that within each of these clusters *Hox* genes are arranged in the same relative order as their homologues in HOM-C (Duboule & Dollé 1989, Graham et al 1989, Boncinelli et al 1991). This structural resemblance between HOM-C and *Hox* gene clusters has led to the idea that there was a common ancestor to insects and mammals which existed some 600 million years ago and that the four paralogous murine *Hox* clusters (A, B, C and D) are the result of a duplication of this ancestor (McGinnis & Krumlauf 1992).
Figure 4: Evolutionary relationship and organisation of HOM-C and Hox Homeobox complexes.

This figure is adapted partly from Keynes & Krumlauf (1994). Alignment of C.elegans (top) and Drosophila HOM-C complex (second from top) with the four identified mouse Hox cluster based on regions of homology. Boxes represent genes. Solid vertical lines indicate that there is a clear homologous relationship between the genes. Boxes representing homologous genes are in the same colour. Dashed vertical lines indicate that the true relationship is not determined yet (grey and white boxes). The number of the paralogous groups is above the each horizontal line representing a cluster.

The arrow at the bottom indicates the colinear relationship between the gene order and antero-posterior expression boundaries, temporal order of expression and responsiveness to retinoic acid.
The more 3’ a gene is positioned within HOM-C in *Drosophila*, the more anterior its expression limit during development. This relationship between expression domain along the antero-posterior axis and the position of a Hox gene within the cluster is also observed during vertebrate development (Gaunt et al 1988). In the vertebrate hindbrain, for example, anterior expression limits of 3’ members of the Hox clusters coincide with rhombomere boundaries (Murphy et al 1989, Hunt et al 1991). Furthermore, Hunt et al. (1991) demonstrated that in the mouse all paralogous genes, with the exception of the *labial* related ones (Hoxa, b-1), share the same anterior expression limit. Expression of *Dfd* paralogues (Hoxa, b, d-4) have their expression limit at the r6/r7-boundary, transcripts of *Zen/pb* related genes (Hoxa, b, d-3) at the r4/r5- and pb (Hoxa, b-2) like genes at the r2/r3 boundary. Further studies have shown however that Hoxa-2 is expressed up to the r1/r2 boundary and does not share the same anterior limit with its paralogue Hoxb-2 (Prince and Lumsden 1994). Although anterior expression limits are shared in most of the cases by paralogous genes groups their expression levels varies along the antero-posterior axis (Hunt et al 1991).

In contrast, paralogues of the *lab*-like genes display differences in expression patterns within their own group and with respect to the rest of the cluster (Murphy & Hill, 1991). According to their position within their cluster (most 3’), Hoxb-1 and Hoxa-1 should be expressed at a more anterior level than any other Hox gene in the same cluster. This is however not the case: Both genes are initially expressed up to the r3/r4 boundary, and later, whilst Hoxa-1 is downregulated, Hoxb-1 expression is elevated in a stripe at r4 level and posteriorly, up to the r6/r7 boundary (Murphy & Hill 1991).
Although pairs of rhombomeres expressing specific combinations of Hox genes are out of register with the pairs of rhombomeres innervating specific cranial nerves it is conceivable that a unique combination of Hox gene expression imposes segmental identity on each individual rhombomere (Wilkinson 1993).

Hox genes are also expressed in the neural crest and it may be that they govern the development of cranial ganglia and branchial arch derived structures. The combination of Hox gene expression in neurogenic and branchial crest matches the rhombomeric one, so that the blend of Hox genes of a rhombomere is ‘passed’ to the crest cells arising from it. Here again there are exceptions to the rule. Hoxa-1 is not expressed in the neural crest, whereas Hoxb-1 is only expressed in neurogenic crest (Hunt et al 1991) and Hoxa-2 is expressed in rhombomere 2 but not in its crest derivatives (Prince & Lumsden 1994).

3.2.1.2 Regulation of Hox genes

The expression of Hox genes during development is very dynamic and complex. By generating transgenic mice carrying different portions of Hox gene regulatory regions fused to a lac-Z reporter gene it was possible to dissect regions responsible for a specific aspect of Hox gene expression (e.g. Sham et al 1993, Marshall et al 1992, Whiting et al 1991). The observed Hox expression pattern in the hindbrain seems to be the result of modular regulation. For example, expression of pb related genes in r3 and r5 is driven by different regulatory regions to those that direct expression in r4 (Sham et al 1993, Nonchev et al 1996). Krox-20, a zinc-finger containing transcription factor is expressed first in r3 (0-3 somites) and later also in r5 (4-7 somites), well before morphological segmentation, and was postulated to be a candidate for regulating Hox
expression (Wilkinson et al 1989). The regulatory elements necessary for \textit{Hoxa-2} and \textit{Hoxb-2} expression in r3 and r5 possess \textit{Krox-20} binding sites which are necessary (in transgenic lines at least) to drive their r3/r5 specific expression pattern (Sham et al 1993, Nonchev et al 1996). Furthermore, ectopic expression of \textit{Krox-20} results in ectopic activation of the Lac-Z fusion gene driven by the same \textit{Hoxb-2} regulatory region described above (Sham et al 1993) and expression of both genes is downregulated in r3 and r5 in a mouse with a disrupted \textit{Krox-20} gene (Schneider Manouri et al 1993, Nonchev et al 1996). Taken together these data suggest that \textit{Krox-20} may well regulate some aspects of \textit{Hox} gene expression in the vertebrate hindbrain.

Another candidate for regulating \textit{Hox} gene expression is retinoic acid. The intracellular level of retinoic acid (all-\textit{trans}) may be regulated by the presence of cellular retinoic acid binding proteins (CRABP), which bind free retinoic acid thus controlling its availability (Balling 1991). The effects of retinoic acid (all-\textit{trans} retinoic acid or the isomer 9-\textit{cis} retinoic acid) are mediated by a family of ligand activated transcription factors termed retinoic acid receptors. Retinoic acid receptors distinguish between the two isomeric forms, where retinoic acid receptor (RAR) binds both and retinoid X receptor (RXR) binds only 9-\textit{cis} retinoic acid. Upon retinoic acid binding (all \textit{trans}) RAR and RXR form heterodimers and are able to bind to retinoic acid response elements (RARE) (Conlon 1995).

Exposing a human embryonal carcinoma cell line to retinoic acid caused an induction of \textit{Hox} gene expression where 3' genes were induced first and genes located at the 5' end of the cluster were induced much later (Simeone et al 1990). The fact that 3' genes, which have the most anterior expression limits, are induced first after retinoic acid exposure has also been observed \textit{in vivo} (Papalopoulu et al 1991b). In general,
retinoic acid has been shown to cause a transformation of anterior hindbrain into more posterior structures (reviewed in Maden & Holder 1992, Kessel 1993). However, another study suggests that retinoic acid may affect individual rhombomeres rather than transforming the whole anterior hindbrain (Marshall et al 1992). Retinoic acid treatment of mouse embryos at midgastrulation (embryonic day (E) 7.5) leads initially to an anterior shift of the \textit{Hoxb-1} expression domain at E8.5 and later (E 9.5) to an ectopic domain at r2 level, as revealed by a \textit{Hoxb-1-lac-Z} reporter construct. Together with the changes in \textit{Krox-20} expression at r3 level (different timing) and the neuroanatomical data revealing an ectopic facial nerve at r2 level, it seems that retinoic acid can lead to a homeotic transformation of segments r2 and r3 into segments r4 and r5 (Marshall et al 1992).

Further evidence that retinoic acid may act directly to regulate \textit{Hox} gene expression comes from the identification of RAREs in the regulatory regions of the \textit{Hoxa-1}, \textit{Hoxb-1} and \textit{Hoxd-4} genes (Langston & Gudas 1992, Popperl & Featherstone 1993, Ogura & Evans 1995a, b). Deletions and point mutations introduced into one RARE of the \textit{Hoxb-1} gene for example, result in the abolition of the early but not the late phase of \textit{Hoxb-1} expression in the neuroepithelium (Marshall et al 1994), whereas mutations in a second RARE led to expansion of its normal expression domain (Studer et al 1994). Multiple RAREs, each with a different function therefore seem to be involved in driving specific aspects of \textit{Hoxb-1} expression. Similar point mutations in the \textit{Hoxa-1} RARE affect only the posterior expression domain (Frash get ref) thereby illustrating the complexity and modular mode of \textit{Hox} gene regulation during development.
The presence of homeobox consensus binding sites in Hox gene regulatory regions, which may also bind their own product (Wu et al 1993) suggests that Hox genes themselves might regulate their segmental expression domains. Similarly, paralogous members of each cluster may regulate each other as illustrated by the ectopic upregulation of Hoxb-1 by Hoxa-1 in selected regions (Zhang et al 1994). The selective upregulation of Hoxb-1 expression by Hoxa-1 might be the result of a restricted presence of a cofactor, required to drive Hoxb-1 expression normally and also in the ectopic domains. Popperl et al (1995) discovered the existence of a conserved enhancer region necessary and sufficient to drive the r4 specific expression pattern of Hoxb-1 in vivo and to which HOXB-1 may bind in presence of PBX (a vertebrate homologue of the Drosophila homeodomain protein extradenticle) as a co-factor in vitro.

3.2.1.3 Function of Hox Genes

The function of Hox genes in mouse has been analysed by genetic studies involving the generation of mutants for a specific Hox gene and also the generation of mice ectopically expressing Hox genes (e.g. Chisaka & Capecchi 1991, Lufkin et al 1991, Rijli et al 1993, Gendron-Maquire et al 1993, Condie & Capecchi 1993, Zhang et al 1994). In this section I will focus on reports describing functional implications of Hox gene expression in hindbrain and head development only.

Hoxa-1 is expressed up to r4 at E 8.0 and regresses later. Hoxa-1 mutant mice embryos have only five rhombomeres instead of seven and this has been attributed to the partial 'loss' of r4 and r5 (Mark et al 1993, Carpenter et al 1993). The lack of Hoxa-1 during development may have either respecified r4 and r5 cells to become r3
and/or r6 cells or caused the simple loss of two segments. The first interpretation suggests that \textit{Hoxa-1} is a homeotic selector gene whilst the second implies that \textit{Hoxa-1} might function as a vertebrate segmentation gene (Mark et al. 1995). Driving widespread expression of \textit{Hoxa-1} with a human \(\beta\)-actin promoter induces rhombomere transformation in the mouse hindbrain, thereby supporting the first interpretation (Zhang et al. 1994). Furthermore, ectopic expression of \textit{Hoxa-1} partly phenocopies the effect of retinoic acid on hindbrain development in mouse and zebrafish and this could imply that \textit{Hoxa-1} may also specify some aspects of hindbrain development normally, perhaps in response to retinoic acid (Zhang et al. 1994, Alexandre et al. 1996).

\textit{Hoxa-2} is the only analysed \textit{Hox} gene which is expressed in r2. Interestingly, it is expressed in r4-, but not r2-derived crest, populating second and first branchial arches respectively. The first branchial arch, populated by r2-derived neural crest, normally develops into Meckel's cartilage, whilst the second branchial arch, populated by r4-derived neural crest, forms Reichert's cartilage. In \textit{Hoxa-2} \(-/-\) mice Reichert's cartilage was lost while a partial duplication of Meckel's cartilage was observed. This implies the existence of a 'ground state' (Meckel's cartilage), defined by the absence of \textit{Hox} gene expression, which is normally respecified by \textit{Hoxa-2} acting like a homeotic selector gene (Rijli et al. 1993, Gendron-Marigue et al. 1993).

\textit{Hoxa-3} is normally expressed up to the r6/r7 boundary and also in r6- and r7-derived crest which will migrate into third and fourth branchial arches. Disruption of \textit{Hoxa-3} does not cause a homeotic transformation but rather results in severe defects of third branchial arch structures which are even more pronounced in mice lacking the two \textit{zen/pb} - related paralogues, \textit{Hoxa-3} and \textit{Hoxd-3} (Chisaka & Capecchi 1991, Condie & Capecchi 1994). This indicates that the activity of the two paralogues
requires mutual interactions. Condie & Capecchi (1994) point out that Hox gene activity might involve the regulation of proliferation rather than specification of identity. Similarly, r4 and r5 may fail to develop due to lack of proliferation of these specific cells in Hoxa-1 mutant mice. The link between growth and Hox genes gains further support from studies where fibroblasts (NIH 3T3) transfected with an activated Hoxb-8 gene cause tumors in nude mice (Aberdam et al 1991). In general, Condie & Capecchi (1994) highlight that the role of Hox genes may well include both control of proliferation and specification.

The different phenotypes observed in ‘knock-out’ mice and also transgenic mice highlight three important features of Hox gene action. First, the extent of their expression domain does not necessarily mirror their functional domains. In many cases however the functional domain coincides with the most anterior expression domain within a tissue or a subpopulation of that tissue (Chisaka et al 1992, Lufkin et al 1991, Rijli et al 1993, Gendron-Maquire et al 1993, Condie & Capecchi 1993). Second, Hox genes act on a modular basis by imposing identity on a subset of cells within a tissue. In Hoxa-2 and Hoxa-3 mutant mice for example, only r4 and r6 derived mesencephalic crest respectively are affected whilst neurogenic crest from that same origin remains unrefined (Chisaka and Capecchi 1991, Gendron-Maquire et al 1993). Third, paralogous genes within the four Hox clusters act synergistically, as demonstrated by the comparison between the phenotypes described in single knock outs and mice lacking two paralogous genes (Condie & Capecchi 1994).
3.2.2 Other Genes

The *kreisler* mutation was identified in an X-ray mutagenesis experiment and the characterisation of the mutated gene revealed that *kreisler* encodes a basic domain leucine-zipper protein (Cordes & Barsh 1994). *kreisler* is expressed in r5 and r6, and mutants display segmentation abnormalities in the posterior hindbrain which have been attributed to the loss of r5 and r6 (McKay et al 1994). They also display defective inner ear development. *kreisler* might therefore represent a mouse segmentation gene (Cordes & Barsh 1994).

Cell-to-cell interactions play an important role in hindbrain segmentation and several signalling molecules and receptors have been identified and found to be expressed in the vertebrate hindbrain. For example, FGF3, a member of the fibroblast growth factor family for example is expressed early in rhombomeres 5 and 6 and later in boundary regions (Wilkinson et al 1988, Mahmood et al 1995). Expression of another signalling molecule, *Cwnt-8C*, is found in presumptive r4 and precedes the up-regulation and restriction of expression of *Hoxb-1* (Hume & Dodd 1993). *Neuronatin*, a putative transmembrane protein, was found to be expressed from E8.5 onwards in r3 and later (E9.0) in r3 and r5 of the developing vertebrate hindbrain (Wijnholds et al 1995).
**Figure 5: Gene expression in the developing hindbrain**

On the left side a hindbrain with 8 rhombomeres is drawn next to bars representing gene expression. *Hox*-gene (A,B,D) and expression of other genes in the developing hindbrain (for references see text) are shown on the right side of the diagram. Where the expression pattern is not uniform it is indicated by the use of different colours (where darker and lighter tones are used within a bar representing expression of a single gene weaker expression is represented by a lighter tone).
3.2.3. The Function of Eph Receptors during Hindbrain Segmentation

Good candidates for mediating cell to cell signalling are the members of the Eph receptor family. Eph receptors and at least one of their ligands are expressed in a spatially restricted manner in the hindbrain. High levels of Sekl expression are restricted to r3 and r5, similar to the pattern observed for Krox-20, whilst lower levels can also be found in r2 and r4 in the mouse (Gilardi-Hebenstreit et al 1992; Nieto et al 1992). Sek2 is transiently expressed in prospective r4. Sek3 is initially expressed in r2 and r3 and, later, also in r5. Sek4 expression is first seen in r2 and r3 and then becomes restricted to r3 and r5. ELF-2, a ligand for Sek3 and Sek4 is restricted to r1, r2, r4 and r6 from E8.5 onwards (Bergemann et al 1995).

In zebrafish and Xenopus Xu et al (1995) have used the dominant negative approach to assay the function of rtkl. mRNA encoding a truncated form of mouse or Xenopus Sek-1, lacking the kinase domain, were injected into Xenopus and zebrafish oocytes. Blocking of the endogenous homologue of Sekl in Xenopus and zebrafish led to ectopic expression of Krox-20 in even rhombomeres. Interestingly, this phenotype could be rescued by co-overexpression of a wild-type full-length Sekl, which on its own had no effect on segmental gene expression. The authors suggest that in the normal embryo, restricted domains of Krox-20 expression might be maintained by local interactions acting in a community effect or by the partial restriction of cell movement across rhombomere boundaries. Two possible mechanisms are then suggested to explain the mechanism by which blocking rtkl/Xsek1 could lead to cells expressing Krox-20 in even numbered rhombomeres. First, increased cell mixing
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occurs due to lack of a contact dependent repulsion of r3/r5 cells by r2/r4/r6 cells. This scenario suggests that in the normal embryo expression domains of Krox-20 could be sharpened by repulsive events, probably mediated in part by the Sek1/X receptor ligand-pair (where X has yet to be found). Second, blocking rtkl /Xsekl interferes with the signal transduction events necessary to execute the community effect. In the normal embryo, some cells cross boundaries even after the observation of sharp Krox-20 domains (Birgbauer & Fraser 1994). This implies that some cells which have been expressing Krox-20 and rtkl /Xsekl will switch those genes off and that a dynamic regulation of cell identity may be mediated through a community effect. Contact between receptor and ligand expressing cells will quickly change the fate of r3/r5 cells into r2/r4/r6 cells. The ligand which might interact with rtkl /Xsekl in the hindbrain has yet to be identified. The role of rtkl /Xsekl in the hindbrain might then be the maintenance of segmentation, although it is not yet clear how this is achieved (Xu et al 1995).

4 Limb Development

4.1 Development of the Chick Limb

A fully grown limb is a complex, polarised structure comprising many different cell types, including dermis, epidermis, muscle, bones, cartilage, nerve cells, blood vessels and loose connective tissue. Initially this structure arises from a small number of apparently identical mesenchymal cells encased in ectoderm. Experiments in the embryonic chick limb have identified the signalling mechanisms guiding limb development. The mechanisms operating during limb development are best explained by referring to the three axes: the proximo- distal axis (shoulder to finger tip), the
dorso-ventral axis (back of the hand to palm) and the antero-posterior axis (thumb to little finger).

4.1.1 Proximal-Distal Axis
4.1.1.1 The Apical Ectodermal Ridge

Limb buds develop at specific positions along the body axis due to a maintenance of proliferation of lateral plate mesenchyme at that specific level (Searls & Janners 1971). The apical ectodermal ridge (AER) is a thickened rim of epithelium which marks the tip of the limb bud. It develops from surface ectoderm in the limb-forming region and consists of pseudo-stratified elongated cells that are closely packed and linked by extensive gap junctions (Fallon & Kelly 1977). An AER is induced when presumptive limb mesenchyme is grafted to the flank (Kieny 1968), suggesting that the formation of the AER is normally induced by its underlying mesoderm.

The AER is essential for proper development of the limb. Removing the AER at stage 18 results in severely truncated limbs with only the most proximal part of the humerus remaining, while AER removal at later stages (28) leads to the loss of the most distal phalanx of digit 3 only (Summerbell 1974b). Skeletal structures along the proximo-distal axis thus develop in a sequence, where proximal structures are laid down first and distal structures last. The information for proper sequential development along this axis is intrinsic to the limb mesenchyme, since AERs of all stages up to stage 29 are able to promote normal outgrowth and proximo-distal patterning when recombined with stage 18-20 limb bud mesenchyme (Rubin & Saunders 1972). Signalling is maintained even in reorganised AERs (Errick & Saunders 1976) and are required to promote limb
outgrowth. Furthermore, if the AER is grafted on a dorsal part of a chick wing bud a secondary outgrowth is induced (Saunders et al 1976).

4.1.1.2 The Progress Zone

The ridge signal maintains a zone of undifferentiated mesenchyme cells confined to the tip of the limb, approximately 300 μm beneath the AER. The mitotic index of these cells at stage 24 is higher than that of more proximal cell populations (Hornbruch and Wolpert 1970) and cells in the progress zone use an autonomous timing mechanism to assess their position along the proximo-distal axis (Summerbell et al 1973). Thus when the distal tip of a stage 24 limb bud was replaced with a whole stage 19 limb bud duplicated elements were generated along the proximo-distal axis, whereas grafting of the distal tip of a late bud to a young stump led to loss of some of these structures. The time that cells spend in the progress zone thus specifies which structures they will form: the shorter the period of time that they spend in the progress zone the more proximal their fate. The positional value along the proximo-distal axis of cells changes autonomously in the progress zone with time (Summerbell 1973). As soon as cells leave the progress zone, the autonomous change stops. Cells thus measure the length of time they spend in the progress zone and this provides them with positional information. This mechanism is based on a continuous presence of an AER, i.e. continuous ectodermal-mesenchymal interaction. Removal of the AER freezes the positional value of the cells in the progress zone.
4.1.2 Antero-Posterior Axis

4.1.2.1 The Polarising Region

The polarising region is a group of cells located in the posterior margin of the wing bud, which, when grafted to the anterior part of a host wing bud causes a full mirror image duplication of the digits (Saunders and Gasseling, 1968). The normal digit pattern in the chick wing is 234 (from anterior to posterior) and the digit pattern in the manipulated wing is 432234. Grafts of mouse polarising regions to the chick wing bud showed that newly induced digits derive from the host tissue thus indicating that the polarising region is a conserved signalling region (Tickle et al 1976).

The polarising region (or zone of polarising activity (ZPA) is defined by its activity to induce supernumerary digits. This activity has been detected in the lateral plate mesenchyme even before any appearance of a limb bud (Hombruch & Wolpert 1991). Once the limb bud appears the polarising activity extends into both the posterior wing bud and the posteriorly adjacent body wall (MacCabe et al 1973, Honig and Summerbell 1985). From stage 23 onwards, maximal activity solely resides in the posterior distal margin of the wing bud, just proximal to the progress zone, and may be found there up to stage 29.

Further characterisation of the ZPA revealed that gradual 'dilution' of actively signalling ZPA cells with anterior limb bud cells leads to a gradual decrease of polarising activity. This activity may be carried out by as few as 30 cells provided that these cells are in close contact with the AER (Tickle 1981). Thirty ZPA cells are able to induce a digit 2, 70 cells induce a digit 3 and 100 cells may induce a digit 4 immediately adjacent
to the graft (Tickle 1981). Removing the AER reduces the polarising activity of the ZPA (Vogel & Tickle 1993). Similarly, treatment of the ZPA with increasing doses of $\gamma$-irradiation led to a gradual attenuation of the polarising signal (Smith et al 1978). The action of the ZPA is not only ‘dose’ dependent but also time dependent (Smith 1980). When a ZPA graft is removed after 15 h a digit 2 is induced, whereas a digit 3 is formed when the graft remains in the host for 24 h. Digit 2 is always the first one to be specified by the ZPA. Higher concentration of activity and also longer time of exposure to that signal induces development of more posterior digits and the most posterior digits are always induced next to the ZPA (Tickle et al 1975).

By grafting ZPAs to the anterior margin of successively older limb buds Summerbell (1974) demonstrated that the action of the ZPA was limited to the cells in the progress zone. An anteriorly grafted ZPA does not induce a new ZPA in adjacent tissue (Smith 1979) and the ZPA can act at a distance as shown by performing double grafting experiments where non polarising leg tissue was transplanted in between an anteriorly placed chick wing ZPA and the rest of the chick wing bud (Honig 1981). These grafts always patterned the leg tissue and, depending on the width of the intercalated leg tissue, also led to mirror image duplication of wing digits, thus indicating that the ZPA can act over a distance of twenty cell diameters (Honig 1981).

4.1.2.2 The Morphogen Model

One model explaining above results is that the ZPA is considered as source of a morphogen (Tickle et al 1975). A morphogen is a substance which has a concentration dependent effect on recipient cells. In the limb bud, the ZPA might release such a morphogen which then diffuses from its source and establishes thereby a field with a
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continuous range of concentrations. The fate of the cells depends upon their position along the gradient (Wolpert 1969). As the concentration falls through certain threshold levels the limb bud tissue is instructed to form specific digits. Cells responding to the highest concentration of morphogen develop the most posterior structure (digit 4) and cells exposed to the lowest concentration morphogen will develop as more anterior structures (digit 3, digit 2). This model explains how ZPA grafts to the anterior margin of the limb bud interfere with normal limb development: A graft of ZPA cells to the anterior part of a limb bud at stage 20 changes the concentration of the postulated morphogen locally and a symmetrical distribution is established. The result is a symmetrical duplication (figure 6).

4.1.3 Dorso-ventral axis

The dorso-ventral axis is determined from stage 11 onwards (Chaube 1959) and may be controlled by factors from the ectoderm, though not from the AER (Saunders et al 1976). Inverting the dorso-ventral axis of the ectoderm with respect to the mesoderm by recombining the ectoderm of a right limb bud with the mesoderm of a left limb bud led to development of ‘hybrid’ limbs, where more proximal structures display the orientation of the mesenchyme while distal structures adopt the dorso-ventral polarity of the ectoderm (Patou & Kieny 1973, MacCabe et al 1974). Recently similar grafting experiments performed by Akita (1996) confirmed these results and also demonstrated that the dorsal ectoderm modifies the ventral mesoderm before the ventral ectoderm affects the dorsal mesoderm. Akita (1996) therefore proposes a “two way signalling model” where first a factor from the dorsal ectoderm specifies a dorsal state opposing
the ventral “ground-state”, and second a symmetrical positional signal derived from the AER specifies the nature of the tissue differentiating along the dorso-ventral axis.

4.1.4 Summary

In summary, grafting experiments have demonstrated that limb development requires continuous interactions between the ectoderm and the underlying mesenchyme. The progress zone seems to be the site of integration of information where signals from the ectoderm, the AER and the ZPA are interpreted. The time cells spend in the progress zone determines their proximo-distal level and their distance from the ZPA during that period establishes their antero-posterior level. Similarly control of the dorso-ventral polarity is thought to be exerted by the ectoderm over progress zone mesenchyme.

4.2 Molecules Implicated in Limb Development.

To date, some of the patterning activities described above have been assigned to the activity exerted by some molecule or genes. Any inductive process requires a signalling tissue and a responsive tissue. By the same token, the molecules implicated in patterning the three axes described above may be categorised in to two classes: signalling molecules and response molecules.

4.2.1 Limb Initiation

FGF (fibroblast growth factor) is involved in limb initiation. Remarkably, application of FGF1, 2, 4 or 8 to the presumptive flank between the wing and the leg of
chick embryos leads to development of an additional limb (Cohn et al 1995, Ohuchi et al 1995, Crossley et al 1996). Thus a single protein (FGF1, 2, 4 or 8) can, when present at the right time and position, trigger a whole cascade leading to limb development. FGF8 might be that endogenous signal (Crossley 1996), since it is expressed prior to limb formation in the adjacent intermediate mesoderm.

It is unclear what controls the position of limbs, but it may be that Hox gene expression in the lateral plate mesenchyme determines limb position: Forelimbs of mice lacking Hoxb-5 activity are shifted anteriorly (Rancourt et al 1995) and an anterior shift of the anterior expression limit of Hoxb-8 in presumptive forelimb mesenchyme results in a duplication of the ZPA (Charité et al 1994). The identification of factors which in combination with Hox gene expression determines limb position is an important issue and the analysis of the mouse mutant Strong's Luxoid (Chan et al 1995) led to the possible identification of one such factor. The limbs of mutant Strong's Luxoid (1st D) mouse are polydactylous and result from an ectopic ZPA (Chan et al 1995). The authors propose that the 1st D gene product cooperates with Hoxb-8 (in the forelimb) or alternatively inhibits a ZPA formation anteriorly thereby creating an asymmetry distribution of polarising activity.

4.2.2 Proximo-Distal Axis

4.2.2.1 FGFs Mediates the Function of the AER.

Recently, three members of the FGF-family have been found to be expressed in the AER of the developing limb (FGF2, 4 and 8). The FGF-family consists currently of nine known members (FGF-1 to FGF-9). They bind to high affinity fibroblast growth factor receptor (FGFR1-4) and also to low affinity receptors such as heparan sulphate
proteoglycans (reviewed in Baird 1994). Of the FGFRs only FGFR1 (mesenchyme) and FGFR2 (AER) but not FGFR3 and FGFR4, have been found to be expressed in the limb bud (Peters et al 1992, Peters et al 1993, Stark et al 1991). FGF-8 is expressed throughout the AER from very early on in development (Crossley and Martin 1995), whereas FGF4 expression is detected slightly later and is restricted to the posterior part of the AER (Niswander et al 1994). FGF2 protein is found in the AER and also in the underlying mesenchyme (Savage et al 1993). FGF-4 and FGF2 can both mimic the chemical signal from the ridge (Niswander et al 1993, Fallon et al 1994). When the AER is surgically removed and beads loaded with either FGF2 or 4 are applied to the limb bud, complete outgrowth occurs. A single protein may thus promote limb outgrowth just as the AER normally does. Both FGF4 and FGF2 can maintain polarising activity in culture (Vogel & Tickle 1993, Anderson et al 1993) and FGF4 may perform this function also in vivo. Furthermore, application of both FGF2 and FGF4 can lead to complete regeneration of a "competent" amputated chick wing bud stump (Taylor et al 1994, Kostakopoulou et al 1996).

In another study it was shown that cells carrying a replication defective retrovirus expressing FGF2 may cause duplication of the humerus and radius and also extra digits when grafted on the anterior margin of a host wing bud (Riley et al 1993). It has been suggested that ectopic FGF2 stimulates growth in anterior tissue and leads thereby to duplications of anterior skeletal elements, rather than respecifying the anterior wing bud. Interestingly beads loaded with FGF2 protein never result in any kind of duplication.
4.2.2.2 Genes Expressed in the Progress Zone

Cells in the progress zone are maintained by FGF signals, emanating from the AER. Of all genes expressed in the progress zone (*Msx-1, Msx-2, AP2, Evx-1* and *Wnt5a* - reviewed in Tickle and Eichele 1994) *Msx*-genes are good candidates for interpreting the growth signals of the AER. *Msx*-genes are vertebrate homologues of the Drosophila *msh* (muscle segment homeobox) gene and they possess a homeobox (Hill et al 1989) that differs from the Antennapedia-like homeodomain described in the first chapter. In the early stages of limb bud formation, *Msx-1* transcripts were found abundantly within the entire limb bud. Later on in development, at stages 20-22, *Msx-1* RNA is localised mainly in the mesenchyme underneath the AER, while *Msx-2* RNA is found in the AER itself and is also particularly concentrated in the anterior mesenchyme (Coelho et al 1991).

When non expressing proximal mouse limb bud mesenchyme that lacks *Msx-1* and *Msx-2* expression is grafted under the AER at the tip of a chick limb bud, *Msx-1* and *Msx-2* gene expression was activated in the graft (Davidson et al 1991). Within the grafts, transcripts were distributed in a gradient with the highest level present in mesenchyme immediately under the AER. This mirrors the situation in the host and is consistent with the idea that the AER provides signals that activates expression of these genes. Furthermore, these signals must be functionally conserved between chick and mouse, since the chick AER regulates gene expression in mouse derived mesenchymal tissue.
In the chick mutant *limbless*, a recessive mutation leading to animals lacking fore- and hindlimbs, the cessation of outgrowth in mutant limbs, due to a non functional AER, is correlated with decreasing *Msx-1* expression from stage 20 onwards. This finding suggests that *Msx-1* might be under the regulation of the AER which seems to maintain but not to initiate its expression. Robert et al (1991) demonstrated that rescue of *limbless* mutants by AER grafts led to formation of a new progress zone and coincides with the onset of *Msx-1* and *Msx-2* expression in the AER and the underlying mesenchyme.

Further evidence that *Msx-1* is under the regulation of the AER has been provided in an *in vitro* system. In micromass, distal limb bud mesenchyme cells will differentiate into cartilage if cultured very densely in the absence of any ectoderm. There is no *Msx-1* expression in such cell cultures. If an apical ectodermal ridge is grafted to this culture *Msx-1* transcripts become abundant underneath the graft and no cartilage differentiation occurs in this region. The interaction between AER and mesenchymal cells seems to activate *Msx-1* expression.

The exact function of these genes in limb development is not understood yet, but experiments described by Song et al (1992) highlight their potential importance. Song et al (1992) overexpressed *Msx-1* in a myogenic cell-line by stably transfecting them with full length *Msx-1* cDNA. These cells are unable to differentiate into myotubes upon induction and the level of MyoD1, an early muscle marker, is much reduced. This finding is consistent with observations *in vivo* where *Msx-1* expression and muscle differentiation are found to be incompatible. Msx-1 expressing cells may proliferate but not differentiate (Song et al 1992), and this could also be true for Msx-1 expressing cells in the progress zone of the developing limb.
4.2.3 Antero-Posterior Axis

All-trans retinoic acid is the first molecule which has been shown to mimic the polarising activity of a ZPA when applied to the anterior margin of a developing chick wing bud (Tickle et al 1982). An increase in retinoic acid concentration induces the development of more posterior digits, and it has been suggested that retinoic acid could be the morphogen produced by the ZPA (Tickle et al 1985). Indeed, retinoic acid is enriched in the ZPA of the vertebrate limb bud and retinol can be converted to retinoic acid by limb bud cells. The amount of retinoic acid required to induce a complete duplication is in the same range as the dose of retinoic acid found in the limb bud (reviewed in Eichele and Tickle 1994). In addition, nuclear receptor that transduce retinoic acid signalling called RARs and RXRs (retinoic acid receptor, retinoic X receptor), have been identified and found to be expressed in the vertebrate limb bud. Whereas RARα, γ and RXRα, β, γ are expressed throughout the limb bud, RARβ expression is confined proximal regions (reviewed in Eichele and Tickle 1994, Schofield et al 1992, Thaller et al 1993, note that RXRγ is not expressed in the chick limb and that RXRβ is not known in chick). Cellular retinol binding proteins (CRBP I and II) and cellular retinoic acid binding proteins (CRABP I and II) are also expressed in limb buds (reviewed in Mendelsohn 1992) and mice homozygous for a CRABP II null-mutation develop postaxial polydactyly in the forelimbs (Fawcett et al 1995). Retinoic acid was a good candidate for being a morphogen released by the ZPA. However, in contrast to an anterior ZPA graft, a bead soaked in retinoic acid and applied to the anterior wing margin induces a ZPA in adjacent mesenchyme and also induces ectopic expression of RARβ (Noji et al 1991). These latter findings predicts a role for retinoic acid in establishing a ZPA, rather than being the morphogen released by the ZPA.
The importance of retinoid signalling in limb development is further demonstrated by studies where other retinoids like a all-\textit{trans},3,4- didehydro-retinoic acid (Thaller & Eichele 1990) and 9-\textit{cis} retinoic acid (Thaller et al 1993) have also been found to induce a dose dependent mirror image digit duplication when applied to the anterior wing margin. All-\textit{trans},3,4- didehydro-retinoic acid but not 9-\textit{cis} retinoic acid has also been detected in chick wing buds, with higher levels posteriorly (Scott et al 1994).

Recently \textit{Sonic hedgehog} (\textit{Shh}), a vertebrate homologue of the Drosophila segment polarity gene (\textit{hh}), has been isolated and transcripts of this gene found to colocalise with the ZPA (Riddle et al 1993). \textit{Shh} expression is induced in adjacent mesenchyme distal to the anteriorly applied retinoic acid bead and ectopic expression of \textit{Shh} maps the ectopic polarising activity associated with that tissue (Riddle et al 1993). Furthermore when \textit{Shh} expressing cells or recombinant \textit{SHH} protein are applied to the anterior margin a mirror image digit duplication is induced, mimicking the effect of a ZPA graft or a retinoic acid bead to that location (Riddle et al 1993, Lopez-Martinez et al 1995). \textit{Shh} is also expressed in Hensen’s node, the notochord and the floorplate, all structures known to have polarising activity (Riddle et al 1993).

\textit{SHH} is proteolytically processed and leads to two major, N-and C-terminal, cleavage products. The N-terminal, but not the C-terminal cleavage product or the uncleaved \textit{SHH} precursor, is necessary and sufficient to mimic a ZPA activity (Lopez-Martinez et al 1995). If \textit{SHH} is the ZPA morphogen one would expect a graded distribution of the actively signalling domain across the limb bud. However, the distribution of the N-terminal product of \textit{SHH} colocalises with the ZPA and the exact nature of \textit{SHH} signalling remains to be further investigated. Marigo et al (1995) propose
that the level of \textit{patched} expression, a transmembrane protein involved in transducing the SHH signalling activity, reflects the range over which SHH acts. Another possibility is that SHH induces \textit{Bmp2} (bone morphogenetic protein-2) expression (Laufer et al 1994), whose protein product in turn may mediate the polarising effect. However, when recombinant BMP2 is applied ectopically to the anterior wing margin, no digit duplication occurred. Interestingly BMP2 expressing cells can lead to duplication, but only of digit 2, when applied to the anterior margin of the chick wing. (Duprez et al 1996). Taken together these results rule out a polarising signal relay function for BMP2 (Francis et al 1994)

Recently a positive feedback loop between growth (FGF) and patterning (\textit{Shh}) signals was shown to operate in the developing chick wing bud (Niswander et al 1994, Laufer et al 1994). When a retinoic acid bead is grafted to the anterior wing margin, FGF4 expression is first induced after 18 hours and later, at 24 hours, \textit{Shh} transcripts are detected. When \textit{Shh} expressing cells were grafted to the anterior wing margin \textit{Fgf4} expression is induced within 24 hours in the anterior AER. FGF4 on its own cannot induce \textit{Shh} expression in anterior mesenchyme, but maintains \textit{Shh} expression in the ZPA in the absence of an intact AER. When the AER was removed, \textit{Shh} expression could be detected in the anterior mesenchyme only when RA and FGF4 where applied simultaneously anteriorly.

When \textit{Bmp2-} expressing cells are grafted to the anterior wing margin \textit{Fgf4} is ectopically expressed in the anterior AER and later \textit{Hoxd-11} and \textit{Hoxd-13} expression is induced (Duprez et al 1996). Taken together these results suggests that FGF4 may normally induce SHH, which in turn, through BMP2 maintains FGF4, thus establishing and constantly maintaining the ZPA. Whether FGF8 is involved in that loop
is not clear yet as gene expression analysis of the limb deformity (Id) mutant limb buds and also the work of Mahmood et al (1995) suggest that FGF8 is sufficient to maintain the outgrowth of the limb bud not to maintain a ZPA (Haramis et al 1995).

4.2.4 Dorso-Ventral Axis

The dorsal ectoderm directs the dorso-ventral patterning of the underlying mesoderm. WNT7a, a member of the WNT family of secreted proteins, is expressed in dorsal ectoderm of the chick limb bud. It is first detected in the pre-limb ectoderm at stage 15 and remains expressed in the dorsal ectoderm at least until stage 23 (Riddle et al 1995). WNT7a may mimic the signalling activity of the dorsal limb ectoderm (Yang & Niswander 1995) and mice lacking WNT7a-activity have in many aspects double ventral limbs (Parr & McMahon 1995). Expression of Lmx-1, a member of the LIM homeobox gene family, is found to be confined to the limb mesenchyme from stage 15 onwards. Lmx-1 is expressed in the mesenchyme just beneath the Wnt7a expressing ectoderm at stage 17 and is later confined to the dorsal half of the limb bud. Riddle et al (1995) showed that WNT7a is sufficient to induce Lmx-1 in vivo and in vitro (and to maintain Lmx-1 in vitro). Furthermore ectopic expression of Lmx-1 in the ventral mesoderm induces double dorsal limb patterns (Riddle et al 1995, Vogel 1995). Ectopic expression of WNT7a in the ventral ectoderm does not induce double dorsal pattern, thus indicating the existence of a ventral factor which represses dorsal differentiation. Engrailed-1, a homeodomain-containing transcription factor, is expressed in the ventral ectoderm and loss of its function in mice leads to dorsal transformation of ventral paw structures (Loomis et al 1996). Furthermore Wnt7a is expressed also in the ventral ectoderm of these mutant mice. It seems thus that Engrailed-1 might be the dorsal repressing, ventralising molecule.
Adapted from Tickle and Eichele 1994. (A) From a wing bud (3d) to a wing (10d). 
(B) The morphogen model: The ZPA might release a putative morphogen which then 
diffuses from its source and establishes thereby a field with a continuous range of 
concentrations. The fate of the cells depends upon their position along the gradient. 
As the concentration falls through certain threshold levels the limb bud tissue is 
instructed to form specific digits. Cells responding to the highest concentration of 
morphogen develop the most posterior structure (digit 4) and cells exposed to the 
lowest concentration morphogen will develop as more anterior structures (digit 3, 
digit 2). (C) A graft of ZPA cells, Shh producing cells or a retinoic acid bead to the 
anterior part of a stage 20 limb bud alters the concentration of the postulated 
morphogen locally and a symmetrical distribution is established. The result is a 
symmetrical duplication. (D) The interactions between the three signalling regions, the 
AER, the dorsal ectoderm and the ZPA in the developing chick limb bud. (E) Gene 
expression in a stage 20 chick limb bud. 
Numbers refer to digits (unless otherwise indicated); A, anterior; P, posterior; D, 
dorsal; V, ventral.
Recently Yang and Niswander (1995) demonstrated that interactions between the signalling molecules WNT7a (dorso-ventral), SHH (antero-posterior) and FGF4 (proximo-distal) are necessary for the coordinated growth and patterning of the vertebrate limb. WNT7a and FGF4 are sufficient to maintain and enhance the ZPA (Shh expression). Removal of the dorsal ectoderm (WNT7a source) leads to downregulation of Shh expression and consequently to loss of the most posterior skeletal elements. If Wnt7a or Shh expressing cells are grafted on to the posterior distal part of the chick wing bud, stripped of its dorsal ectoderm, the ZPA is maintained and no structures are lost. The authors propose that WNT7a and FGF4 positively regulate SHH. SHH then positively regulates Fgf4 (feedback loop). The ZPA thus integrates the signals for positional information along all three axes (Yang & Niswander 1995).

4.2.5 Hox Genes

The organisation and characteristics of Hox genes were described above (3. 2.1. Hox genes). In the vertebrate limb the expression of the most 5' genes of the Hoxa- and Hoxd- gene cluster have been described. The most 5' members of the Hox D cluster (Hoxd-9-13) are expressed in the developing limb, where Hoxd-13 is confined to the posterior part of the limb, and more 3' members are expressed in progressively more anterior regions of the developing bud. From stage 24 onwards the initial distal posterior domain of Hoxd-13 shifts anteriorly and correlates with the branching process of precartilagenous condensations starting at stage 28 (Yokouchi et al 1991). The time of onset of expression of individual Hoxd- genes also correlates with their position within the cluster, so that Hoxd-9 is the first and Hoxd-13 the last gene to be expressed in the developing limb. And this spatial and temporal pattern of activation is also observed in the anterior mesenchyme when retinoic acid or a ZPA are grafted to the anterior wing.
margin (Izpisua-Belmonte et al 1991). Ectopic expression of Shh at the anterior wing margin also induces ectopic Hoxd gene expression. The induction of a mirror image Hoxd gene expression in the limb bud after anterior retinoic acid application is restricted to the progress zone and depends on the continuous presence of an intact AER (Izpisua Belmonte et al 1991, 1992b). FGF4 application to the posterior mesenchyme is sufficient to maintain Hoxd-13 expression after AER removal (Vogel et al 1995). However FGF4 on its own is not able to induce Hoxd genes (Niswander et al 1994). Thus the existence of a maintained (via FGF4) ZPA seems to regulate Hoxd gene expression.

Expression of three of the most 5' members of the Hoxa gene cluster has also been detected in the developing limb. Hoxa genes are expressed in proximo-distal restricted domains in chick and mouse limbs and their expression boundaries are collinear with their position within the cluster (Hoxa-10, 11 & 13, Haack & Gruss 1993, Yokouchi et al 1991). By stage 28 the proximal expression boundary of Hoxa-13 maps the border between the autopodal (digits) and zeugopodal (humerus) elements. The distal Hoxa-11 expression is confined to the zeugopodal elements and Hoxa-10 is expressed throughout the limb bud. Cells integrated into precartilagenous condensation stopped expressing the analysed Hoxa genes.

Based on this observations it was suggested that in the limb, as in the hindbrain, a Hox code may exist, where cells expressing various combinations of Hox proteins may translate these differences by producing distinct structures (Yokouchi et al 1991, Duboule 1992). To test this hypothesis Morgan et al (1992) altered the Hoxd “code” in the chick limb by infecting them with a replication competent retrovirus expressing Hoxd-11. Hoxd-11, rather then being restricted to the posterior half of the limb, is now
also transcribed and in more anterior regions of the bud. This gave rise to a homeotic transformation in the leg where the most anterior digit (I) has been transformed to a more posterior fate (digit II) in 30% of the cases. In the wing an ectopic anterior digit was observed and the authors argue that anterior cells undergoing cell death have been transformed (by the new \textit{Hox} gene combination) to give rise to a digit.

Disruption of individual \textit{Hoxd-} and \textit{Hoxa-} genes however did not lead to the anticipated homeotic transformations in the limb, thus shedding a new light on the role of \textit{Hox} genes in limb development. Disruption of \textit{Hoxd-11, Hoxd-13} or \textit{Hoxa-11} for example lead to partly overlapping phenotypes, none of which included a homeotic transformation (compared in Davis & Capecchi 1994). Disruption of \textit{Hoxd-13}, for instance resulted in delayed ossification, loss of certain phalanges and also development of an additional posterior digit (Dollé et al 1993). These results do not support a role for \textit{Hoxd-13} in conveying positional information onto posterior limb bud cells and the authors suggest that \textit{Hox genes} may control limb patterning by regulating the timing and extent of local growth rates. One interesting observation is that the more 3' the position of the disrupted gene, the more proximal the extent of the observed abnormal limb phenotype (Dollé et al 1993, Davis & Capecchi 1994, Small & Potter 1993, Favier et al 1996, Fromental-Ramain et al 1996). The results of Morgan et al (1992) have now been reinterpreted (Morgan and Tabin 1994) and the initial proposed \textit{Hox} code was rejected in favour for a role of \textit{Hox} genes in growth stimulation. Ectopic expression of \textit{Hoxd-11} (as the ectopic application of FGF2 to the anterior wing margin. Riley et al 1993) led to stimulation of limb outgrowth thereby creating a new chondrogenic center.

Ectopic expression of \textit{Hoxa-13} in the chick limb bud induced severe truncations of the long bone cartilage in the zeugopod (Yokouchi et al 1995). \textit{Hoxa-13} is normally
expressed in zeugopodal mesenchymal cells but not in the chondrocytes. Although Hoxa-13 was ectopically expressed in the whole limb bud, i.e. in zeugopodal as well as autopodal chondrocytes (and mesenchymal cells), the observed truncation was limited to the zeugopod. The authors thus interpreted this specific truncation as homeotic transformation, where, in the zeugopodal cartilage, the long bone was transformed to the short bone. Another intriguing finding of Yokouchi et al (1995) was that, in tissue culture experiments, cells expressing Hoxa-13 sorted from non-expressing cells. Hoxa-13 may be involved in controlling cell adhesion in the limb mesenchyme.

This specificity of Hox gene action was also demonstrated by Davis et al (1995) who generated mice mutant for both Hoxa-11 and Hoxd-11. Remarkably those mice were lacking radius and ulna (zeugopod) in the forelimb, and this phenotype could not be anticipated by simply adding the abnormalities observed in each of the single knock-outs. These results suggest that paralogous Hox genes act together by specifying limb outgrowth and patterning along the proximo-distal axis. Interestingly, this functional cooperation is not limited to paralogous genes (Favier et al 1996).

5 Work Described in this Thesis

In this thesis I describe the expression pattern and the regulation of Cek-8 during chick limb development. Cek-8, a cell to cell signalling receptor, is the first molecule of its kind described in limb development and the findings reported in chapter three may suggest that cell to cell signalling, next to long and short range signalling, is a third way of cell-communication during limb development.
Cek-8 is also expressed in rhombomeres (r) 3 and 5 in the developing chick hindbrain and, initially, I wanted to compare the regulation of Cek-8 expression in the limb to that in the hindbrain. Local application of retinoic acid to the chick hindbrain led to down-regulation of Cek-8 expression in r5 and, later in development, to loss of boundaries in the posterior hindbrain. In chapter four of this thesis I describe the molecular and morphological changes and also the cellular dispersal (antero-posterior spread) observed in retinoic acid-treated, boundary-less hindbrains.
CHAPTER TWO: Materials and Methods

1 Chick embryos

Fertilised chicken embryos were purchased from Poyndon Farm, Herts, England, and were incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951). Following surgical manipulation embryos were dissected in cold phosphate buffer (PBS), fixed in 4% paraformaldehyde (PFA) at 4°C and processed for whole mount in situ hybridisation or antibody staining.

2 Application of FGF-2, FGF-4 and BMP-2 on beads

Recombinant FGF-2 was obtained from R and D Systems and Recombinant FGF-4 was a kind gift from John Heath. FGFs were applied to heparin acrylic beads. Beads with a diameter from 200-250 μm were selected, washed in PBS and soaked in 2μl FGF2 or FGF4 (0.7mg/ml).

Recombinant human BMP-2 was provided by Genetics Institute, Cambridge, Massachusetts at a concentration of 2mg/ml. This stock was diluted 4-fold in 0.02 M sodium acetate, pH 5.0, containing 0.2% w/v BSA before use. Heparin acrylic beads of 200-240 μm diameter (Sigma) were soaked in 0.5 mg/ml BMP-2 for a minimum of one hour at 37°C. Beads were placed at different antero-posterior levels under the AER (see chapter three) of a stage 20 chick wing bud.
3 Application of Retinoic Acid on Beads

All \textit{trans} retinoic acid was obtained from Sigma (UK). AG1X2 beads were soaked for 20 minutes in 0.1 or 0.05 mg/ml retinoic acid in dimethylsulphoxide (DMSO) in the dark. Beads were then briefly washed and incubated for 30 minutes at 37°C in fresh tissue culture medium consisting of 90% Minimum Essential Medium (MEM), 10% foetal calf serum (FCS), 1/100 antibiotic-antimycotic and 2mM L-Glutamine (all from Gibco-BRL) in the dark. Beads were then implanted into stage 20 chick wings underneath the AER at different levels as described chapter three.

4 Experimental Manipulation of Chick Wing Buds

All manipulations were performed at stage 20 unless otherwise stated. For apical ectodermal ridge removal, a fine tungsten needle was employed. Beads were secured at apical sites using a fine platinum wire staple or by placing them under the apical ectodermal ridge. Beads were applied to proximal sites by inserting them into tissue in which a fine slit had been cut with a tungsten needle. For polarising region grafts tissue was dissected from the posterior margin of a stage 20 chick embryo and trypsinised at 4°C in 2% Trypsin (1: 250 Gibco-BRL) to remove the ectoderm. The tissue was subsequently grafted under the anterior ridge of a stage 20 host chick embryo. One embryo from each series of manipulations was allowed to develop for six days in order to validate the treatment.
5 Grafting of Mouse Limb Tissue to Chick Wing Buds

Forelimb buds from 10.5 day old C57/BL/Ha mouse embryos were dissected from the body wall and trypsinised for 20 minutes at 4°C to remove the ectoderm. Mesenchymal tissue was dissected using tungsten needles and grafted proximally, as described above, or distally under the apical ridge of a stage 20 chick embryo wing.

6 Wholemount in situ Hybridisation

A 420bp antisense RNA probe corresponding to nucleotides 394-813 was used to detect Cek-8 (Saddique and Pasquale 1993). The entire coding region for Sek-1 (3.5kb) (Gilardi-Hebenstreit et al 1992) was used after size reduction to 700 bp. Msx-1 probe was a kind gift of Dr S. Wedden (Brown et al 1993). Follistatin transcripts were detected using antisense RNA for the whole cDNA (1.1kb, Conolly et al., 1995). The Hoxb-4 antisense riboprobe (1.3 kb) is complementary to the homeobox and sequences 5' and 3' to it (Burke et al. 1995). The riboprobe to detect Krox20 transcripts spanned 0.23kb of the Krox20 cDNA. The Hoxa-2 probe was generated as described in Prince and Lumsden (1994) and Hoxb-1 expression was screened using an antisense riboprobe stretching over 2kb.

6.1 DIG-11-UTP-labelled RNA Probes

All the procedures described below were carried out using RNase-free reagents. The linearised DNA template of interest was purified from a low melting point
agarose/TAE gel (50 x TAE (2 M Tris, 50 mM EDTA, adjusted to pH 8.0 with glacial acetic acid) using Gene Clean (see below). The probe was made according to a protocol provided by Dr. Ketan Patel. The reaction mixture was set up as follows: 1 μl of DNA template (1 mg/ml stock), 5 μl of 5x transcription buffer, 10.5 μl of double distilled (dd) water, 1.3 μl of 10mM UTP, 2 μl of 10mM ATP, 2 μl of 10mM CTP, 2 μl of 10mM GTP, 0.7 μl of DIG-11-UTP, 1 μl of RNase Inhibitor (40 units), 1.5 μl of either T7 or T3 RNA polymerase (20 units). The mixture was incubated at 37°C for 2 hours. After the addition of 2 μl of RNase-free DNase I and 1 μl of RNase Inhibitor, the reaction mix was incubated for a further 15 minutes at 37°C. The RNA was then precipitated for 20-30 in at -70°C with ethanol and 0.4M lithium chloride. The RNA pellet was then washed in 70% ethanol, air-dried and resuspended in 100 μl of double distilled water and stored at -20°C until use. The reaction was monitored by loading 1 μl of the reaction mixture, before the DNase I step and after ethanol precipitation, on to a TBE (0.1M Tris, 0.1M boric acid, 2mM EDTA, pH 8.35) agarose gel and visualising the riboprobe after brief electrophoresis.

### 6.2 Pretreatments

All chick embryos were washed in PBS and fixed overnight in 4% (w/v) paraformaldehyde at 4°C. All steps were performed at 4°C on a shaking platform unless otherwise stated. The embryos were then rinsed twice in PBT (0.001 % (v/v) of Triton X-100 in 1x PBS) for 10 minutes before incubating them with Proteinase K (20 mg/ml in PBT) for a variable duration (depending on size and stage of the specimens) at room temperature. After a wash in PBT, embryos were re-fixed in 4% (w/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde for 20 minutes. The embryos were then rinsed in PBT and prehybridised overnight at 60°C. A 50 ml volume of prehybridisation
solution contained 25ml of deionised formamide, 12.5 ml 20x SSC pH 4.5, 1g of Blocking Reagent (Boehringer Mannheim), 50µl of Triton X-100, 0.5ml of 10% (w/v) CHAPS solution (Sigma), 50µl of 50mg/ml Heparin lithium salt, 0.5ml of 0.5M EDTA pH 8, 250µl of 20mg/ml phenol/chloroform extracted yeast total RNA.

6.3 Hybridisation and Post-hybridisation Treatments

All steps were carried out whilst shaking unless otherwise stated. The probe was thawed to room temperature, heated for three minutes at 80°C and added to fresh hybridisation mixture (identical to prehybridisation mixture). The amount of probe added was optimised for each different probe used. Embryos were then hybridised for 48 hours at 62°C. The embryos were washed two times in 2x SSC, 0.1% (w/v) CHAPS, at 60°C for 1 hour each, and then two times in 0.2x SSC, 0.1% (w/v) CHAPS. After a washing in KTBT (0.05M Tris pH 7.5, 0.15M NaCl, 0.01M KCl, 0.1% v/v Triton X-100), embryos were incubated for 3 hours at 4°C in 20% (v/v) heat-inactivated lamb serum in KTBT. Then embryos were incubated at 4°C, overnight, in fresh 20% v/v lamb serum in KTBT, supplemented with anti-digoxigenin antibody (Boehringer Mannheim) to a concentration of 1µl per 1ml of solution. Embryos were washed 5 times at room temperature in KTBT and overnight in KTBT at 4°C. Subsequently embryos were treated with alkaline phosphatase buffer (50ml stock solution contained 5ml of 1M Tris pH 9.5, 2.5ml of 1M MgCl2, 1.25ml of 4M NaCl, 0.5ml of 10% (v/v) Triton X-100, 50µl of 1M levamisol). The 'colour reaction' was performed in the dark, in BM purple AP-substrate precipitating (Boehringer Mannheim) until the desired colour intensity was obtained, when the embryos were washed in KTBT for 30 minutes, fixed in 4% (w/v) of paraformaldehyde in PBS for 2 hours, and rinsed twice in PBS. The embryos were stored up to 18 months at 4°C in PBS and sodium azide.
7 **Wholemount Antibody Staining**

7.1 **Anti-SEK-1 antibody**

Stage 26-28 chick embryos were dissected into two along the dorsal mid-line. One half was processed for wholemount *in situ* hybridisation and the other for wholemount antibody staining with an affinity purified anti-SEK-1 antibody that cross-reacts specifically with its avian homologue (Irving et al 1995). The dissected embryos were washed in ice cold PBS and then fixed in 2% (w/v) Trichloroacetic acid for 2h at 4°C. They were then washed 3 times for 10 minutes each in PBS, bleached with 0.05% (v/v) $\text{H}_2\text{O}_2$ for 30 minutes at 4°C, washed for 30 minutes PBS at room temperature and then incubated for 1h in 10% (v/v) sheep serum to prevent non-specific protein binding. The embryos were then incubated with anti-SEK-1 antibody (1:1000) for 12 hours at 4°C. Subsequently embryos were washed 3 times for 1 hour each with PBT before incubating for 12 hours with an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:200). The embryos were then washed 3 times for 10 minutes each before visualising antibody binding with NBT/BCIP.

7.2 **3A10 Antibody Staining**

All steps were performed with rocking at 4°C, unless otherwise stated. After three hours of washing in PBS, endogenous peroxidase was blocked using 0.05% (v/v) hydrogen peroxide in PBS and 1% (v/v) Triton (PBTTr) overnight. After 3 days' incubation with monoclonal 3A10 antibody (1:3000) in 10% (v/v) Newborn calf serum in PBTTr, embryos were washed extensively in 1% (v/v) normal goat serum in PBTTr. Secondary antibody, peroxidase-conjugated goat anti-mouse (Jackson laboratories) was
added at a dilution of 1:100 to 1% (v/v) normal goat serum in PBTr overnight. After extensive washing in 1% Normal Goat Serum in PBTr and PBS, embryos were soaked for 1 hour in 0.5 mg/ml 3,3'-diaminobenzidine tetra hydrochloride (DAB, Sigma) and the reaction was initiated by adding hydrogen peroxide (0.003%) at room temperature. Once the colour had developed, the reaction was stopped using PBS with sodium azide.

8 Frozen section

Embryos used for wholemount in situ hybridisation were refixed overnight in 4% (w/v) paraformaldehyde and then washed in ice cold PBS. Embryos were then placed in increasing concentrations of sucrose in PBS up to 30% (w/v). Embryos were then embedded in OCT and slowly frozen in liquid nitrogen and stored at -70°C until sectioning with a cryostat. Sections of 10-15 μm thickness were collected on to slides and mounted with glycerol before analysis under the microscope.

9 Retinoic Acid Application into Chick Embryo Hindbrains.

All-trans retinoic acid was obtained from Sigma (UK) and dissolved at 0.01 mg/ml in dimethylsulphoxide (DMSO). Retinoic acid was loaded on to AG1-X2 beads of approximately 100μm diameter as described above (3 application of retinoic acid on beads). Control beads were soaked in DMSO only. The beads were placed into the fourth ventricle of the hindbrains through a small slit in the roof plate of stage 10-11 chick embryos. Eggs were then resealed and returned to the incubator. Embryos were harvested at different time points.


10 Iontophoretic Application of DiI and DiAsp

Small deposits of the lipophilic membrane dye DiI or DiAsp (Molecular Probes D-282, D-3883) were applied in ovo to the developing hindbrain by iontophoresis. Microelectrodes with a tip diameter of approximately 2μm were filled at their tips with a small quantity of DiI or DiAsp (3mg/ml dimethyl formamide) and then filled with 1M lithium chloride. These were inserted into an electrode holder connected to the positive pole of a 9 volt battery. The electrode was micromanipulated into the neural tube and the dye driven out of the electrode by completing the circuit with a second silver wire electrode placed in the egg albumen and attached to the battery’s negative terminal. Completing the circuit for about 2-3 seconds was sufficient to label a small patch of cells. Dye application was done under a dissecting microscope and the position of the labelled cells was checked on a fluorescence microscope and imaged and measured using a cooled CCD camera and Biovision software. The embryos were then allowed to develop for 48 hours and examined under a fluorescence microscope.

11 Semithin Sections

Embryos were fixed in 0.1 M sodium phosphate buffer pH 7.2 overnight and subsequently rinsed for 5 minutes in 0.1 M cacodylate buffer. Specimens were then osmicated in 1 % aqueous OsO₄ for one hour at 4°C before rinsing them again in 0.1 M cacodylate buffer. After gradual dehydration, embryos were placed for 30 minutes in propylene oxide before bathing them 30 minutes in a 1:1 mixture of propylene oxide and resin (10g Araldyte, 10g Hardener, 0.8g plasticiser (Dibutyl Phtalate), 0.4 ml accelerator, mixed on a heated stirring plate). Then embryos were orientated in resin and left at room
temperature for 4-5 hours before hardening the resin for 24-48h in a 60°C oven. Semithin sections (1μm) were collected on glass slides and stained with toluidine blue.

12 Preparation of Plasmid DNA

Large Scale Preparation

500ml LB-Amp (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl. The pH was adjusted to 7.2 with NaOH, 0.1mg/ml of ampicillin) was inoculated with a bacterial glycerol stock, or a colony containing a desired plasmid and grown for 18 hours in a shaking incubator at 37°C. The culture was spun at 3,000 rpm for 20 minutes at 4°C. The cells were resuspended in 20ml of ice-cold solution I (50 mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA), placed for 30 minutes on ice, mixed with 40ml of freshly prepared solution II (0.2N NaOH, 1% (w/v) SDS), and incubated for 10 minutes at room temperature. Then, 20ml of ice-cold solution III (3M potassium acetate, pH 4.3) was added and the mixture was placed on ice for 10 minutes. The suspension was spun at 3,000 rpm for 20 minutes at 4°C and filtered through four layers of nylon. Nucleic acids were precipitated with 0.6 volumes of propan-2-ol, and spun at 6,000 rpm for 15 minutes at room temperature. The pellet was washed with 70% (w/v) ethanol and resuspended in 3ml of autoclaved dd water. High molecular weight RNA was precipitated by adding the same volume of ice-cold 5M LiCl, and spinning at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and the DNA was precipitated at room temperature for 10 minutes with 2.5 volumes of ethanol. Following centrifugation at 10,000 rpm for 10 minutes at 4°C, the pellet was washed with 70% ethanol, and
resuspended in 750µl of autoclaved dd water. Remaining RNA was digested by adding boiled RNase-A (1µl of 50mg/ml) and incubating at room temperature for 1 hour. Plasmid DNA was then precipitated by adding the same volume of ice-cold PEG buffer (1.6M NaCl, 13% (w/v) polyethylene glycol 8000) and spinning at 13,000 rpm for 10 minutes at 4°C. The pellet was resuspended in 400µl autoclaved dd water, phenol/chloroform- extracted and precipitated with 2.5 volumes of ethanol at room temperature for 5 minutes. Following centrifugation, the DNA pellet was washed twice with 70% (w/v) ethanol air dried at room temperature, and resuspended in 400 µl of autoclaved dd water and stored at -20°C.

13 DNA Purification with "Gene Clean"

The procedure described was that recommended by the manufacturer (Bio-101, La Jolla, USA). The gel area containing the linearised DNA fragment was cut out, and the agarose was dissolved by addition of 3ml of NaI per 1g of excised gel, and incubation at 50°C for 5 minutes. After the agarose was dissolved, 5ml of "glass milk" were added followed by vortexing and incubation on ice for 10 minutes, to allow binding of the DNA to the "glass milk". The mixture was then spun for 15 seconds at 13,000 rpm and the pellet was washed twice with fresh ice-cold "new wash". This was followed by resuspension of the pellet in 10ml of dd water and incubation at 50°C for 5 minutes. The "glass milk" was then precipitated by spinning at 13,000 rpm for 30 seconds. The supernatant, which contained, the DNA was transferred to a fresh tube.
14 Agarose Gel Electrophoresis of DNA

Normal or low melting point agarose was used for the preparation of gels. Restriction enzyme digests were analysed on agarose gels containing between 0.7 and 1.2% (w/v) agarose, in 1x TBE (Tris-Borate-EDTA) or 1x TAE (Tris-Acetate-EDTA) with 0.5 mg/ml of ethidium bromide. Samples were mixed with 10% (v/v) gel loading buffer (0.25% w/v bromophenol blue, 25mM EDTA, 50% v/v glycerol.) before loading on the gel. The gel was run at 50-80 V. The DNA was visualised on a short wave (254nm) ultraviolet transilluminator.

15 Preparation of Competent Bacteria

A single bacterial colony of the E.coli strain JM83 was grown in a small volume of LB-broth overnight at 37°C with shaking. 5 µl of that culture were used to inoculate 50ml of fresh LB-broth. This culture was incubated in a shaking incubator at 37°C until an OD600 of 0.3-0.4 was reached. The culture was then placed on ice for 15 minutes and spun at 2,500 rpm for 10 minutes at 4°C. The pellet was resuspended in 25ml of ice-cold 50mM CaCl₂ and, after 10 minutes on ice, spun again at 2,500 rpm for 10 minutes at 4°C. The cells were then resuspended in 3-4 ml ice cold 50 mM CaCl₂, according to the OD600 measurements, and incubated for 1 hour on ice before transformation.

16 Transformation of Competent Bacteria

Plasmid DNA (5µl) was chilled on ice and 100 µl of freshly prepared competent cells were added. This mixture was incubated for 10 minutes on ice and heat-shocked for
2 minutes at 42°C. Then cells were incubated for 10 minutes at room temperature. 1ml of LB-broth was added and the tube was incubated while shaking at 37°C for 30 minutes. The cells were spun for 3 minutes at 3,000 rpm, most of the supernatant was removed and the cells were resuspended in approximately 200μl of LB-broth. The bacteria were then plated on pre-warmed LB-Amp agar plates and incubated overnight at 37°C. White colonies were picked and subjected to small scale plasmid preparation and restriction enzyme digestion to confirm their identity.
CHAPTER THREE: *Cek-8* and Limb Development

1 Introduction

*Cek-8* (Sajjadi and Pasquale 1993), a member of the *Eph* class of receptor tyrosine kinases, displays a restricted expression pattern during hindbrain development. Preliminary results suggested that *Cek-8* transcript were found during chick limb development and this was the starting point for this thesis. First, the expression pattern of *Cek-8* during chick limb development was analysed. The expression pattern of *Cek-8* in older chick limbs was then compared to the late expression pattern of *Sek-1* (the murine homologue of *Cek-8*) in wild-type and also in *Wnt7a* -/- (“double ventral”) mouse limbs. Second, factors regulating *Cek-8* expression were analysed using grafting and wholemount in situ hybridisation techniques. *Cek-8* transcript distribution in limb buds of the polydactylous chick mutant *talpid* was also examined to gain an insight into its position in the signalling cascade that governs patterning and outgrowth.

2 Results

2.1 Expression of *Cek-8* in Limb Buds

To analyse *Cek-8* expression in the developing chick limb bud *wholemount in situ* hybridisations were performed using a digoxygenin labelled RNA against a non conserved extracellular coding region of the receptor tyrosine kinase. During early embryogenesis, stages 6-20, *Cek-8* transcripts were detected in a number of sites previously described in the mouse (Nieto et al 1992), including presumptive somites and hindbrain. In addition, *Cek-8* expression in limb buds was detected and this has not been
previously described in detail. *Cek-8* transcripts were first detected in the posterior part of emerging wing bud and in body wall at stage 17. Subsequently weak expression of *Cek-8* extended throughout the wing bud (Fig. 1A, arrows). As the limb buds became more distinct, expression of *Cek-8* became confined to the distal tip by stage 22/23 and in a stripe at the base of the bud which extends into the flank (Fig. 1B). Dynamic expression of *Cek-8* was also observed in leg buds, although slightly delayed compared with wing buds. At stage 22, *Cek-8* was transcribed in mesenchyme cells but not ectoderm. As the limb bud grew out further (stage 23-29), *Cek-8* expression was confined to the distal apical part of the bud with a sharp posterior demarcation and fading anteriorly (e.g. see control side in Fig. 8A, 8B; this antero-posterior gradient is obscured in strongly stained specimens). Transversal sections through the distal limb bud revealed that higher levels of expression occur in peripheral mesenchyme (Fig. 1E). During stages 24-29, transcripts at the bud apex gradually decreased but were still detectable up to stage 29 (Fig. 1F).

New sites of *Cek-8* expression were detected as tissues of the limb began to differentiate. *Cek-8* transcripts were observed at stage 24-25, in regions where cells were condensing to give rise to the humerus (Fig. 1C). In transverse sections of the proximal part of a stage 25 limb, *Cek-8* expressing cells were located subectodermally (Fig. 1D, arrow) and also near dorsal and ventral edges of pre-cartilage cell condensations (Fig. 1D, open arrow). As development proceeded, *Cek-8* expression was up-regulated in distal structures and down-regulated proximally, although expression was also maintained between long bones in joint regions. By stage 27/28 expression was not observed over previously expressing long bones, but was now prominent in the developing hand and foot plates. In the foot plate, for example, the initial metacarpal-like zone of distal expression pointed towards the fading posterior distal site of *Cek-8*.
mesenchymal expression (Fig. 1F). Cek-8 mRNA was initially detected in posterior metacarpals, then in more anterior ones. Cek-8 expression was detected earlier and was subsequently stronger ventrally in the foot plate compared to dorsally. By stage 31, the earlier posterior region of Cek-8 expression was no longer detectable but there was expression of the gene over distal structures including developing phalanges (Fig. 2A).

Transverse sections of stage 27-31 foot plate revealed that Cek-8 expression was associated with development of tendons. Asymmetrical expression of Cek-8 was initially observed in a broad domain in the ectoderm and its immediate underlying mesenchyme, with stronger expression ventrally than dorsally at stage 27 (Figs. 1G and 1H). As cartilage condenses, the broad domain of Cek-8 expression concentrated into and became predominantly mesenchymal, first ventrally then dorsally. The ventral mesenchymal zone of Cek-8 expressing cells gradually became transformed into tight knots residing a few cell diameters under the ectoderm. Figures 2D and 2E show the expression of Cek-8 in formation of tendons in relation to anterior-posterior and dorsal-ventral axes at stage 31. Ventrally, three zones of Cek-8 expression were seen, with the posterior zone being associated with a tight mesenchymal condensation and the most anterior zone being more diffuse and including ectoderm and mesenchyme. Dorsally, two broad regions of expressing cells were found posteriorly, with a third anterior zone of cells only just beginning to express Cek-8. Eventually, tight knots of Cek-8 expressing cells were seen below the cartilage elements, whereas above, groups of Cek-8 cells were more flattened and these tendon-like structures became localised towards the centre of the limb. At proximal levels Cek-8 transcripts were found adjacent to part of the tibia perichondrium which is closer to the ectoderm and which marks the tendon attachment site to the cartilage element (Figs. 2B and 2C).
Figure 1  Distribution of Cek-8 during chick limb development

(A) Dorsal view of Stage 18 embryo showing Cek-8 expression throughout the wing bud (arrow) but restricted to the posterior leg bud (open arrow). (B) Stage 22/23 wing shown in dorsal view showing strong expression distally with a sharp edge posteriorly but fading anteriorly. (C) Stage 24/25 wing shown in dorsal view. Weak expression is observed in proximal regions (arrow) and strong expression at tip (open arrow). The white dotted lines indicate the level of the transverse sections shown in (D) and (E). (D) Transverse section of the proximal part of a stage 24/25 limb. Cek-8 expressing cells were located subectodermally (arrow) and also at the part of dorsal and ventral edges of pre-cartilage cell condensations (open arrow). (E) Transverse section of the distal part of a stage 24/25 limb through the distal Cek-8 expressing part of the wing. A graded distribution of Cek-8 transcript is seen with reduced levels of transcripts in core mesenchyme (arrow) and high levels in the subectodermal mesenchyme (open arrow). (F) Stage 27/28 leg in ventral view. High levels of transcripts are observed in regions associated with metacarpal development (open arrow) and there is a remnant of the distal expression domain at the posterior tip (arrow). The white dotted line indicates level of transverse section shown in (G). (G) Transverse section of metacarpals of a stage 27/28 leg. Dorsally (d) three regions of weak mesenchymal expression (arrows), ventrally (v) strong continuous mesenchymal expression but with zones of decreased expression (open arrows). Dotted lines indicate area shown in high power in (H). (H) High power of a transverse section of metacarpals of a stage 27/28 leg (turned 90° with respect to G) showing Cek-8 transcripts in mesenchyme (m) and ectoderm (e). (a) anterior, (p) posterior, (d) dorsal, (v) ventral.
**Figure 2** Distribution of *Cek-8* during chick limb development and in tendons and feathers.

(A) Stage 31 leg in ventral view. Strong expression is observed in stripes fanning out over metacarpals (open arrow) and phalanges (arrows) and also at more proximal regions (arrowhead). White dotted lines indicate level of transverse sections shown in (B), (C), (D) and (E). (B) Transverse section of the proximal part of a stage 31 leg. *Cek-8* transcripts are found adjacent to the part of tibia perichondrium (t) closest to the ectoderm and marks the tendon attachment site to cartilage element (arrow) - fibula (f). Dotted lines indicate area which is shown in high power in (C). (C) High power of a transverse section of proximal part of a stage 31 leg (flipped 180° with respect to B). *Cek-8* expressing cells are adjacent to tibia perichondrium (arrow). (D) Transverse section of a stage 31 leg at metacarpal level. Dorsally (d) expression is observed in three discontinuous sheets of mesenchymal and ectodermal tissue (arrows). Ventrally (v) tissue condensation is most advanced posteriorly (p) (open arrows). Dotted lines indicate area which is shown in high power in (E). (E) High power of a transverse section of metacarpals of a stage 31 leg show one of the dorsal zones of *Cek-8* expressing cells. (F) Stage 33 wing shown in dorsal view. Expression is detected at low levels in ectoderm and but higher levels in placode cylinders (open arrow). Placodes appear to bud away from the medial aspect of each cylinder (solid arrowhead). *Cek-8* expression is confined to posterior and proximal regions of the feather bud (arrows). (G) and (H) show matching distribution of *Cek-8*-mRNA and CEK8 protein respectively of a right and left leg of the same embryo (stage 27, dorsal view).

(a) anterior, (p) posterior, (d) dorsal, (v) ventral. (t)tibia, (f)fibula.
In the chick wing, between stages 29-37, Cek-8 was also expressed in a restricted pattern in feather primordia (Fig. 2F). In the least differentiated anterior regions of the wing, Cek-8 was uniformly expressed in the ectoderm but gradually became restricted, giving an appearance of hollow stripes of expressing tissue. Circular zones of cells seemed to bud away from the most posterior region of the Cek-8 expressing stripe and eventually formed a ring at the edge of each feather placode. As the feather appendage began to emerge, Cek-8 expression was upregulated at the posterior margin of the developing bud. At more advanced stages of feather development, Cek-8 expression was down-regulated in the anterior region but markedly raised at the posterior region and confined to the base of the growing appendage. Expression was not sustained beyond stage 36.

The distribution of CEK8 Protein was compared with that of Cek-8 mRNA distribution in right and left limbs of embryos at stages 20,24,27 and 31. In all cases, staining pattern of the antibody corresponded with the Cek-8 transcript pattern (compare Fig. 2F with 2G).

### 2.2 Expression of Sek-1 (Murine Homologue of Cek-8) in Wild-type and Wnt7a Mutant Mouse Limbs.

Cek-8 expression is observed before tendon condensations are distinct in the embryonic chick leg and thus represents an early marker for tendon development (Figs 2A, D). The restricted expression of this receptor tyrosine kinase may give an insight into cellular mechanism underlying tendon formation. Although dorsal and ventral tendons have different shapes and functions the expression pattern of Cek-8 suggests that the
same mechanism acts to form both dorsal and ventral tendon. WNT7A is a dorsalising signal and mice lacking functional Wnt7a gene have partly "double ventral" limbs (Parr & McMahon 1995). These limbs possess double ventral tendons and early stages of tendon development can now be studied by analysing Sek-1 (murine Cek-8 homologue) transcript distribution in these mutants.

Sek-1 expression was analysed in embryonic 14.5d old limbs of wild-type mice and compared with that in Wnt7a mutant limbs of the same age. Sek-1 is expressed in tendons of the hand- and footplate of the mouse embryo (Figs. 3A,C,E,G). As in the chick leg, tendon development is more advanced proximally compared with distally in mouse toes. The pattern of expression in digit two of the forelimb illustrates the general features of the pattern found in all the digits in both fore- and hindlimbs. At the distal tip of the digit, expression was found in the dorsal and ventral ectoderm and also in the underlying mesenchyme. Transcripts were also detected in a thin stripe of cells adjacent to the cartilage precondensation. At more proximal level, the ventral domain in the ectoderm has disappeared whereas the dorsal Sek-1 expression is sustained and in the mesenchyme a ring of cells around the cartilage condensation now expresses Sek-1. Even more proximally, the dorsal domain in the ectoderm becomes weaker and finally disappears, whilst, in the mesenchyme, Sek-1 is expressed around the ventral tendon precondensation (Figs. 3A,F; 4A,C). Dorsally, as in the chick, tendon development is delayed and the mesenchymal Sek-1 expression domain, although distinct, is not as defined as the ventral one (Figs. 3C,G and 4A,C). The expression around the cartilage condensation, initially linked to the joints, becomes also weaker and is seen, at least anteriorly, down to the most proximal levels. Expression at the perimeter of tendon condensation also becomes weaker at more proximal levels. The precise distribution of
transcripts around the circumference of the cartilage condensation varies slightly among different digits.

In *Wnt7a* -/- mouse limbs, mesenchymal and ectodermal distribution of *Sek-1* transcript is in certain aspects strikingly different from that in wild type mouse limbs. In certain digits in 14.5d mouse limbs, tendon development dorsally is more advanced in the distal part of mutant limbs compared with control limbs. In forelimbs, synchronised appearance, on both dorsal and ventral sides, of a symmetrical *Sek-1* expression domain of similar size and shape can be seen in digit two and three, whilst no expression at all is seen in digit one (compare Fig. 3A with 3B and Fig. 4A with 4B, Fig. 3C with 3D and Fig. 4A with 4B). In contrast, the rest of the digits display a comparable *Sek-1* transcript distribution to that of wild-type forelimb digits. In the mutant hindlimb, it is only in digit three that *Sek-1* is expressed in two symmetrical domains whilst a wild-type-like expression pattern is seen in the rest of the digits (compare Fig. 3E with 3F and Fig. 4C with 4D; Fig. 3G with 3H and Fig. 4C with 4D). In contrast to its limited ectodermal distribution in normal limbs, *Sek-1* expression is prominent in the entire ectoderm of mutant limbs, from distal to proximal levels in all toes (compare Fig. 4A with 4B and 4C with 4D).
Figure 3 Sek-1 expression in limbs of 14.5d wild-type and Wnt7a -/- mice

All figures are wholmount hybridisations showing the Sek-1 expression pattern in 14.5d old mouse limbs. Anterior is always to the top of the page. (A) Ventral view of a wild-type forelimb. Strong expression is seen over the metacarpals. (B) In the forelimb of the Wnt7a mutant the ventral expression pattern is similar to the one observed in the wild-type limb. (C) Dorsal view of a wild-type forelimb. Sek-1 expression seems broader at dorsal than at ventral level. (D) In contrast, digits two and three of the dorsal forelimb of the Wnt7a mutant displays a ventral Sek-1 expression pattern. Digit 1 does not express Sek-1 dorsally and expression in digit four and five seems comparable to the one seen in wild-type limbs. (E) Ventral view of a wild-type hindlimb. As in the forelimb, strong expression is seen over the metacarpals. (F) Ventral view of a Wnt7a -/- forelimb. The expression pattern of Sek-1 is similar to the one detected ventrally in wild-type hindlimbs. (G) Dorsal view of a wild-type hindlimb. (H) Dorsal view of a Wnt7a -/- hindlimb. The general distribution of Sek-1 resembles its dorsal distribution in normal hindlimbs. +/+, wild-type; WT, wild-type; -/-, Wnt7a mutant;
Forelimbs

WT

ventral

Dorsal

Hindlimbs

WT

ventral

dorsal

/- Wnt7a

/- Wnt7a

ventral

dorsal
Figure 4  Transverse sections of wild-type and Wnt7a -/- forelimbs

Transverse sections of the distal part of the fore- and hindlimbs shown in figure 3 (A), (B), (E) and (F). Digits 2 to 5 are visible and, due to the plane of the section (transversal), each digit is at a different developmental stage. Digit 3 is the most developed, followed by digit 4, digit 2 and digit 5. The detailed description in the text is based on analysis of a series of adjacent transverse sections and the figures only illustrate part of that analysis. (A) Wild-type forelimb: a distinct Sek-1 expression domain is seen around the ventral tendon condensations in digits three and four. Ventrally there is no expression in the ectoderm. (B) Wnt7a mutant forelimb: digit three displays a mirror image Sek-1 expression pattern around ventral and dorsal tendon condensations. In the mutant limb, Sek-1 is expressed in the entire ectoderm of all toes. Similar, but not identical observations were made by comparing Sek-1 expression in (C) wild-type hindlimbs to that in (D) Wnt7a mutant hindlimbs. +/-, wild-type; WT, wild-type: -/-; Wnt7a mutant, numbers indicate digit identity; A, anterior; P, posterior; D, dorsal; V, ventral.
2.3 Response of \textit{Cek-8} Expression to AER Removal

The expression pattern of \textit{Cek-8} in the early wing bud suggests that it may be regulated by the AER. To test this possibility the AER was removed from a stage 20 limb bud using a fine tungsten needle and \textit{Cek-8} expression was monitored at several time-points after the manipulation. Removal of the apical ridge from early wing buds resulted in a dramatic down-regulation of \textit{Cek-8} expression. 24 hours after the operation, wing buds were severely truncated and no \textit{Cek-8} transcripts were detectable ($n=2$, Fig. 5C). A detailed time-course study revealed that \textit{Cek-8} expression was only slightly reduced 3-4 hours after the operation ($n=2$) but lost completely between 5.5 ($n=2$) and 6 hours ($n=2$) after ridge removal (Fig. 5A,B). The localisation of the signal emanating from the ridge which is required for \textit{Cek-8} expression was investigated by removing various portions of the ridge along the anterior-posterior axis. Removal of anterior AER resulted in a general down-regulation of \textit{Cek-8} expression in adjacent anterior mesenchymal tissue but there was also a slight down-regulation of \textit{Cek-8} expression in posterior mesenchyme ($n=2$, Fig. 6A). Similarly, removal of posterior ridge resulted in down-regulation of \textit{Cek-8} in adjacent posterior mesenchyme but also, albeit to a lesser extent, in anterior mesenchyme ($n=2$, Fig. 6B). Neither manipulation on its own could mimic the ablation of the entire ridge.

Recent experiments have intimated that FGF-2 and FGF-4 can substitute for signalling activity originating from the ridge (Niswander et al 1993). I therefore investigated the relationship between FGF and \textit{Cek-8} expression by applying FGF-2 to the limb after having removed the AER. Figs. 7 A,B show that application of FGF after ridge removal does indeed maintain \textit{Cek-8} expression. The zone of \textit{Cek-8} expression...
maintained by FGF was dependent upon the site of application. When FGF-2 was applied at the anterior margin of the bud, expression was predominantly induced around lateral and posterior rims of the bead (n=3, Fig. 7A) but when applied in more posterior regions Cek-8 was expressed anteriorly, proximally and posteriorly to the FGF source (n=2, Fig. 7A). In both cases there was a zone of non-expressing tissue directly adjacent to the bead. These results imply that limb tissue displays an anterior-posterior gradient of competence in its ability to express Cek-8 in response to FGF-2. This idea was further tested by applying beads soaked in FGF-2 into proximal regions of the bud where Cek-8 is not normally expressed (n=11). A gradient of competence to respond to FGF by expressing this receptor tyrosine kinase exists along both antero-posterior and proximo-distal axes such that cells in the postero-proximal region can be influenced by FGF-2 to express Cek-8 (Fig. 7C) whereas cells in anterior-proximal regions cannot.

In order to further investigate the ability of distal signals to induce cells to express Cek-8, proximal, non-expressing tissue from mouse forelimb was grafted into distal chick wing bud (n=3). Using a mouse-specific probe for Sek-1, the murine homologue of Cek-8, it was found that expression of the gene had been induced throughout the grafted tissue 24 hours after grafting (Fig. 7D). In the complementary experiment where Sek-1 expressing distal forelimb tissue from mouse embryos was transplanted in proximal regions of the developing chick wing bud no expression was detectable in the transplanted tissue after 24 hours (n=3, data not shown).
Figure 5  Maintenance of Cek-8 expression in the progress zone
requires continuous presence of the apical ectodermal ridge

All manipulations were performed at stage 20. In each case the entire ridge was removed from the right wing bud as illustrated by the cartoon. (A) Dorsal view of wing 5.5 hr after ridge removal. Weak posterior expression is still detectable (arrowheads). (B) Dorsal view of wing bud, 6 hr after ridge removal. There is no detectable Cek-8 expression. (C) Dorsal view wing of bud at 24 hr after ridge removal shows severe truncation of the limb and loss of Cek-8 expression.
Maintenance of Cek-8 expression in the progress zone requires continuous presence of the apical ectodermal ridge
Figure 6  Partial down regulation of \textit{Cek-8} expression after localised apical ectodermal ridge removal

(A) Dorsal view of wing bud 24hr after anterior ridge removal (area delineated by arrowheads). There is a slight decrease in \textit{Cek-8} expression near the anterior edge of remaining ridge (arrow). (B) Dorsal view of wing 24 hr after posterior ridge removal. \textit{Cek-8} expression is down-regulated both adjacent to the site of ablation and anteriorly (arrow).
Partial down-regulation of Cek-8 expression after localised apical ectodermal ridge removal
Figure 7  FGF2 maintains and induces Cek-8 expression

(A) Anterior application of FGF-2 after AER removal. Cek-8 expression is maintained proximally and posteriorly around the bead (arrowheads). (B) Posterior application of FGF-2 after AER removal. Cek-8 expression is maintained both anterior and posterior to the bead (arrow, arrowheads). (C) Proximal application of FGF-2. Upregulation of Cek-8 expression distal to bead (arrow). (D) Ventral view of wing after proximal mouse limb bud tissue was grafted to the distal region of a chick wing. Upregulation of Sek-1 is observed in grafted tissue (arrow). E. Application of a retinoic acid bead together with a FGF4 bead to the anterior wing margin after AER removal. Cek-8 expression is maintained posterior and induced anterior to the FGF bead. Compare this result with Fig 5C.

FGF-2, FGF4 were used at 1mg/ml, retinoic acid was used at 0.05mg/ml in DMSO.
FGF2 maintains and induces Cek-8 expression
2.4 Effect of Polarising Signals on Cek-8 Expression.

Expression of Cek-8 in distal mesenchyme of the limb bud was asymmetrical across the antero-posterior axis with a blunt edge posteriorly next to the polarising region and fading anteriorly. In order to determine whether the polarising region influences the expression domain of Cek-8, a polarising region was grafted to the anterior margin of a developing wing bud. Fig. 8A shows that transplantation of the polarising region led to anterior enhancement of Cek-8 expression resulting in symmetrical expression at tip of the bud (n=3) although expression did not appear to extend right up to the transplanted tissue. Interestingly the graft itself did not appear to express Cek-8.

Retinoic acid has been previously shown to mimic signalling of the polarising region (Tickle et al 1982) and therefore its effect on Cek-8 expression was examined by applying beads soaked in retinoic acid to anterior margin of developing wing buds. Like polarising region grafts, retinoic acid beads, at 0.1mg/ml, shifted the limit of Cek-8 expression more anteriorly (n=3, Fig. 8B) but not immediately up to the bead. Thus, both polarising region and retinoic acid applied anteriorly induce a very similar response with regards to Cek-8 expression.

This elicited response could be a result of an extension of the Fgf4 expression domain after retinoic acid application to the anterior margin of the wing bud (Niswander et al, 1994). However, when the AER is removed and two beads, one soaked in 0.05 mg/ml retinoic acid (n=3) and another one soaked in FGF4, were placed at the anterior wing margin, Cek-8 expression is partly maintained posteriorly and induced anteriorly to the FGF4 bead (arrows, Fig. 7E). This response differs to the result shown in Fig. 7A.
where only FGF2 (or FGF4, not shown) was applied anteriorly after AER removal, and no new site of expression was detected. It therefore seems that the polarising signals, in conjunction with growth signals, regulate Cek-8 expression in the early wing bud.

When beads soaked in higher concentrations of retinoic acid (1mg/ml, n=4) were applied anteriorly, Cek-8 expression did not appear to be extended but instead the anterior limit of expression was shifted posteriorly.

2.5 Cek-8 Expression in the Polydactylyous Mutant Talpid

Homozygous talpid chickens have limbs with an increased number of morphologically identical digits. In early limb buds of talpid chickens, Cek-8 expression was seen to extend anteriorly across the tip (Fig. 8C) reminiscent of the response of normal limb buds to application of either retinoic acid or a polarising region graft (Figs. 8A,B). In older talpid wing buds, not only an anterior extension of the expression domain but also a weak signal throughout the bud was observed suggesting a possible disruption in distal as well as posterior restriction mechanisms.

2.6 Inhibitory Action of Retinoic Acid on Cek-8 Expression

Retinoic acid, when applied to the anterior margin of a stage 20 wing bud, did not induce Cek-8 expression in directly adjacent tissue. To examine further this effect on Cek-8 expression, beads soaked in retinoic acid were placed into the domain of expression at wing bud tips. When a retinoic acid soaked bead (0.1mg/ml) was applied to
the bud apex, there was considerable down-regulation in *Cek-8* expression (n=2, Fig. 9A). However, this down-regulation was not complete and there was residual expression both anterior and posterior to the bead. When a bead soaked in the same concentration of retinoic acid was placed posteriorly there was almost complete down-regulation of *Cek-8* expression (n=2, Fig. 9B) even anteriorly at the tip.

### 2.7 Response of *Cek-8* Expression to BMP-2 Application

Recently it has been shown that *Bmp-2* expression is activated by retinoic acid (Francis et al. 1994) and so it was examined whether this molecule exerts the same effect on *Cek-8* expression as retinoic acid. When a bead soaked in 0.5mg/ml of BMP-2 was applied to the anterior margin of the developing wing bud, *Cek-8* expression was down-regulated around it but, none the less, gene expression was maintained in more posterior regions of the bud tip (n=3, Fig. 10B). When BMP-2 was applied in the region corresponding to the polarising region it led to a complete down-regulation in *Cek-8* expression, similar to the effect induced by retinoic acid where inhibition of expression occurred over long distances (n=3, Fig. 10A). Thus BMP-2 can mimic the inhibitory effects of retinoic acid on *Cek-8* transcription. In addition, I determined the effect of anterior BMP-2 on expression of *Msx-1*, another gene whose expression is regulated by factors originating from the ridge. Unlike *Cek-8*, the expression profile of *Msx-1* was not altered by the application of BMP-2 to wing buds (n=4, Fig. 10C).
Figure 8  *Cek-8* is regulated signals from the polarising region and *Cek-8* expression in wing buds of the polydactyous mutant *talpid*³.

All manipulations were performed at stage 19-21. (A) Anterior graft of the polarising region (arrow) or (B) anterior application of beads soaked in 0.1mg/ml retinoic acid (arrow) results in an anterior extension of the normal expression pattern after 24hr (arrow). (C) *Cek-8* expression in a stage 21talpid³ wing showing an anterior extension (arrowhead).

Note: A and B are reversed.
Cek-8 is regulated by signals from the polarising region
Cek8- expression in wing buds of the polydactylous mutant talpid3
Figure 9  Down-regulation of $Cek-8$ expression after retinoic acid application to the apical or posterior part of a stage 20 chick wing bud

All manipulations performed were performed at stage 20. (A) Ventral view. Apical application of a bead soaked in 0.1 mg/ml retinoic acid for 5hr results in down-regulation of $Cek-8$ expression but slight expression remains in posterior regions. (B) Dorsal view. Posterior application of retinoic acid results in almost complete down-regulation of $Cek-8$ expression after 24 hours.
Down-regulation of Cek-8 expression after retinoic acid application to the apical or posterior part of a stage 20 chick wing bud
Figure 10  

BMP2 down-regulates *Cek-8* but not *Msx-1* expression

(A) Dorsal view after posterior application of BMP-2 for 24 hr results in down-regulation of *Cek-8* both in adjacent and anterior regions (arrow). (B) Ventral view after anterior application of BMP-2 for 24 hr results in down-regulation of *Cek-8* near the BMP-2 bead (arrow). (C) Ventral view of wing after anterior application of BMP-2 (25 hr) probed with *Msx-1*. BMP-2 did not affect *Msx-1* expression (arrow).

BMP-2 was used at 0.5 mg/ml.
BMP-2 down-regulates Cek-8, but not Msx-1 expression
3. **Discussion**

3.1 **Expression Profile of the Receptor Tyrosine Kinase**

The expression profile of the receptor tyrosine kinase *Cek-8* suggests that the receptor has roles in both early and late chick limb development. Limb structures are generated in sequence from undifferentiated mesenchyme at the tip of the early bud and *Cek-8* expression is found in distal mesenchyme and regulated by outgrowth and patterning signals. Later on, transcripts of the gene are associated with cell condensations that form tendons and their attachments to cartilage rudiments and with developing feather buds. Another member of the *Eph* family, *Eck*, has also been found to be expressed in distal mesenchyme and in forming cartilage although, unlike *Cek-8*, *Eck* appears not to show a posterior restriction in distal mesenchyme (Ganju et al. 1994). Factors regulating *Eck* expression have not been examined, but ECK and CEK-8 may have overlapping or cooperative roles in the limb.

3.2 **Expression in the Early Limb Bud**

Expression of *Cek-8* during early wing development overlaps with the expression domains of a number of genes thought to play a pivotal role in patterning of the limb. *Cek-8* expression overlaps at the posterior part of the limb bud tip with expression domains of both *Shh* (Riddle et al., 1993) and the gene encoding BMP-2 (Francis et al. 1994). *Shh* and *Bmp-2* transcripts remain confined to the posterior margin of the limb bud. In contrast, *Cek-8* expression extends much more anteriorly, probably up to but not into the anterior expression domain of the gene encoding BMP-4. *Cek-8* expression also overlaps at the distal tip with expression of three transcription factors, *Msx-1*, *Msx-2*
(Davidson et al 1991) and Evx-1 (Niswander and Martin 1993b) which are regulated by ridge factors.

Distal expression of Cek-8 in apical and distal mesenchyme is modulated by ridge and polarising region signals. When proximal cells are placed at the tip of the limb beneath the apical ridge, they can respond to AER signals by re-expressing Cek-8. Ridge removal results in the loss of detectable expression of Cek-8 after 6 hours but expression can be maintained by application of FGF. Msx-1 expression is also maintained by FGF but is down-regulated very rapidly after ridge removal (Ros et al 1992). One possibility suggested by these data is that Cek-8 expression is downstream of Msx-1. With beads soaked in FGF2, Cek-8 expression apparently starts in tissue a few cell diameters away from the bead whereas Msx-1 can be expressed in cells immediately next to a bead soaked in the same concentration of FGF2 (Vogel et al, 1995). However when BMP-2 is applied anteriorly, expression of Cek-8 and Msx-1 are differentially affected.

Anterior application of retinoic acid or grafts of polarising region can also modulate Cek-8 expression and extended the normal domain of Cek-8 expression anteriorly. These manipulations would be expected to lead to anterior expression of Fgf-4 (Niswander et al 1994) which in turn could regulate Cek-8. Although application of FGF alone to anterior mesenchyme in the absence of the ridge does maintain Cek-8 expression this occurs predominantly in mesenchyme posterior to the bead. Furthermore, only cells in posterior two thirds of proximal limb bud but not anterior proximal cells can activate Cek-8 in response to FGF. This suggests that another factor is present in the posterior part of the bud which together with FGF regulates Cek-8 expression. Recently Yang and Niswander (1995) have demonstrated that FGF-4 can induce Shh expression in proximal posterior regions of the bud. It is also possible that in addition to requiring inductive
signals to activate and maintain the expression of Cek-8 (and Shh) in posterior and distal regions, the antero-distal part of the limb is rich in inhibitory molecules that can be overridden by polarising signals. A finding not accounted for by these possibilities is that expression of Cek-8 is always found distal to the FGF bead placed in posterior proximal mesenchyme. Yet when an additional bead is placed more proximally this mesenchyme is quite capable of expressing Cek-8 (not shown).

Several molecules are known to be regulated by co-operation between polarising and outgrowth signals including transcripts of Bmp-2, Bmp-7 and Hoxd-13 genes. All of these molecules lie downstream of SHH signalling and are expressed uniformly across the antero-posterior axes of the broad limb buds of polydactylous talpid³ mutant embryos (Izpisua-Belmonte et al 1993; Francis-West et al 1995). Cek-8 is also expressed uniformly in early limb buds of this mutant, which, together with its restricted expression pattern, suggests that Cek-8 expression is involved in coordination of responses to polarising and outgrowth signals.

3.3 Expression of Cek-8 in Mesenchymal Condensations

Cek-8, is transiently expressed in condensing mesenchyme that will form tendons and their associations with the perichondrium of cartilage elements. Cellular localisation of Cek-8 in developing tendons is first found ventrally and then dorsally and this fits with anatomical observations (Wortham 1948) that ventral tendons develop before dorsal ones. Cek-8 is expressed in a sub-ectodermal layer of cells, which then condenses just below the ectoderm. Ventrally this condensation then becomes a discrete knot of cells expressing Cek-8. This progression appears to parallel the morphology of tendon development described by Hurle et al (1989) and Ros et al (1995). Ros et al
(1995) identified six stages (I-VI) of tendon morphogenesis, from day 6 to day 11, in the long autopodal tendons of the chick foot. At day 6 of development, the mesenchyme surrounding the cartilage appears homogenous but the interface between the ectoderm and the mesoderm of the digital rays appears thickened dorsally and ventrally (stage I). The presence of a lamina of extracellular matrix, continuous with the ectodermal basement membrane is first seen in the digital rays at stage II. This lamina has been termed mensenchymal lamina (Hurle et al 1989) and Ros et al (1995) suggest that, during the next stage (stage III, day 7), it is used as a substrate by mesenchymal cell aggregates to form a tendon blastema. The cellular condensations along each digital ray are irregular and without defined border at that stage. These condensations appear more pronounced at stage IV and later, by stage V (day 8), a peripheral layer of flattened cells can be seen around these condensations. At stage VI the tendon blastema consists of a central core of cells with extensive deposits of extracellular matrix and is surrounded by a layer of flattened cells referred to as the epitenon (Ros et al 1995).

*Cek-8* expression also bears a striking resemblance to the expression patterns of tenascin, the extra-cellular matrix proteins Collagen I, III and VI (Ros et al 1995) and also of murine homologues of the *sine-oculus* genes originally isolated from Drosophila (Oliver et al 1995). One gene member of this family, *Six-2* shows a similar pattern of expression to *Cek-8* with expression initially confined to a broad sheet under the ectoderm that rapidly condenses into a mesenchymal layer. One difference in expression patterns of the two genes is that distal expression of *Six-2* only extends as far as cartilage condensations whereas *Cek-8* expression extends more distally and fans out from the digit primordia. A detailed study comparing timing and levels of expression of the mentioned markers during tendon morphogenesis may give an insight about the cellular mechanism underlying tendon formation.
Although Sek-1 expression in 14.5 d mice limbs is less restricted than the Cek-8 expression pattern observed in the stage 31 chick leg, the analysis of transverse sections revealed a clear correlation between Sek-1 expression and tendon formation. Sek-1 expression therefore represents an outstanding marker for mouse tendon development and this was demonstrated by screening limbs of 14.5d Wnt7a -/- mice for Sek-1 expression.

3.4 Sek-1 Expression, a Marker for Tendon Development

The limbs of Wnt7a -/- mice display double ventral features at most distal levels (Parr & McMahon 1995). By analysing transverse section through 15.5 d mutant limbs double ventral tendons have been identified in the original work (Parr & McMahon 1995). The analysis of Sek-1 expression in 14.5 d wild-type and Wnt7a mutant mouse limbs described in this thesis provides a more detailed description of tendon development. Interestingly, a limited “double ventral” Sek-1 expression pattern in the mutant has been observed, where only a few digits display the anticipated double ventral Sek-1 expression pattern. Interestingly, the ventralisation of dorsal Sek-1 expression domains closely linked to tendon formation was detected to a greater extent in the forelimb (in both digits two and three) than in the hindlimb (just digit three). Analysis of Sek-1 transcript distribution in mutant limbs of a series of stages should show whether this limited “ventralisation” of the dorsal tendon specific Sek-1 expression pattern is stage or limb- (i.e. fore- or hindlimb) specific.

Dorsally or ventrally restricted expression of a signalling molecule (Wnt7a) or transcription factors (Engrailed, Loomis et al 1996; Lmx-1, Riddle et al 1995, Vogel et al 1995) in the ectoderm and mesenchyme (Lmx-1) of the developing limb suggests that
dorsal and ventral tendons are differently specified. The interpretation of different positional values may give rise to the same differentiated cell type and the correlation of the expression of the receptor tyrosine kinase Sek-1 and both, dorsal and ventral, tendon formation however, reflects a common cellular mechanism involved in tendon development. Sek-1 is downstream of the dorso-ventral patterning event but may be important during early stages of tendon formation. Determining factors which regulate Sek-1 expression at dorsal and ventral levels may identify the regulatory events which lead to the use of the same developmental program by two different -dorsal and ventral- cell populations. One possibility is that the dorsal and ventral pathway converge at a certain factor which will then activate the common “tendon pathway” and consequently Cek-8 expression. Alternatively the dorsal and ventral pathways are completely divergent, and Cek-8 expression is regulated differently in the dorsal and in the ventral part of the limb. One possible way to distinguish between these two pathways is to analyse the Sek-1 promotor activity in mice. If different promotor elements drive the dorsal and ventral tendon Sek-1 expression domain, Sek-1 expression might the converging factor. On the other hand, if the dorsal and ventral tendon Sek-1 expression domains are driven by the same promotor element this suggests the existence of convergent pathways upstream of Sek-1. The complexity of the regulatory pathways involved in this process may be illustrated by the comparison of the ectodermal expression domain of Sek-1 in normal and in mutant hindlimb. In normal mice limbs the Sek-1 expression regresses ventrally at more distal level than dorsally. In mutant limbs, non-functional Wnt7a in the dorsal ectoderm results in a change of Sek-1 regulation not only in the dorsal but also in the ventral ectoderm.
3.5 Expression of Cek-8 in Developing Feather Buds and Scales.

Cek-8 is expressed in ectodermal placodes that will form feathers and scales. Many of the same genes are expressed in both the early limb bud and the feather buds and the same signalling network may produce budding in both cases (Choung et al 1990). Transcripts of Cek-8 are localised to feather ectoderm whereas in early limb bud transcripts are found in mesenchyme. A similar switch in tissue expression is also found for Shh which is mesenchymally expressed in the early limb bud but epithelially expressed in the feather buds (Nohno et al 1995). In feather buds Cek-8 is found in epithelium associated with mesenchyme expressing tenascin. It is intriguing that a similar association may also occur in developing tendons.

3.6 Potential Role of Cek-8 Expression.

Previous work has identified diffusible molecules e.g. retinoic acid and retinoids that potentially mediate signalling with a range of several to many cell diameters in the developing limb. Cek-8 is a member of the Eph family of receptor tyrosine kinases that recent work suggests are activated by membrane bound ligands (Bartley et al 1994; Davis et al 1994; Beckmann et al 1994; Cheng and Flanagan 1994). Thus the finding that Cek-8 is expressed in the chick limb bud suggests a role for contact mediated signalling in limb patterning. In addition, cell-cell communication via gap junctions has been observed between limb bud cells (Coelho and Kosher 1991) and thus all three mechanisms of signalling may operate in developing limbs.
Cek-8 may mark cells that have the ability to respond to short range signals presented by neighbouring cells. Cek-8 can bind to both classes of ligands (GPI-linked and transmembrane ligands, Gale et al 1996) in vitro but it is unclear which ligand interacts with Cek-8 in vivo. It is also not clear whether Cek-8 expressing cells constitute a population of mutually interacting cells or whether interactions only take place at the borders of Cek-8 expressing domains. In the first case, cells could express both receptor and ligand and this would coordinate activities such as cell proliferation at the tip of the early limb bud or cell differentiation in developing tendon. On the other hand, if cells expressing Cek-8 only interact with neighbouring cells at the boundaries of expression domains, interactions could occur at the proximal edge of the progress zone and at the sharp boundary of expression at the posterior margin of the progress zone adjacent to the polarising region. Later in development, an interaction at the boundary of condensing cells in tendons could perhaps control formation of the tendon sheath and connection with skeletal elements. This idea was recently reinforced results from Gale et al (1996) which demonstrated that the GPI-linked ligands and their subclass of receptor tyrosine kinases are expressed in complementary regions in the developing mouse limb bud and the authors suggest that interactions occur at the interface of the two expression domains.

Recent studies implicated ligands for receptor tyrosine kinases of the Eph family as good candidates for positional labels in the retinotectal system (Drescher et al., 1995; Cheng et al., 1995). Gradients of those ligands on the tectum provide positional information that is interpreted by the receptor tyrosine kinases on the axons to direct topographical projections. By analogy Cek-8 could perhaps be important in interpreting positional information in the limb progress zone.
Factors identified in this study that regulate \textit{Cek-8} expression in the early limb bud could potentially modulate expression elsewhere in tendons, featherbuds and even the hindbrain. Key components (or related molecules) that modulate \textit{Cek-8} expression in the limb are also found in the developing hindbrain; notochord is a source of retinoic acid as well as Shh (Riddle et al 1993), Fgf-3 is expressed in rhombomeres 5 and 6 (Wilkinson et al 1989), and Bmp-2 and Bmp-4 as well as Msx-1 and Msx-2 are expressed in a restricted manner during embryogenesis (Graham et al 1994). The fact that the same molecules are expressed during limb and hindbrain development was the starting point for the next series of experiments. Initially it was planned to analyse whether \textit{Cek-8} expression during hindbrain development may be regulated by the same factors as during limb development. Preliminary results showed that local retinoic acid application of retinoic acid leads to a partly unsegmented hindbrain and the analysis of that phenotype is described in detail in chapter four.
CHAPTER FOUR: Boundary-less hindbrains

1. Introduction

Cek-8 expression was found in the progress zone and is regulated by factors from the ridge (FGF) and the ZPA (Chapter three). In the developing hindbrain, Cek-8 expression is initially found in r3 and later also in r5. The work described in this chapter was initiated to compare the regulation of Cek-8 expression in the limb to that in the hindbrain. To investigate the effect of retinoic acid on Cek-8 expression beads soaked in retinoic acid were applied to hindbrains of stage 10 (Hamburger and Hamilton, 1951) chick embryos. It was found that local application of retinoic acid to the hindbrains of chick embryos, just after the initial events of morphological segmentation, leads to downregulation of Cek-8 expression in r5 and to loss of boundary cells in the posterior part of the hindbrain. This phenomenon allowed the investigation of the possible role of rhombomere boundaries in the chick hindbrain.

2 Results

2.1 Retinoic Acid Leads to Loss of Hindbrain Boundaries

Retinoic acid was applied to the hindbrains of stage 10-11 chick embryos by using AG1X2 beads (100 μm diameter) soaked in 10 μg/ml retinoic acid. Beads of 200 μm diameter release retinoic acid continuously over at least 20 hours as shown by Eichele et al. (1985) and this period is probably reduced for beads with half the diameter as used here (Eichele et al 1984). The embryos were allowed to develop for 24h or 48h, up to stages 15/16 or 19/20 respectively, and their hindbrains were then dissected and
flatmounted. Phase contrast optics revealed that this local retinoic acid application causes the loss of boundaries between posterior rhombomeres. In 60% of cases (Fig. 1), only rhombomeres 1 to 3 (r1 to r3) were distinguishable, as boundaries posterior to r3 were missing (Fig. 1A,B); in 26% of cases one or two rhombomeres were visible and boundaries posterior to r1 or r2, respectively, were missing; in the remaining cases (14%) either 4 or 5 rhombomeres were visible. The identity of rhombomeres was deduced from their relationship with the cranial nerve roots. Parasaggital semi-thin araldyte sections of the hindbrain revealed that the characteristic fan-shaped array of boundary cells and the increased extracellular spaces normally associated with boundaries (Heyman et al 1993, 1995) were also lost (Fig. 1C,F).

Morphological loss of posterior boundaries was associated with the absence of follistatin transcripts which mark rhombomere boundaries in normal embryos at stages 18-20 (Conolly et al., 1995). In normal embryos, 5 stripes of follistatin expression domains co-localised with morphological boundaries from the r2/3 boundary down to the r6/7 boundary, whereas a maximum of 2 stripes could be seen in retinoic acid treated embryos (n=4/4 Fig 1D,E; the apparent stripe between r1 and r2 is due to a sharp expression border at that level, of the low follistatin expression domain observed throughout the hindbrain).

In normal embryos, staining with the monoclonal antibody 3A10 revealed that neuronal differentiation in r2 and r4 is ahead of differentiation in r3 and r5, and that boundaries contained more circumferential axons than non-boundary regions (Fig 1G). In retinoic acid treated hindbrains, at stage 15 (i.e. 24 hours after treatment) there was virtually no sign of the usual alternating levels of neuronal differentiation in r4, r5 and r6.
and the periodic axon accumulations normally associated with boundaries was lost at these and subsequent stages (Fig 1H,I).

2.2 *Cek-8* and *Krox-20*

*Cek-8* and *Krox-20* are first expressed in the chick hindbrain at stages 8\* and 8 respectively (Irving et al 1995) and are both expressed in r3 and r5 from stage 10 onwards (Fig 2A,B). *Cek-8* expression lasts until at least stage 22 whereas *Krox-20* is present until at least stage 18. At 24 and 48 hours after the retinoic acid bead implants (stages 15/16 or 19/20 respectively), *Cek-8* was no longer expressed in r5, correlating with lack of boundaries at r5 level but was still usually expressed at r3 level (n=9/9 Fig 2B). In embryos with three or more rhombomeres, the presence of morphological boundaries correlated with sharp expression boundaries of *Cek-8* in r3 (n = 9/9 (24 h+ 48h) Fig 2D). In specimens where only two rhombomeres were clearly distinguishable, *Cek-8* had a sharp expression boundary at the anterior part of r3, where a morphological boundary was still visible, but not at the posterior part, where *Cek-8* expression was downregulated and the morphological boundary was abolished ( n=2/2 Fig 2C,E). In two specimens, an r4/5 boundary was also present and in both cases this correlated with residual *Cek-8* expression in r5 although this expression was not immediately adjacent to the boundary.

In contrast to *Cek-8*, *Krox-20* was completely downregulated in both r3 and r5 at 24 hours after retinoic acid treatment irrespective of the number of remaining rhombomeres (n=4/5 not shown, see below) except for one specimen in which weak residual expression of *Krox-20* was observed in r3 (1/5).
Figure 1  Retinoic acid leads to loss of hindbrain boundaries

All hindbrains shown are flatmounts from stage 18-20 chick embryos. (A) Control hindbrain with 7 identifiable rhombomeres (1-7). (B) Retinoic acid treated hindbrain, where only 3 rhombomeres are visible (1-3) and the posterior hindbrain is unsegmented (arrow).© Parasaggital section of a normal stage 18 hindbrain showing two rhombomere boundaries with their characteristic increase in extracellular space near the pial surface and bulges at the ventricular surface. (D) Follistatin expression in a control hindbrain. Five transversal stripes correlating to rhombomere boundaries 2/3 to 6/7 are visible. (E) Low level of follistatin expression is seen in the retinoid treated hindbrain, with a sharp expression boundary at the ventral side of the r1/r2 boundary. Only two transversal stripes of follistatin transcripts corresponding to r2/3 and r3/4 boundaries can be seen in a retinoid treated hindbrain. The arrows indicate the lack of the more posterior domains of follistatin expression. (F) Parasaggital section through a retinoic acid treated hindbrain which shows no periodic boundary specialisations. (G) 3A10 neurofilament staining of a normal hindbrain. Neuronal differentiation is advanced in r2, r4 (arrowheads) and from r6 onwards and axons accumulate in boundaries (*). (H) 3A10 neurofilament staining of a retinoic acid treated hindbrain. Neuronal differentiation is only advanced at r2 level (arrowhead) and seems to be uniform up to a more posterior level. (I) There is no axon accumulation at any site in the posterior hindbrain.

Numbers 1-7 indicate individual rhombomeres; ros, rostral (anterior); cau, caudal (posterior); d (dorsal); v (ventral); fp (floor plate); Scale bar = 200 μm A,B,D,E,G,H,I and 100 μm C,F.

Note: C and F are reversed
Figure 2  Cek-8 expression and boundaries

All hindbrains shown are flatmounts from stage 18-20 chick embryos. (A) Cek-8 is expressed in r3 and r5 in a control hindbrain. (B) Loss of the r5 expression domain of Cek-8 in a retinoid treated hindbrain with 3 remaining rhombomeres. (C) Cek-8 expression in retinoid treated hindbrain with 2 remaining rhombomeres. (D) High power of r3 of a retinoid treated hindbrain with 3 rhombomeres analysed for Cek-8 expression. A sharp expression boundary on either side of r3 is seen and this highlighted by the arrow and arrowhead. (E) r3 level of a retinoid treated hindbrain with 2 rhombomeres analysed for Cek-8 expression. The expression boundary of Cek-8 at r3 level is sharp anteriorly (arrow) and blurred posteriorly (arrowhead).

Numbers 1-7 indicate individual rhombomeres. b, boundary; ros, rostral (anterior); cau, caudal (posterior). Scale bar= 200 μm in A,B,C and 50 μm in D,E.
2.3 Changes in Cek-8 and Krox-20 Expression Precede Morphological Changes

Loss of morphological segmentation in the posterior hindbrain was mirrored by changes in gene expression. To test the timing of these events gene expression at a series of times after retinoic acid treatment was examined. At 4 to 6 hours after placing a retinoic acid bead at any position between r1 and r6, Cek-8 expression was unaffected in r3, but abolished in r5 in all treated embryos (n=10/10, Fig 4 C,D). At this stage all boundaries were still visible. Thus the change in gene expression occur before morphological alterations. When the retinoic acid bead was placed anterior to r1 or posterior to r6, Cek-8 expression in r5 as well as in r3 remained unaffected (n= 3, Fig 4A,E).

The effect on Krox-20 expression is complex and varies with the position of the bead. When the retinoic acid bead was placed anterior to r2, Krox-20 expression was unaffected in r3 and in r5( n=2/2, Fig 4F). A bead placed more posteriorly, at r2 or r3 level, led to maintenance of Krox-20 expression in r3 but downregulation in r5 (n=2 Fig 4G). However retinoic acid application at r4 level abolished Krox-20 expression in both r3 and r5 (n=1, Fig 4H). Beads located even more caudal, at r5 or r6 level, abolished Krox-20 transcripts at r5 level only ( n=2/2, not shown). A bead placed posterior to r6 did not affect Krox-20 expression at all (n=1, Fig 4I).

To compare the timing of changes of Cek-8 and Krox-20 expression following retinoic acid treatment, a bead was placed at r4 level and changes in expression of those two genes were monitored at a series of times after the manipulation. Complete loss of
Cek-8 expression in r5 was observed 4 hours after treatment (n=2/2, Fig 5A), whilst Krox-20 is still weakly expressed in r3 but completely downregulated in r5 (n=2/2 Fig 5B). When the retinoic acid bead, at r4 level, was removed after 4 hours and embryos were allowed to develop a further 4 hours, the response of Krox-20 (and also Cek-8) expression was similar to the one described above (n=4/4 Fig 5C,D). However, Krox-20 expression was lost in both r3 and r5 after six hours of continuous retinoic acid treatment.

Retinoic acid affects expression of Cek-8 in r5 only when applied in a certain proximity (between r1 and r6) and this suggests that this effect is dose dependant. With beads soaked in lower concentrations of retinoic acid (0.001 mg/ml) and placed at r4 level, Cek-8 expression was unchanged at r3 level and only slightly downregulated in r5 4 hours after treatment (Fig 5E) and this is consistent with a dose dependant response.

In general, Cek-8 expression is maintained in r3 and abolished in r5 after retinoic acid application, whereas Krox-20 can be abolished in both rhombomeres r3 and r5. Furthermore, at the time examined, expression of both Cek-8 and Krox-20 in r5 is more sensitive to retinoic acid than expression in their respective r3 domains. The expression of Cek-8 in r3 is maintained despite the loss of Krox-20 transcripts from stage 11/12 onwards, i.e. 6 hours after implanting the bead. Boundary maintenance is strictly correlated with Cek-8 expression.
Figure 3  *Cek-8* and *Krox20* mRNA expression in normal and retinoic acid treated hindbrains

The top row shows the normal expression patterns of *Cek-8* (A, stage 10) and *Krox20* (B, stage 10). (A) *Cek-8* is normally expressed in r3 and r5 and in neural crest derived from r6. (B) Normal embryo; *Krox20* transcripts are detected in r3 and r5 and in neural crest derived from r6. (C) *Cek-8* expression is down-regulated at r5 level 6 hours after retinoic acid application. The bead was damaged after fixing. (D) *Krox20* expression is down-regulated in r3 and r5 6 hours after retinoic acid application.

ros, rostral; cau, caudal; OV, otic vesicle; b, bead. Scale bar = 500 μm in A,B and D and 150 μm in C.
Figure 4  Position dependent effect of retinoic acid on *Krox20* and *Cek-8* expression

All experimental embryos were fixed 6 hours after implanting the bead unless otherwise indicated. (A) *Cek-8* expression is unchanged at r3 and r5 (arrows) level when the retinoic acid bead (*) was placed at forebrain level. (B) Expression of *Cek-8* is slightly reduced (arrow) when the bead was placed at r1 level. (C) r5 domain of *Cek-8* expression was lost (arrow) when the bead (*) was placed at r4 or r5 (D) level. (E) Expression was unchanged when the bead (*) was placed at somite 1 level. (F) Placing a retinoic acid bead (*) at r1 level does not change *Krox20* expression in the neural tube. When a retinoic acid bead (*) was applied at r3 level, *Krox20* expression is downregulated in r3 and even more so in r5 (arrows). (H) In contrast *Krox20* is strongly downregulated in r3 and r5 (arrows) when retinoic acid was applied at r4 level and any remnant of expression is lost by stage 14 (24 hours after retinoic acid treatment, not shown). (I) *Krox20* expression at r5 is down-regulated to a very limited extent when a retinoic acid bead (*) is placed at somite 1 level.

r3 and r5, rhombomere 3 and 5; *, position of the bead; ant, anterior (rostral); post, posterior (caudal). Scale bar = 500 μm in A,B,E,F,G,H and 100 μm in C,D.
Figure 5  The response of Cek-8 and Krox20 expression to retinoic acid is time and dose dependent

A retinoic acid bead was placed at r4/r5 level in all shown embryos. (A) Cek-8 expression in r5 is downregulated as soon as 4 hours after retinoic acid (0.01 mg/ml) treatment. (B) Krox20 expression is also downregulated in r5 but still visible in r3. Note that Krox20 expression, in contrast to Cek-8, is normally lost in both r5 and r3, 6 hours after retinoic acid treatment at r4 level (see 4H). The same response as in (A) and (B) was obtained when retinoic acid was applied only for 4 hours, the embryos were allowed to develop a further 4 hours (C and D). (E) Cek-8 in r5 is still visible after 4 hours treatment with a low dose of retinoic acid (0.001 mg/ml). Compare this result to 5 (A).
2.4 *Hoxa-2, Hoxb-1 and Hoxb-4*

Hox gene expression in retinoic acid-treated hindbrains was examined as these genes are thought to confer segment identity and are known to be responsive to retinoic acid. *Hoxb-4* is normally expressed in the neural tube up to the r6/r7 boundary [Fig 6D]. At 16 hours after the bead implants when *Cek-8* and *Krox-20* expression were already affected, *Hoxb-4* expression remained unchanged (n=3/3) but by 24 hours a change in the domain of *Hoxb-4* expression was visible. The domain has shifted anteriorly to the last visible boundary at the posterior limit of r3 (n=3/3, [Fig 6E]).

Expression of *Hoxb-1*, at stage 15 was detected at low level up to the r6/7 boundary and at high level in r4 ([Fig. 6A]). Furthermore *Hoxb-1* is known to be responsive to retinoic acid treatment (Marshal et al 1992, 1994, Studer et al 1994). As with *Hoxb-4*, *Hoxb-1* expression appeared unchanged 5-6 hours after retinoic acid treatment (n=3) but was also severely affected at 24 hours after treatment. In general *Hoxb-1* expression in r4 was down-regulated and its caudal domain shifted anteriorly. Up to 24 hours after treatment *Hoxb-1* was still expressed to a variable level at the r4 level in a width equivalent to one rhombomere (n=5). The caudal domain has shifted anteriorly to the region of the r5/6 boundary (n=5, [Fig. 6B]). At 48 hours *Hoxb-1* at the r4 level was further down-regulated and the caudal domain had again shifted anteriorly to lie close to the r3/4 boundary (n=3 [Fig 6C]). Within the hindbrain expression of *Hoxb-1* was reduced to a very low level.
Figure 6  \textit{Hox} gene expression

All hindbrains shown are flatmounts from stage 15-20 chick embryos. Top row (A,D,F) Expression of all analysed \textit{Hox} genes in normal embryos. (A) Normal embryo: \textit{Hoxb-1} expression in a stage 15 control hindbrain. \textit{HoxB1} is expressed up to r7 and also in r4. The bidirectional arrow labels the gap between the two expression domains. (B) \textit{Hoxb-1} is still expressed in r4 in a retinoic acid treated (24 hours after treatment, stage 15) hindbrain with 4 rhombomeres, but the gap to the more posterior expression domain is reduced (bidirectional arrow). (C) \textit{Hoxb-1} expression is generally downregulated and the caudal domain has shifted anteriorly to lie close to the r3/4 boundary (arrow) 48 hours after retinoic treatment. (D) Normal stage 15 embryo; \textit{Hoxb-4} is expressed in the neural tube up to the 6/7 rhombomere boundary (arrow). (E) The anterior limit of \textit{Hoxb-4} expression is shifted anteriorly up to the last visible boundary, only 24 hours after retinoic acid treatment (stage 14/15, arrows). (F) Normal stage 20 embryo; the anterior expression limit of \textit{Hoxa-2} lies at the r1/r2 boundary. (G) The anterior expression limit is unchanged 48 hours after retinoic acid treatment (stage 19/20).

Numbers 1-7 indicate individual rhombomeres; d, (dorsal); fp (floor plate); v (ventral).

Scale bar = 200 \text{\mu m}.
From stage 11 the normal anterior limit of *Hoxa-2* expression was at the r1/2 border and this remained visible until stage 24 (Prince and Lumsden 1994). *Hoxa2* was expressed at high levels immediately adjacent to the floor plate in r2, r3 and r4 (Fig. 6F) and was also in the crest that emanated from r4 to populate the second branchial arch. The anterior limit of *Hoxa-2* expression remained unchanged at the r1/2 border in retinoic acid-treated hindbrains. There was uniform expression from posterior to the r3 boundary and the high level of expression immediately adjacent to the floor plate in r4 was lost while those adjacent to the floor plate in r2 and r3 were maintained (Fig 6G). *Hoxa-2* continued to be expressed in the second branchial arch of retinoic acid-treated embryos (n=4).

In summary, both *Hoxb-4* and *Hoxb-1* expression shift anteriorly in retinoic acid treated embryos whereas the anterior expression limit of *Hoxa-2* is unaltered. In contrast to *Cek-8* and *Krox-20*, the changes in *Hoxb-4* and *Hoxb-1* expression domains occur after the loss of posterior boundaries, 24 hours after retinoic acid treatment.

### 2.5 Precursor Dispersal

Retinoic acid treatment abolishes morphological and molecular characteristics of posterior boundaries and also changes the expression of two segmentally restricted genes *Cek-8* and *Krox-20*. It was therefore investigated whether these changes would lead to increased cell mixing between cells of former neighbouring rhombomeres in retinoic acid treated embryos.

The antero-posterior spread of groups of labelled cells in individual rhombomeres was first established. Cells in rhombomeres 3, 4 and 5 were injected with DiI or DiAsp at
stage 10/11 and some embryos were treated with retinoic acid and others were used as controls. All injections were performed as close as possible to a boundary (Fig 7 A,B) and usually label 5-10 cells. Embryos were fixed after 48h and the position of the descendant cells examined. Labelled cell clusters of various shapes and sizes were seen, ranging from elongated stripes of cells to small oval patches of cells in both control and retinoic acid treated specimens. In control embryos, the maximal antero-posterior spread of cells ranged from 16 to 155µm. Similar measurements in retinoic acid-treated embryos ranged from 16µm to 181µm. Figure 9 summarises the results obtained (the scatter diagram in Figure 8 shows every single measurement) and illustrates that the antero-posterior dispersal of neural precursor cells (78 ± 6 µm) has not changed significantly in retinoic acid treated embryos (85 ± 5 µm) and amounted to 40% of the width of a rhombomere at stage 19. This was true regardless of how many rhombomeres were visible after retinoic acid application.

Since there is no overall increase in antero-posterior spread of neural precursor cells in retinoic acid treated embryos, it may be that r5 precursors remain within their former territory. To explore directly whether cells from former adjacent rhombomeres remain in their usual position DiI was injected into the hindbrain in r3, close to the r3/r4 boundary, and in r5, close to the r4/r5 boundary in the same embryo and then treated those embryos with retinoic acid. The distance between the patches at r3 and r5 level was then measured and the cell clusters were found to be about 220 µm apart (minimal 155 µm and maximal 310 µm). This figure is derived from 15 measurements in 9 specimens. The distance of 220 µm spanned at least across the normal width of rhombomere 4 at stage 19, which ranges from about 145 µm to 224 µm (depending on the dorso-ventral level) as measured on 11 control embryos. Labelled cells thus remained within their original territory.
Figure 7 Precursor labelling studies

(A) Schematic diagram describing two precursor labeling experiments: Cells were labelled with DiI and DiAsp within rhombomere 4 to assess cell mixing within one rhombomere in normal embryos (left drawing). Cells were labelled with DiI and DiAsp respectively either side of the rhombomere 4/5 boundary and a retinoic acid bead was applied at r4 level immediately afterwards (right drawing). (B) Sites of injection are seen at r4 (4) and r5 (5) level immediately after labelling as indicated by the two fluorescent patches of cells. (C) DiI (red) and DiAsp (yellow) labelled clusters were derived from 2 injections within rhombomere 4 of a normal embryo. One injection was performed as close as possible to the r3/r4 and the other as close as possible r4/r5 boundary. Cell mixing was observed, although only to a limited extent. (D,E,F) In retinoic acid treated (i.e. partly unsegmented) hindbrains cells from former r4 and r5 levels do not mix as indicated by lack of any regions containing a mixture of cells labelled with both dyes, despite the absence of a boundary.

RA, retinoic acid; cau, caudal; ros, rostral; b, boundary.
Figure 8  Scatter diagramm of all measurements assessing the maximal AP (antero-posterior) spread of precursor cells labelled in normal and retinoic acid treated embryos

Categories 0-60 in the x-axis represent individual specimens and values in the y-axis (10-190) represent the maximal antero-posterior dispersal of precursor cells in μm. In some specimen two injections in different rhombomeres were performed. The box on the right hand corner is the index key to the scatter diagram. Each symbol represents a measurement within a specific rhombomere.
Maximal AP spread of precursor cells after RA treatment (in μm)
Figure 9  

Comparison of expansion of labelled neural precursor population

The spread of neural precursor cells in control and retinoic acid treated embryos is compared. Categories 2 - 4 in the x-axis refer to the number of remaining rhombomeres in experimental embryos. The presented values are all calculated means (± S.E.) from n specimens. n is indicated above each individual column. Data presented are for labelled cell populations in individual rhombomeres: r3, r4, r5 as indicated. The column total mean represents an average figure for all measurements made in embryos with same number of rhombomeres. Error bars represent standard error.

The measurements displayed in the context of a rhombomere (drawn to scale -200 μm width at stages 19-20) indicate also that neural precursor cells do not spread antero-posteriorly more than 40% of the width of a rhombomere in control and experimental embryos.
Precursor Movements

Rhombomeres counted in retinoic acid treated embryos
(8 = number of rhombomeres in a control)
To assess cell mixing between r4 and r5 cells DiAsp and Dil were injected on either side of the r4/r5 boundary. The distance between the injections was measured using a cooled CCD camera and Biovision software and embryos were then treated with retinoic acid and examined 48 hours later. The two labelled cell patches were initially between 8μm and 76μm apart. Since the average antero-posterior dispersal in both experimental and control hindbrains is 40% of the width of a rhombomere the descendants of "average" cells that were less than this distance apart and not separated by a boundary should mix. At the time of injection rhombomeres are about 140μm wide and 40% is equivalent to 56μm. Of 17 measured injection pairs only 2 were more than 56μm apart and these were not scored in this analysis. After 48 hours only those embryos where the boundary between r4 and r5 was abolished were scored (n=14/15). In only one embryo did the two labelled cell patches overlap; in every other case, despite the absence of a morphological boundary, the labelled clusters in r4 and in r5 did not mix (n=13/14). In five cases, the two cell clusters were adjacent to each other and no cell mixing could be seen. In the remaining eight embryos, the two labelled clusters were separated by a stripe of unlabelled cells (Fig 7 D, E, F).

To assure that the injections are close enough not only to each other but also to the boundary, injections were performed close to the r4/r5 boundary in untreated embryos to analyse how many of these abut onto the boundary over the subsequent 48 hours. In 13/23 cases fluorescent cells spread at least up to the boundary. Furthermore cell mixing within a normal rhombomere was tested by injecting Dil towards the anterior and DiAsp towards the posterior boundary of r4. Cell mixing within a single rhombomere occurred in 50% (4/8) of the cases although not extensively (Fig 7 C). In all cases where no mixing was seen the two labelled cell clusters were more than 56 μm (40% of a rhombomeres width) apart at the time of injection. Taken all these results
together, I believe that increased cell mixing would have been detected if it had occurred in retinoic-acid treated hindbrains.

2.6 Organisation of Branchiomotor Nuclei

The anterior shifts in \( Hoxb-4 \) and \( Hoxb-1 \) expression suggest that the posterior hindbrain may have been posteriorised and the lack of boundary cells raises the possibility that the usual segregation of branchiomotor nuclei may be disrupted. To examine these questions in terms of neuronal organisation retrograde labelling studies were performed from the cranial nerves at 48 hours (when embryos have reached stage 19/20) and 3A10 antibody staining at 48 h and 72 hours (stage 23-25 embryos) after retinoic acid treatment. At these times cells at former r4, r5 and r6 levels have been expressing \( Hoxb-4 \) for 24 and 48 hours respectively. In normal embryos the VIIth (facial) nerve root is located at r4 level and recruits its motor neurons from r4 and r5, whilst cell bodies of motor neurons contributing to the IXth (glossopharyngeal) nerve are found in r6 and r7. There is normally little or no spatial overlap between these two branchiomotor nuclei.

The retrograde labelling study after retinoic acid application was performed by Dr. Jon Clarke and Yogish Joshi. I briefly mention these results, with permission of Dr. Clarke, since they are relevant to this thesis and result of a collaboration. In retinoid treated hindbrains there was a slight increase in the spatial overlap of the facial and the glossopharyngeal nuclei. Furthermore the number of contralateral-vestibulo-acoustic (CVA) neurons located in the floor plate specifically at the level of r4 was reduced in retinoic acid-treated embryos and a significant number of r3
motor neurons were often labelled by Dil application to the nerve root on r4. Normally r3 motor neurons exit from r2 in the trigeminal root.

Normally the exit point of the facial nerve is restricted to a tightly defined region on r4 but in retinoic acid treated embryos both 3A10 staining (compare Fig 10A with 10B) and retrograde labelling demonstrate that the facial nerve exits from a line stretching from r4 down into r5. The facial exit point has became an "exit line" (7/8) and in one case even fused with the trigeminal exit point.

2.7 Organisation of Cranial Nerves in the Periphery

The peripheral organisation of the cranial nerves was analysed by studying normal and retinoic acid treated embryos stained with the monoclonal antibody 3A10. In general the location of trigeminal, facial/ vestibulocochlear, abducens, glossopharyngeal and vagal roots appeared normal in retinoic acid treated hindbrains (Fig 10 A, B, C, D, E). However, the normally compact exit point of the facial root has split into series of rootlets in retinoic acid treated embryos and this confirmed the finding of an “exit line” in the retrograde analysis performed by Dr Jon Clarke. Anastomoses between glossopharyngeal and vagal nerves were commonly seen (Fig 10B, C n=4/8). Occasionally a nerve bridge between the facial root and trigeminal ganglion was observed. In summary the overall peripheral nerve pattern seemed unperturbed in retinoid treated hindbrains whilst there was an effect on the fine detail of specific peripheral nerves.
Figure 10  3A10 staining

(A) to (E) 3A10 antibody labelling reveals the peripheral nerves at facial level at stage 20 to 23. (A) Control embryo. (B) and (C) retinoic acid-treated embryo. The arrow in (B) and the asterisk in (C) indicate the exit line of the facial (VII) motor nerves (compare with (H)). The arrowhead in (B) and the arrowhead (blue) and arrow in (C) indicate disorganisation at level of motor nerves IX (glossopharyngeal) and X (vagus). (D) The abducens (VI) is present in retinoic acid treated embryos (boxed area). (E) Enlargement of the boxed area seen in D.

V, trigeminal nerve; OV (otic vesicle).
3 Discussion

At the time of retinoic acid application to the hindbrain (stage 10/11), rhombomere boundaries bordering r2, r3, r4 and r5 were already visible, and the remaining ones would normally appear over the next 4 to 5 hours. However, local application of retinoic acid led to loss of posterior boundaries. Lack of boundaries and segmental gene expression (Cek-8 and Krox-20) did not lead to increased antero-posterior spread of neuronal precursor cells and, more importantly, no increase of cell mixing between former adjacent rhombomeres could be detected in the unsegmented posterior hindbrain. At late stages (20), downregulation of Cek-8 expression was correlated with loss of boundaries. In contrast to Cek-8 and Krox-20 expression changes in Hox gene expression were apparent only after loss of segmentation and did not affect hindbrain regionalisation at the examined time. Retrograde labelling also revealed that some precursors, however, may have been respecified. These observation after local retinoic acid treatment are in some aspects different to those previously reported (Gale et al 1996) and, at first sight, resemble the phenotypes of mutant mice (McKay et al 1994, Mark et al 1993) and quails (Maden et al 1996).

3.1 Precursor Movements in Boundary-less Hindbrains

With a few exceptions (Birgbauer and Fraser 1994), cells of adjacent rhombomeres do not mix in normal embryos (Fraser et al 1990, Guthrie et al 1993). The question of whether loss of boundary cells and segmental gene expression in retinoic acid-treated embryos leads to increased mixing between cells that derive from adjacent rhombomeres was explored. This question has not been addressed in previous reports of
unsegmented hindbrains. An increased amount of cell mixing between former r4 and r5 cells was not detected and there was no general expansion in anterior-posterior spread of neuronal precursors in the posterior hindbrain in retinoic acid-treated embryos. The data presented show that over the stages examined here the descendants of cells disperse on average 40% of a rhombomere’s width (see also Birgbauer and Fraser 1994) and that, in retinoic acid- treated hindbrains, pairs of labelled cells were sufficiently close to each other and to the boundary to detect some mixing if it occurs. However, in many labellings of two groups of cells, on either side of the boundary, the two cell clusters ended up being separated by an unlabelled patch of cells. Cells near boundaries therefore seem to disperse in a preferred direction, and that is away from a (former) boundary. This implies that after the initiation of segmentation the role of boundaries is not to limit the freedom of precursors to disperse in the antero-posterior axis (Wingate et al 1996) and more significantly that boundaries are not required to keep cells of adjacent rhombomeres separated. Alternatively cells at the former boundary region expanded more than cells in neighbour regions thus filling the space between the two labelled cell clusters.

Boundaries normally develop at the interface of two cell population with divergent cell surface properties (Guthrie and Lumsden 1991). No boundaries form when rhombomeres of the same kind (odd or even respectively, Guthrie and Lumsden 1991) are juxtaposed. This suggests that, in the retinoic acid treated hindbrain, posterior rhombomeres might have similar cell surface traits and hence boundaries are not maintained. This in turn would lead to increased cell mixing or dispersal (Guthrie et al 1993) at former boundary regions. However the absence of cell mixing in boundary-less hindbrains suggests that certain distinct cell surface properties of individual rhombomeres must have been maintained. Boundary formation requires a minimal surface of two different cell types interacting but cells with different surface properties
may be segregated without a boundary (Guthrie et al 1993). Retinoic acid might therefore reduce the cell surface property differences of adjacent rhombomeres sufficiently to cease maintenance of boundaries but not sufficiently to allow cell mixing. Alternatively, retinoic acid interferes only with the signalling required for boundary maintenance and does not alter the cell surface properties of individual rhombomeres at all. The persistence of rhombomeres with alternating adhesion properties would then be responsible for the absence of increased cell mixing in boundary-less hindbrains.

3.2 Cek-8, but not Krox-20 is Correlated with Boundary Maintenance

Retinoic acid treatment led to partly unsegmented hindbrains by abolishing posterior boundaries and this was preceded by loss of segmental gene expression of Cek-8 and Krox-20 in the posterior rhombencephalon. Downregulation of Cek-8 mRNA expression in both r3 and r5 was always correlated with lack of boundary cells (Fig. 2B-E). This indicates that after their initial formation the maintenance and further differentiation of boundaries is not autonomous but may require continuous signalling and that Cek-8 might be involved in conveying this activity (Xu et al 1995).

In contrast, changes in Krox-20 mRNA expression did not always correlate with changes in segmentation. A complete loss of Krox-20 expression was observed in both r3 and r5 but an r2/r3 and usually also an r3/r4 boundary (depending on the number of remaining rhombomeres) was still found in retinoid treated hindbrains. Krox-20 is required for maintenance of r3 and r5 (Schneider-Manouri et al., 1993) and also to control early phases of Cek-8 expression (Thiel et al unpublished results). However, the divergent effect of retinoic acid on Krox-20 and Cek-8 expression in r3 suggests that the
maintenance of Cek-8 expression in r3 is independent from Krox-20 at that time and that, maintenance of Cek-8 expression in r3 and r5 are regulated independently.

3.3 Hox Gene Expression and Neuronal Organisation in Boundary-less Hindbrains

Hox genes are thought to impart segment identity to individual rhombomeres. In the experiments reported in this chapter the domains of Hoxb-1 and Hoxb-4, in contrast to those of Cek-8 and Krox-20, were not altered until after morphological segmentation was lost. Then, Hoxb-4 shifted anteriorly up to the posterior boundary of r3 and an anterior shift in the caudal domain of Hoxb-1 also occurred together with the down-regulation of its r4 domain. It is tempting to speculate that the normal expression domains of Hox genes are held constant by the presence of rhombomere boundaries; boundary cells have reduced junctional communication (Martinez et al 1992) and this has been proposed to limit the spread of small inductive signalling molecules (Martinez et al 1995). Stability of Hox gene expression may be important as neurogenesis continues, and the regional complexity of the hindbrain continues to increase, beyond the stages studied here (Marin and Puelles 1995).

The spatial organisation of the facial and glossopharyngeal branchiomotor nuclei was surprisingly normal despite these changes in Hox gene expression. This suggests that the regional organisation of facial and glossopharyngeal nuclei is established early and is up to the stages examined largely resistant to molecular and morphological changes. In contrast to this stability, the loss of CVA neurons from r4 territory correlates with downregulation of Hoxb-1 expression in r4 (Gale et al 1996) and also the
respecification of some motor neurons in r3 (Marshall et al 1992, Guthrie and Lumsden 1992) demonstrate that some aspects of regional identity are labile under these conditions.

3.4 The Effect of Retinoic Acid Is Variable, Depending on Dose, Time and Method of Application

Using non-localised applications of retinoic acid during gastrulation and early neurulation commonly results in a dose dependent disruption and/or respecification of segmentation specifically in the anterior hindbrain of several species (see for example, Papalopulu et al 1991, Marshall et al 1992, Wood et al 1994, Hill et al 1995). Between gastrulation and the stages of hindbrain segmentation the regional sensitivity to retinoic treated shifts more posterior and thus only the posterior part of the hindbrain is affected in this study (Conlon 1995).

The method of delivery of retinoic treated to the hindbrain appears to be influential for the resulting phenotype as these results differ to those previously reported by Gale et al. (1996). In both studies the treatment was performed at the same time (stage 10) and at the same location (r4). However the use of different concentrations and delivery approaches has led to different phenotypes. Whilst Gale et al (1996) injected a pulse of retinoic acid, retinoic acid was applied from slow-release beads which provide a continuous steady state release for a prolonged period in this study. In their retinoic-acid-treated embryos, segmentation was unaffected and *Krox-20* was expressed in r3, r4 and r5 and *Hoxb-1* expression was diminished in r4 but normal posteriorly. However in agreement with these results they also saw a reduction of CVA neurons at r4 level and suggested that r4 is neither lost nor respecified, but rather that there are some alterations of its individual characteristics.
Figure 11 Summary diagram

Segmentation, branchiomotor nuclei organisation, lineage restriction and gene expression in a normal (right) and a retinoic acid-treated hindbrain (left). The posterior part of the retinoic acid treated hindbrain is unsegmented due to loss of posterior boundaries. Some motor neurons from r3 exit via the facial “exit line”. There is only little overlap between the facial and the glossopharyngeal motor neurons at the level where the r5/r6 boundary is present in normal embryos. *Krox20* expression is completely downregulated in r3 and r5, whilst *Cek 8* expression is only downregulated in r5. *Hoxb-1* expression is partly downregulated but is expressed up to the last visible boundary. *Hoxb-4* expression shifts anteriorly to the last visible boundary.

RA, retinoic acid; cau, caudal (posterior); fp (floor plate); mVII and mIX, exit points of the facial and glossopharyngeal branchiomotor nerves; ros (rostral, anterior).
3.5 Lack of Segmentation

Lack of segmentation in the posterior hindbrain described in this work is morphologically strikingly similar to the phenotype of unsegmented hindbrains observed in *kreisler* and *Hoxa-1* mutant mice (McKay et al 1994, Mark et al 1993) and also in vitamin A-deficient quail embryos (Maden et al 1996). *Kreisler* and *Hoxal-/-* mice as well as vitamin A-deficient quails possess partly unsegmented hindbrains due to the lack of development of some or all posterior rhombomeres. In contrast, it seems that in this study loss of morphological segmentation is a direct consequence from loss of boundary cells.

4. Conclusion

Boundaries do no act as barriers between adjacent rhombomeres to assure cell lineage restriction, but it could be that they are necessary to define a field by limiting the spread of *Hox* gene expression. Alternatively, maintained boundaries could be remnants of an early, transient embryonic field, reflecting an early event of lineage restriction which has already been translated into alternating cell surface properties.
CHAPTER FIVE : General Discussion

1. Regulation of Cek-8 Expression

Results presented in this thesis show that Cek-8 expression is initially restricted to the progress zone of the chick limb bud and is regulated by factors from the AER and the ZPA. Signals from the AER, the ZPA and the ectoderm are all integrated by cells in the progress zone. Cells outside the progress zone stop expressing Cek-8. Fgf2, Fgf4 and Fgf8 are expressed in the AER and FGF4 and FGF2 can substitute for the AER (Niswander et al 1994; Fallon et al, 1994). As reported here, Cek-8 expression is induced in proximal, non-expressing tissue after FGF2 application and also when such tissue is transplanted beneath the AER. This demonstrates that tissue proximal to the progress zone is able to respond to the AER and to FGF and suggests that these cells stop expressing Cek-8 because they are not within the normal range of that signal.

In the developing hindbrain, Cek-8 expression is restricted to r3 and r5. Cek-8 expression domains are initially fuzzy (Irving et al 1995) and are observed before boundary formation (lineage restriction), but later the borders of the Cek-8 expression-domains become sharper. Interestingly, the border of the expression domain of Cek-8 in the early limb and also during tendon development is initially fuzzy and only later becomes sharp and restricted to the progress zone and the tendons respectively. In the limb, a progress zone cell may constantly review the strength of the AER signal. This is necessary because cells move away from the AER and finally leave the progress zone. The proximal boundary of the progress zone does not constitute a definite cell population. In the hindbrain, in contrast, the boundaries of the Cek-8 expression
domains in r3 and r5 are distinct cells. The morphologically indistinct proximal boundary of the progress zone (fluid boundary) and the morphologically distinct rhombomere boundaries (static boundary) thus represent two different mechanisms of limiting a region in which signalling is integrated. The fluid boundary may involve the use of a threshold concentration, below which cells will leave the progress zone. The static boundary, in contrast, may limit the spread of any putative inductive signal (Martinez et al 1992) and thus represent a physical barrier to signalling. The nature of the Cek-8 expressing cell population observed during tendon development is initially unclear, but cells that later express Cek-8 are part of morphologically distinguishable tendons.

The different nature of these cell populations (progress zone, tendon and rhombomere cells) implies that Cek-8 expression within each population is regulated by a different mechanism. This is not necessarily true. In all systems the same local signal may be required to maintain or induce Cek-8 expression. In the early limb FGF2 or FGF4 may be that signal. In the hindbrain, another member of the FGF-family, Fgf3, is initially expressed in presumptive r4, r5 and later also in r6. By stage 13, Fgf3 expression is restricted to the boundaries (Mahmood et al 1995). Fgf3 may thus initially regulate the expression of Cek-8 at r5 level whilst a different factor may control Cek-8 expression at r3 level. Results in this thesis imply that Cek-8 expression in r3 and r5 are indeed differently regulated, as shown by the divergent responses after local retinoic acid treatment. Retinoic acid application always leads to downregulation of the r5 domain, whilst the r3 domain remained unaffected. It remains to be shown whether FGF3 regulates Cek-8 during hindbrain development and also whether FGFs are involved in controlling Cek-8 during tendon development.
2 Cek-8 Signalling and Possible Functions

The analysis of the way the receptor operates in the hindbrain, the progress zone and during tendon development may help to define the function of Cek-8. Eph-receptor activation requires binding by membrane-associated ligands (Davis et al 1994). The “true” ligand of Cek-8 has not yet been identified, but SEK-1 (the murine homologue of CEK-8) can bind to both GPI-anchored and to transmembrane ligands in vitro (Gale et al 1996). Determining the distribution of possible ligands, in combination with a receptor binding assay, will shed light on the possible modus operandi of the ligand-receptor pair. Briefly, at least two different scenarios may be envisaged. First, CEK-8 is only activated at the edge of its expression domain and, second, CEK-8 may be activated throughout its domain. Gale et al (1996) reported that members of an Eph-receptor subclass and its respective ligands are distributed in reciprocal and apparently mutually exclusive domains in the developing embryo. The authors argue that receptors seem to encounter their ligands at their mutual expression boundary as outlined in the first model (Gale et al, 1996). This is interesting because it suggests that, in vertebrates like in insects, the borders of the compartment-specific gene expression are important for the function of these genes. For example, the patterning of the antero-posterior axis of the wing disc in Drosophila is good example to illustrate that principle. Briefly, the Drosophila wing is divided into two lineage compartments along the antero-posterior axis. engrailed expression specifies the posterior lineage compartment while absence of engrailed expression specifies the anterior compartment. Elegant experiments where engrailed expression was switched off in clones of cells within the posterior compartment demonstrated that a “boundary” that can organise pattern forms where
engrailed expressing cells meet non-expressing cells (reviewed in Lawrence and Struhl 1996).

In the vertebrate limb, for example, if the function of Cek-8 is confined to the edge of the progress zone, the expression domains of Cek-8 and its ligand should be complementary. CEK-8 activity at the intersection of these domains may define a boundary between the progress zone and more proximal cells. Cells whose receptor has been activated by ligand binding may leave the progress zone and their positional value becomes locked. Cell-to-cell signalling activity via Cek-8 would therefore mark the limit of the influence of FGF emanating from the AER. Alternatively CEK-8 activity and also the expression of its ligand could be intrinsic to the progress zone and cells cease to belong to the progress zone when the receptor fails to be activated.

The function of the receptor-ligand pair could be analysed by interfering with the signal transduction pathway downstream of the receptor. This may be achieved by infecting limb tissue with a replication competent retrovirus producing a dominant-negative receptor mutant. For example, a truncated receptor, lacking the intracellular kinase domain, would be able to dimerise with the endogenous wild-type receptors and block their signalling (Xu et al, 1995). If the first model applies then blocking the receptor function would lead to cells unable to transduce the signal at the proximal border of the progress zone. Cells would therefore not know when they cease to belong to the progress zone and a progress zone throughout the limb may be anticipated. It is not clear how a limb with an extended progress zone will develop, but one possibility may be that the absence of a local restricted cell population able to integrate the AER signal, would be equated to the absence of a localised signal and thus lead to a truncated limb. If, in contrast, the second model applies, then the interference with Cek-8
signalling will lead to a limb without progress zone. In the absence of a progress zone, cells might be locked in their positional value much too soon and this may give rise to a truncated limb as well. To distinguish between the two proposed models one would, ideally, wish to direct the expression of the truncated receptor to a certain domain in the developing limb. This would allow one to test whether it is the blocking of CEK-8 signal transduction, within or at the edge of the Cek-8 expression domain, that inhibits its function. For example, if signalling at the border is important then the expression of a truncated receptor within, but not at the border of, the Cek-8 expression domain, will not interfere with the normal Cek-8 function. If, on the other hand, Cek-8 signalling throughout the progress zone is important, the presence of a mutant receptor will inhibit signalling within the progress zone and thus lead to a truncated limb.

In the hindbrain, in contrast to the limb, the distribution of the Eph receptors and their ligands is known and this enables one to predict how they may operate. The binding studies of Gale et al (1996) demonstrated that the ligands expressed in even-numbered rhombomeres of the hindbrain, ELF-2 (Bergemann et al, 1995 and Elk-L3 (Gale et al, 1996)) can bind to the receptors expressed in odd rhombomeres (Sek-1, Sek-3 and Sek-4). Xu et al (1995) found that interference with the Sek-1 (homologue of Cek-8) signal transduction pathway in Xenopus and zebrafish leads to ectopic expression of Sek-1 and Krox-20, normally restricted to r3 and r5 of the developing hindbrain, in r4. Xu et al (1995) propose therefore that Sek-1 function is required for the restriction of Krox-20 and Sek-1 gene expression to r3 and r5. This is either achieved by a dynamic regulation of cell identity where activating SEK-1 leads to down-regulation of Sek-1 and Krox-20 expression or by restriction of cell movement by repulsion after ligand binding. Although this question has not been directly addressed in this thesis, some results shown might argue against Cek-8 restricting cell movement. It
is shown that even in the absence of Cek-8 expression in r5, r4 and r5 precursor do not increasingly mix, thus suggesting that Cek-8 expression is not necessary to restrict cell movement. It remains to be shown whether both Cek-8 mRNA and protein expression are missing in r5 in retinoic acid treated embryos.

The results in this thesis show a correlation between Cek-8 expression and boundary maintenance. Boundaries are loci of circumferential axon accumulation and a loss of boundaries is reflected by the loss of accumulation of circumferential axons at that precise level. However, the significance of axon aggregation in boundaries is not yet understood and remains to be determined. Members of the Eph-receptor family and their respective ligands have been implicated in axon guidance in the optic tectum (Drescher et al, 1995; Cheng et al, 1995). The expression pattern of CEK-8 ligands and the binding profile of CEK-8 in the hindbrain have to be determined and there is an intriguing possibility of a link between CEK-8, boundaries and axon guidance in the hindbrain.
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