Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20

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SUMMARY

Segmentation of the vertebrate hindbrain leads to the formation of a series of rhombomeres (r) with distinct identities. Recent studies have uncovered regulatory links between transcription factors governing this process, but little is known of how these relate to molecules mediating cell-cell signalling. The Eph receptor tyrosine kinase gene EphA4 (Sek-1) is expressed in r3 and r5, and function-blocking experiments suggest that it is involved in restricting intermingling of cells between odd- and even-numbered rhombomeres. We have analysed the cis-acting regulatory sequences of the EphA4 gene in transgenic mice and identified a 470 bp enhancer element that drives specific expression in r3 and r5. Within this element, we have identified eight binding sites for the Krox-20 transcription factor that is also expressed in r3 and r5. Mutation of these binding sites abolishes r3/r5 enhancer activity and ectopic expression of Krox-20 leads to ectopic activation of the enhancer. These data indicate that Krox-20 is a direct transcriptional activator of EphA4. Together with evidence that Krox-20 regulates Hox gene expression, our findings reveal a mechanism by which the identity and movement of cells are coupled such that sharply restricted segmental domains are generated.

Key words: Eph receptor, Krox-20, Hindbrain, Segmentation, Gene regulation, EphA4, Sek-1, Tyrosine kinase, Mouse

INTRODUCTION

The subdivision of tissues into segmental units is a widespread mechanism in animal development. Genetic and molecular analyses in the fruit fly Drosophila melanogaster have provided detailed insights into cascades of transcriptional interactions that underlie the formation and anteroposterior (A-P) specification of segments (reviewed by Lawrence, 1992). A key aspect of this patterning is the expression of genes that regulate A-P identity in parasegments, each composed of a compartment of cells that do not cross segment boundaries. In contrast, relatively little is known regarding mechanisms of segmentation in vertebrates, although progress has been made in elucidating the function and transcriptional regulation of genes involved in segmental patterning of a region of the central nervous system, the hindbrain.

Segmentation of the vertebrate hindbrain is seen morphologically as a subdivision of the neural epithelium by a series of constrictions that demarcate the rhombomeres. This subdivision presages the differentiation of neurons in segment-restricted patterns (McCalie et al., 1986; Hanneman et al., 1988; Lumsden and Keynes, 1989; Clarke and Lumsden, 1993) and relates to the pathways of neural crest migration into the branchial arches (Lumsden et al., 1991; Serbedzija et al., 1992; Birgbauer et al., 1995), indicating a crucial role in craniofacial organisation. Segmentation involves a restriction of cell movement across rhombomere boundaries (Fraser et al., 1990; Birgbauer and Fraser, 1994), such that each of rhombomeres r2-r6 consists of a group of cells that mix little with their neighbours due to alternating cellular properties that cause r3/r5 cells to be immiscible with r2/r4/r6 cells (Guthrie and Lumsden, 1991; Guthrie et al., 1993). The restriction of cell movement is thought to allow each segment to establish and maintain a distinct A-P identity. A key question is thus how mechanisms that restrict cell movement are themselves coupled to cell identity, such that sharp segmental units, each with a distinct and homogenous identity, are established.

Clues to the molecular basis of hindbrain segmentation have come from studies implicating several transcription factors in different aspects of patterning (reviewed by McGinnis and Krumlauf, 1992; Wilkinson, 1995). Members of the Hox gene family are expressed in overlapping segmental domains in the hindbrain and neural crest (Wilkinson et al., 1989; Hunt et al., 1991) that correlate with their general function in specification of A-P identity. Indeed, direct evidence from gene knockouts implicates Hoxb-1 in specifying aspects of neuronal migration in r4 (Studer et al., 1996), and Hoxa-2 in the specification of A-P identity in the branchial neural crest (Gendron-Maguire et al.,...
1993; Rijli et al., 1993). In contrast, the *Hoxa-1* (Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993), *Krox-20* zinc finger (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1997) and *kreisler* basic leucine zipper (Cordes and Barsh, 1994) genes are required for segmentation of the hindbrain. For example, *Krox-20* is expressed in, and required for the formation of r3 and r5. These genes presumably form part of a cascade of transcriptional and intercellular interactions that establishes their expression in specific segmental domains, in order that each rhombomere acquires a distinct and homogenous identity. It is therefore important to identify transcription factors that regulate segment-restricted gene expression. A number of studies have dissected control sequences that drive segmental expression of *Hox* genes and identified several transcriptional regulators, including *Krox-20* (Sham et al., 1993; Nonchev et al., 1996), retinoic acid receptors (Studer et al., 1994), an autoregulation involving *exd/pbx* (Pöpperl et al., 1995) and *kreisler* (Manzanares et al., 1997). The finding that *Krox-20* and *kreisler* are direct regulators of *Hox* gene expression indicates that there is a coupling between the formation and the specification of A-P identity of hindbrain segments. However, target genes for *Krox-20* that are involved in the process of segmentation remain to be identified.

Studies of Eph-related receptor tyrosine kinases suggest that they have key roles in many tissues, including the hindbrain. These receptors constitute a family of at least 14 members, and work in recent years has identified a family of 8 ligands, termed ephrins (reviewed by (Brambilla and Klein, 1995; Xu and Wilkinson, 1997)). Ephrins comprise two related classes of proteins, one anchored in the plasma membrane by a GPI linkage and the other by a transmembrane domain, and each class binds to a largely distinct subset of Eph receptors (Brambilla et al., 1995; Gale et al., 1996b). Unless artificially clustered, ephrins only activate receptor when membrane bound (Davis et al., 1994) indicating that Eph receptors mediate cell-contact-dependent signalling. In situ hybridisation studies indicate that specific Eph receptors and interacting ephrins have complementary expression in the hindbrain: EphA4 (Sek-1), EphB2 (Nuk/Sek-3) and EphB3 (Sek-4) are expressed in r3/r5 (Nieto et al., 1992; Becker et al., 1994; Henkemeyer et al., 1994), and ephrin-B1 (Elf-1), ephrin-B2 (Elf-2) and ephrin-B3 (Elf-L3) are expressed in even-numbered rhombomeres (Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996a) (for nomenclature see Eph Nomenclature Committee, 1997; the name used in the original reference is in brackets). Thus interactions of these signalling molecules occur at rhombomere boundaries.

Clues to the role of Eph receptors in the hindbrain have come from a dominant negative approach in *Xenopus* and zebrafish embryos to inhibit *EphA4* function. Expression of truncated EphA4 disrupts the segmental restriction of gene expression in the hindbrain, such that *Krox-20*-expressing cells, normally only found in r2/r4/r6 territory (Xu et al., 1995). The complementary expression of *EphA4* and interacting ligands may therefore mediate a repulsion that restricts the intermingling of cells between odd- and even-numbered rhombomeres. These findings are consistent with a possible general role of Eph receptors and ligands in repulsion suggested by studies of the retinotectal system (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Monschau et al., 1997), the anterior commissure (Henkemeyer et al., 1996) and migrating neural crest cells (Krrull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997).

In contrast to detailed studies of *Hox* gene regulation, nothing is known regarding upstream factors that directly regulate expression of Eph receptors. The identification of transcription factors that regulate the segmental expression of EphA4 would be a first step towards understanding the coupling between cell-cell interactions and transcriptional control in the hindbrain. We therefore set out to map regulatory sequences that drive segmental expression of EphA4 in the hindbrain. Here, we report the identification of an enhancer element that drives expression in r3/r5 and show that *Krox-20* is an immediate upstream regulator that acts through this enhancer. These data indicate that *Krox-20* has a central role in segmentation, regulating not only *Hox* genes, but also an Eph receptor, and we discuss the implications of this for mechanisms that establish sharply restricted segmental domains.

**MATERIALS AND METHODS**

**Isolation of EphA4 genomic clones, plasmid construction and mutagenesis**

A mouse genomic P1 library prepared from C57Bl6 liver DNA and a mouse λ GEM library prepared from OLA129 liver DNA (generously provided by Anton Berns) were screened using a 1 kb *HindIII* fragment of mouse EphA4 cDNA containing exons 1-4. One clone was isolated from each library and its integrity was confirmed by restriction mapping and Southern blotting. For transgenic analysis, fragments from the P1 clone were inserted into an end-filled SaI site of the lacZ reporter vector pGZ40, upstream of the human β-globin promoter (Yee and Rigby, 1993). The constructs contained the following genomic fragments: construct 1 and construct 2, 7.5 kb *SacI*-SacI in opposite orientations; construct 4, 3.9 kb *Asp718*-SacI; construct 5, 3.7 kb *HindIII*-SacI; construct 6, 3.7 kb *Xhol*-XbaI; construct 7, 2.3 kb *Ncol*-Asp718; construct 8, 1.6 kb *SacI*-Ncol; construct 9, 1.1 kb *BglII*-Ncol; construct 10, 0.47 kb *SacI*-BglII; construct 11, 7.0 kb *BglII*-SacI. To generate construct 3, a *NolI*-Ncol partial digestion fragment from the lambda clone containing 9 kb of 5′ flanking region was introduced into the corresponding sites of pGZ40, placing the lacZ gene under the control of the EphA4 promoter. The 5′ *NolI* site in this clone derives from the lambda polylinker sequence. For the generation of transgenic mice, fragments were purified after *Asp718-NolI* digestion (construct 4) or after *Xhol*-*NolI* digest (all other constructs).

Site-directed mutagenesis reactions of the Krox-20-binding sites were performed on the 3.5 kb *NolI*-Ncol fragment subcloned into the pGEM-SZI vector using the Transformer™ Site-Directed Mutagenesis Kit (Clontech). The oligonucleotides used were A TGCCTAACTTCC- GTTGTTGAGAAA (binding site 1); TGAATTTTCTTGTTC- GACCCAGTGG (binding site 2); CAAAGCCCTCCAGGGTATA- GTGTAACCGACGCCACCGACCACCGACCACCACTCCCTC C (binding sites 3, 4, 5, 6); CCTTCCCCTCCGCCCTCCCTCTTTC (binding site 7); GACAGCTGTGTGTCTGGTGGCAAAAGACA (binding site 8); CCTTCCCATATGCTGACCTGAGG (switch oligonucleotide). After sequencing both strands, the mutated fragments were cloned into the end-filled SaI site of pGZ40 (for the 470 *SacI*-BglII fragment) or used to replace the corresponding region within the 9.5 kb *NolI*-Ncol fragment in pGZ40.

**Generation and analysis of transgenic mice**

Transgenic mice were generated by microinjection of fertilized eggs from crosses between F1 hybrids (CBAxB10) as described previously (Sham et al., 1993) and were identified by a polymerase chain reaction using extraembryonic yolk sac or tail DNA. Expression of the
transgene was analysed by staining mouse embryos for β-galactosidase activity. Embryos were fixed at 4°C for 30 minutes in phosphate-buffered saline (PBS) containing 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, 2 mM MgCl₂ and 5 mM EGTA. After washing three times at room temperature with 0.02% NP-40 in PBS, embryos were incubated in 1 mg/ml X-Gal, 5 mM K₃Fe(CN)₆, 5 mM Fe(CN)₆, 2 mM MgCl₂ and 0.02% NP-40 in PBS for 1-24 hours at 37°C. Stained embryos were postfixed overnight at 4°C in 4% paraformaldehyde. Flat mounts of the hindbrain were prepared by dissection of embryos, equilibrated with 70% glycerol in PBS, with tungsten needles and mounting under a coverslip.

**In vitro DNA-binding assays**

Krox-20 was expressed in *E. coli* as described previously (Chavrier et al., 1990; Sham et al., 1993) and bacterial protein extracts prepared as described (Kadogana et al., 1987). Crude protein extracts were dialysed against 20% glycerol, 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 0.01 mM ZnSO₄, 1 mM dithiotreitol, 0.2 mM PMSF. For DNA I footprinting experiments, the DNA was digested with BglII or SacI, dephosphorylated, 5′-labelled with 32P using T4 polynucleotide kinase and digested with the second restriction enzyme. The labelled fragments were then purified by electrophoresis on 6% polyacrylamide gel. DNA I footprinting was performed as described (Galas and Schmidz, 1978). The products of the reactions were electrophoresed on 6% denaturing polyacrylamide gels. Gel mobility shift assays were performed as described previously (Chavrier et al., 1990).

**RESULTS**

**Identification of an EphA4 r3/r5 enhancer**

*EphA4* expression occurs in a dynamic pattern during segmentation of the mouse hindbrain (Nieto et al., 1992; Irving et al., 1996). Initially, *EphA4* mRNA is detected in a broad domain corresponding to presumptive rhombomeres r3 and r4 in the 7.25 day mouse embryo. An upregulation of expression occurs in pre-r3 at 7.75 days, and in pre-r5 at the 5-somite stage, each initially in narrow stripes that then broaden. Concurrently, there is a downregulation in pre-r4. The r3/r5 domains are initially fuzzy and, by the 12-somite stage, *EphA4* expression has become sharply restricted to definitive r3 and r5, and occurs at a lower level in r2.

To elucidate the transcriptional basis for this expression pattern, we mapped cis-acting regulatory elements of the mouse *EphA4* gene in transgenic mice. Candidate genomic fragments were inserted into a vector that contains the β-galactosidase (*lacZ*) reporter gene under the control of the human β-globin minimal promoter. Fig. 1 depicts the mouse *EphA4* genomic region surrounding the first exon and summarises the regulatory regions examined and their enhancer activity. In this way, a 7.5 kb SacI fragment immediately upstream of the first exon was identified which is sufficient to drive expression of the *lacZ* reporter gene in an identical pattern when in the normal (construct 1) or reverse orientation (construct 2) relative to the minimal promoter (Fig. 2 and data not shown). A similar expression pattern was obtained by use of a 9 kb genomic fragment that includes its own promoter and a further 1.4 kb of 5′ sequences (construct 3; data not shown). At the 16-somite stage, reporter gene expression was found to occur in the hindbrain at high levels in r3 and r5 (Fig. 2A) and, as seen in flat-mount preparations, at lower levels in r2 (Fig. 2F), similar to the expression of the endogenous gene. In addition, expression occurred at low levels in the posterior part of the notochord (Fig. 2B), and this corresponds with the low level expression of *EphA4* in this tissue (Nieto et al., 1992). Thus, regulatory elements within the 7.5 kb SacI fragment can drive expression in specific sites of *EphA4* gene expression, and show characteristics of enhancers as they work on a heterologous promoter and in an orientation-independent manner.

To analyse the time course of expression in detail, transgenic lines were generated that carry the 7.5 kb SacI fragment driving *lacZ* expression (Fig. 2C-F). Analysis of four independent lines led to identical results. In contrast to the endogenous gene which is initially expressed in a broad domain, the reporter gene is first expressed in a narrow stripe of cells corresponding to pre-r3 (3- to 4-somite stage; Fig. 2C). This stripe then widens and staining is detected in a few cells in pre-r5 (7 somites; Fig. 2D). Subsequently, the r5 domain broadens (10 somites; Fig. 2E) and the fuzzy r3/r5 domains become

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**Fig. 1.** DNA fragments used for transgenic analysis. The diagram depicts a restriction map (upper part) of the sequences upstream of the coding region of the *EphA4* gene (the ATG initiation codon and first coding exon are indicated). The bars indicate the restriction fragments which are cloned into vector containing *lacZ* and a minimal promoter, with the exception of construct 3, which includes the endogenous *EphA4* promoter driving *lacZ*. For each of these constructs (1-11), the numbers of transgenic mice that expressed *lacZ* in r3/r5 or in the notochord (No) is indicated. S, SacI; Not, NotI; N, NcoI; A, Asp718; H, HindIII; X, XbaI; B, BglII.
SacI fragment. By this approach, a 470 bp fragment (Fig. 2F; see also Fig. 5A); this may reflect perdurance observed in r4, most frequently in ventral regions in the floor plate (16 somites; Fig. 2F). A few expressing cells are sometimes apparent ectopic expressing cells plus low level expression in r2 at the 16-somite stage reflects the normal expression pattern of EphA4. The localisation of the EphA4 r3/r5 enhancer to a 470 bp fragment enabled us to search for potential upstream transcription factors that mediate the activity of this regulatory element. The upregulation in r3 and r5 is very similar to the early expression pattern of Krox-20 in the hindbrain. Furthermore, in null mutants of Krox-20, in which there is only a transient development of pre-r3 and -r5 (Schneider-Maunoury et al., 1993, 1997), EphA4 is not upregulated in these rhombomeres (Seitanidou et al., 1997). To address the possibility that Krox-20 is a direct transcriptional activator, we searched in vitro for Krox-20-binding sites in the 470 bp r3/r5 enhancer fragment. A DNase I footprinting analysis was performed in the presence of extracts with or without Krox-20 protein. The analysis was carried out on each strand of the 470 bp fragment and revealed the existence of seven regions (labelled A to G) specifically protected by Krox-20 protein (Fig. 3 and data not shown). Analysis of the nucleotide sequences corresponding to these protected regions indicated that, in six out of seven cases, it was possible to identify at least one motif with similarity to the 9 bp Krox-20 consensus binding site 5'-G/C-C/A/T-G-N-G/A-G-G-C/A-T-G/C-3' (Chavrier et al., 1990; Nardelli et al., 1991, 1992; Pavletich and Pabo, 1991; Swirnoff and Milbrandt, 1995): a unique motif in regions A, C, D, F and G and three motifs in region E (Fig. 4). These putative Krox-20-binding sites have been numbered 1 to 8. Surprisingly, in the case of region B, we could not find a convincing potential Krox-20-binding site according to the nucleotide sequence, raising the possibility that Krox-20 might interact with two different types of binding sites. This has not been investigated further.

The r3/r5 enhancer contains numerous Krox-20-binding sites

To investigate the possible involvement of Krox-20 in r3/r5 enhancer activity in vivo we performed a mutational analysis. Since there were many Krox-20-binding sites within the 470 bp fragment, it was important to minimise the modifications introduced within each site. We have shown that changing the central nucleotide of the 9 bp motif (usually a G) into a C leads to severe reduction of in vitro Krox-20 binding (Nardelli et al., 1991) and the elimination of biological activity (Sham et al., 1993; Nonchev et al., 1996). We therefore introduced the same type of mutation into the Krox-20 motifs identified within the EphA4 r3/r5 enhancer. In a first series of experiments, we mutated the five motifs that form a central cluster within the 470 bp fragment and have high similarity to the consensus Krox-20-binding site (sites 3 to 7, Fig. 4). Footprint analysis indicated that this eliminated the sharpened and low levels of expression are upregulated in r2 (16 somites; Fig. 2F). A few expressing cells are sometimes observed in r4, most frequently in ventral regions in the floor plate (Fig. 2F; see also Fig. 5A); this may reflect perdurance of lacZ expression in r3/r5-derived cells that are not segmentally restricted in the floor plate. These data indicate that the 7.5 kb SacI fragment contains an enhancer activity that accurately reflects, both spatially and temporally, the second phase of EphA4 expression in the hindbrain. An enhancer activity characteristic of the early pre-r3/r4 expression was not identified and these two phases of expression therefore appear to be regulated by different cis-acting elements.

To identify a minimal enhancer element involved in r3/r5 expression, a deletion analysis was performed on the 7.5 kb SacI fragment. By this approach, a 470 bp SacI/BglII fragment at its extreme 5’ end (construct 10) was identified which is capable of driving expression in the hindbrain (Figs 1, 5A). All constructs (4,8,10) containing this fragment expressed β-galactosidase in r3/r5, whereas none of the constructs (5-7,9,11) missing this fragment showed any expression in the hindbrain (Fig. 1). Analysis of a line transgenic for construct 10 showed that this fragment drives an identical expression in the hindbrain as the original 7.5 kb fragment (not shown). The 470 bp fragment also drives lacZ expression in the notochord (not shown).

Krox-20-binding sites are required for r3/r5 enhancer activity

To investigate the possible involvement of Krox-20 in r3/r5 enhancer activity in vivo we performed a mutational analysis. Since there were many Krox-20-binding sites within the 470 bp fragment, it was important to minimise the modifications introduced within each site. We have shown that changing the central nucleotide of the 9 bp motif (usually a G) into a C leads to severe reduction of in vitro Krox-20 binding (Nardelli et al., 1991) and the elimination of biological activity (Sham et al., 1993; Nonchev et al., 1996). We therefore introduced the same type of mutation into the Krox-20 motifs identified within the EphA4 r3/r5 enhancer. In a first series of experiments, we mutated the five motifs that form a central cluster within the 470 bp fragment and have high similarity to the consensus Krox-20-binding site (sites 3 to 7, Fig. 4). Footprint analysis indicated that this eliminated the
corresponding protections by Krox-20 (data not shown). Analysis of the enhancer activity of the mutated fragment in transgenic mice revealed that \( r_5 \) expression was abolished whereas \( r_3 \) expression was only reduced (Table 1; Fig. 5B). In contrast, \( \text{lacZ} \) expression was maintained with this construct in the notochord and in \( r_2 \) (at a low level), indicating that the mutations did not affect the corresponding \( \text{cis} \)-acting elements (Table 1).

Since the mutation of the five central motifs did not abolish completely \( r_3 \)-specific enhancer activity, we generated a derivative of construct 10 in which the eight identified putative Krox-20-binding sites had been mutated (Fig. 4). Footprint analysis confirmed that the three additional mutations eliminated the corresponding protections by Krox-20, and gel retardation experiments indicated that its capacity to bind Krox-20 was dramatically reduced (data not shown). Only protected region B was left which does not contain any obvious candidate motif for a Krox-20-binding site. Analysis of the enhancer activity of this mutated fragment indicated that \( \text{lacZ} \) expression was completely eliminated in \( r_3 \) and \( r_5 \), whereas low level expression persisted in \( r_2 \) (12/12 embryos, Fig. 5C). In 2/12 embryos, a few expressing cells were observed in \( r_3 \), but since these were located in the ventral, anterior part these may correspond to floor plate cells that are not segmentally restricted and had crossed from \( r_2 \).

These data indicated that Krox-20-binding sites are required for the \( r_3/r_5 \) enhancer activity of the 470 bp fragment. However, it was important to test the effect of the mutations in the context of a larger fragment to exclude the possibility that the mutations could be complemented by \( \text{cis} \)-acting sequences external to the 470 bp fragment. In particular, gel retardation experiments revealed the presence of additional Krox-20-binding sites between the 470 bp fragment and the \( \text{EphA4} \) coding region (data not shown). Although this region was not capable of driving \( r_3/r_5 \) expression by itself (Fig. 1, construct 11), it was possible that the 470 bp fragment contained binding sites for Krox-20 co-factor(s) able to synergize with Krox-20 bound at sites external to this fragment. Conversely, co-factors bound outside of the fragment might synergize with internal Krox-20. To test these possibilities, the combinations of five or eight mutations described above were introduced into the context of the largest construct (3, Fig. 1), which contains 9 kb of \( \text{EphA4} \) 5’ flanking sequences. Analysis of the construct with the eight mutations in transgenic mice revealed expression in \( r_2 \) and the notochord but not in \( r_3/r_5 \) (Table 1; Fig. 5E, compare with Fig. 5C), indicating that any \( \text{cis} \)-acting sequences outside the 470 bp fragment cannot compensate in this situation. However, in the case of the construct with five mutations, high-level expression was restored in \( r_5 \) while weak expression was maintained in \( r_3 \) (Table 1; Fig. 5D, compare with Fig. 5B). These data suggests that, outside of the 470 bp region, the 9 kb fragment contains binding sites for factor(s) able to

**Table 1. Effect of Krox-20 binding site mutations on enhancer activity**

<table>
<thead>
<tr>
<th>Original Krox-20 construct no.</th>
<th>Sites mutated</th>
<th>( r_3/r_5 )</th>
<th>( r_3 )</th>
<th>( r_5 )</th>
<th>Notochord</th>
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<td>5/9</td>
<td>+++</td>
<td>+++</td>
<td>1/9</td>
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<td>7/15</td>
<td>+++</td>
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<td>5/13</td>
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</table>

The numbers of transgenic embryos expressing \( \text{lacZ} \) in \( r_3/r_5 \) or in the notochord are shown. The relative levels of expression in \( r_3 \) and \( r_5 \) are indicated: no expression (−), low level expression (+), high level expression (+++). The low number of embryos transgenic for construct no. 10 in which \( \text{lacZ} \) was detected in the notochord probably reflects the weak expression in this tissue and the variable efficiency of transgene expression.
synergize in r5 but not in r3 with limiting amounts of Krox-20 bound within the 470 bp fragment. This synergistic effect is sufficient to restore high level enhancer activity in r5. In contrast, the inactivity of the construct with eight mutations indicates that the Krox-20-binding sites located outside of the 470 bp region are unable to compensate for the mutation of the internal sites.

Transactivation of EphA4 by Krox-20

To obtain further evidence for the ability of Krox-20 to upregulate EphA4 through the r3/r5 enhancer, we ectopically expressed Krox-20 in a transgenic mouse line carrying this enhancer driving reporter gene expression (construct 10). We used a regulatory region from the Hoxb-1 gene to direct expression of Krox-20 to r4 (r4/Krox-20 construct) (Marshall et al., 1992; Studer et al., 1994; Pöpperl et al., 1995). Whereas the transgenic line expresses the transgene only in r2, r3 and r5 (Fig. 5F), after introduction of the r4/Krox-20 construct by microinjection into fertilised eggs, reporter gene expression was specifically induced in r4 (Fig. 5G). In addition, we used a neural enhancer from the Hoxb-4 gene (Whiting et al., 1991) to ectopically express Krox-20 in the neural tube posterior to r6, and found that this also led to ectopic activation of the reporter gene (not shown). These experiments demonstrate that Krox-20 is capable of working in trans to activate expression from the r3/r5 enhancer of the EphA4 gene.

DISCUSSION

Krox-20 is a direct transcripational activator of the EphA4 gene

We have shown that a 7.5 kb genomic fragment immediately 5′ to the EphA4 coding region drives reporter gene expression from a heterologous promoter in transgenic mice in r3 and r5, and at lower levels in r2 and newly formed notochord, which correspond to a subset of the sites of EphA4 gene expression. This activity is independent of the orientation of the 7.5 kb fragment, suggesting that it contains a transcriptional enhancer. Further analysis revealed that a 470 bp fragment located at the 5′ end of this region is sufficient to drive expression in all of these sites. In the hindbrain, reporter gene expression coincided temporally and spatially with the upregulation of EphA4 expression in r2, r3 and r5, suggesting that the genomic region analysed contains all the necessary cis-acting regulatory elements involved in controlling this aspect of the expression of the gene in vivo.

This expression in r3/r5 closely parallels that of Krox-20, raising the possibility of a regulatory relationship between the two genes. We obtained several lines of evidence that strongly suggest that Krox-20 is a regulator of EphA4 in r3 and r5 in vivo. First, the 470 bp enhancer contains multiple Krox-20-binding sites that are essential for its activity in r3 and r5. Second, gain-of-function experiments indicate that Krox-20 is capable of upregulating the EphA4 enhancer. Finally, genetic evidence has come from analysis of a null mutant of the Krox-20 gene showing that EphA4 fails to be upregulated in pre-r3 and -r5 (Seitanidou et al., 1997). These latter data indicate that Krox-20 is upstream of EphA4 in the regulatory hierarchy, but do not provide evidence for a direct transcriptional link since an early block to establishing rhombomere identity could indirectly affect the expression of many later genes. However, taken together with the findings described here, this provides strong evidence that Krox-20 is an essential, immediate upstream regulator of EphA4.

Complexity of the regulation of EphA4 expression

The present analysis has revealed several aspects of the complexity of EphA4 regulation. Although reporter gene expression driven by the 7.5 kb enhancer fragment recapitulates the upregulation of EphA4 in r2, r3 and r5, activity was not observed in the early broad domain of EphA4 expression in the hindbrain. This is consistent with a modular control of different temporal and spatial aspects of gene expression, as found for many other genes, including the segmental expression of Hox genes in the hindbrain (Whiting et al., 1991).
Fig. 5. Mutational analysis of the minimal enhancer and effect of ectopic Krox-20 gene expression. The enhancer element within the 7.5 kb SacI-SacI fragment driving expression in the hindbrain was localised by testing subfragments in transgenic mice. (A–E) Mutations in this element were then tested in the context of a minimal enhancer or of the 9.5 kb NotI-NcoI fragment. (A) Wild-type 470 bp SacI-BglII fragment (construct 10). This embryo is mosaic for expression. Reporter gene expression occurs in r2, r3 and r5 in the hindbrain. (B) 470 bp fragment after mutation of five Krox-20-binding sites (sites 3–7). Reporter gene expression in r5 is abolished, but low level expression occurs in r2 and r3. (C) 470 bp fragment after mutation of eight Krox-20-binding sites. r3/r5 enhancer activity is abolished and expression only occurs in r2. (D) 470 bp fragment mutated at five Krox-20-binding sites (3–7) and placed back into the context of the flanking 9 kb region (construct 3). Compared with activity of the 470 bp fragment alone (B), similar low level expression occurs in r2, but high level expression is restored in r5. (E) 470 bp fragment mutated at eight Krox-20-binding sites and placed back into the context of the flanking 9 kb region. Expression occurs in r2 and is still absent in r3/r5. (F) A transgenic line was established using the 470 bp SacI-BglII fragment (construct 10) to drive lacZ expression in the hindbrain. (G) The r4 enhancer of the Hoxb-1 gene (Studer et al., 1994) was used to ectopically express Krox-20 in this transgenic line. Ectopic activation of the lacZ reporter gene occurred in r4.

even though it is not known whether the regulation of EphA4 transcription by Krox-20 in the neural crest is direct or indirect, these data suggest that Krox-20 mediates its activation of EphA4 in the hindbrain and in the crest via distinct cis-acting elements.

The 470 bp enhancer fragment contains numerous Krox-20-binding sites. The mutation of the eight motifs with homology to the consensus site eliminated r3/r5 enhancer activity but did not affect reporter gene expression in r2 and in the notochord, tissues in which Krox-20 is not expressed, indicating that these enhancer activities are mediated by distinct upstream factors. The observation of numerous Krox-20-binding sites in the minimal r3/r5 EphA4 enhancer is in contrast to similar analyses performed on the Hoxa-2 and Hoxb-2 r3/r5 enhancers where only two and three Krox-20-binding sites were identified, respectively. Furthermore, in the case of the Hoxb-2 enhancer, only one of the three Krox-20-binding sites was shown to be necessary and sufficient for enhancer activity (Vesque et al., 1996). The present study suggests that different Krox-20-binding sites make a distinct contribution to expression in r3 and r5, and in cooperating with factors binding outside of the 470 bp fragment. We found that the EphA4 enhancer has weak activity in r3 but no activity in r5 after five mutations that leave the 470 bp fragment. However, analysis of the same mutations in the context of the 9.5 kb fragment indicated that the activity of the enhancer in r5, but not in r3, was restored, whereas no expression occurred after mutation of eight binding sites. These data suggest that there is an r5-specific element within the 9 kb region outside of the 470 bp fragment that can cooperate with the remaining Krox-20-binding sites, but is not sufficient on its own for enhancer activity. This element presumably binds a factor that is present or active in r5 but not in r3 and can synergize with limiting amounts of DNA-bound Krox-20. In addition, there is a context-dependent effect of

Fig. 6. Hierarchical relationships between genes expressed in r3/r5. The diagram depicts the regulatory relationships and functions of genes expressed in r3/r5 in the hindbrain. Solid lines indicate that evidence has been obtained for a direct transcriptional regulation and dashed lines that the gene is known to be downstream based on analysis of mutants. Krox-20 has a central role in hindbrain patterning, being required for segmentation by maintaining r3/r5 territory and directly regulating Hoxa-2, Hoxb-2 and EphA4 in r3/r5. This regulatory relationship therefore couples A-P identity to the segmental restriction of cell movement. For references see the text.
mutation of two sites (sites 3 and 6): in the 9 kb context, it does not affect the activity of the r3/r5 enhancer while, in the 470 bp context, it behaves like the five-site mutation (data not shown). A more detailed study will be required to determine the contribution of each individual site and ability to synergise with factors outside the 470 bp fragment.

**Organisation of the transcriptional hierarchy in r3 and r5**

The finding that Krox-20 transcriptionally activates EphA4 provides a further understanding of the regulatory cascade controlling r3 and r5 delimitation and specification. Two transcription factors have been shown to play key roles in this process: Krox-20 and kreisler (Fig. 6). The inactivation of the corresponding genes leads to the early disappearance of r3 and r5 in the case of Krox-20 (Frohman et al., 1993; Schneider-Maunoury et al., 1993; Swiaterk and Gridley, 1993; Cordes and Barsh, 1994; McKay et al., 1994), and r5 and r6 in the case of kreisler (Frohman et al., 1993; Cordes and Barsh, 1994; McKay et al., 1994). In addition to EphA4, Krox-20 has been shown to directly regulate the expression of Hoxa-2 and Hoxb-2 in r3 and r5 (Sham et al., 1993; Nonchev et al., 1996) and at least indirectly to control the expression of Hoxb-3 in r5 (Seitanidou et al., 1997). Hoxb-3 has also been shown to be a direct transcriptional target of kreisler in r5 (Manzanares et al., 1997). Finally, analysis of the Hoxa-2 mutant has indicated that the Hoxa-2 protein is required for expression of another member of the Eph family in r3 and r5, EphA7 (Mdk1/Ebk), though it is not known whether this is a direct or indirect effect (Tanega et al., 1996). The significance of this latter link for the phenotype of Hoxa-2 mutant mice is unclear as the role of EphA7 is not known but, taken together with our data, this suggests that different Eph receptors are regulated at distinct levels of the transcriptional hierarchy: whereas EphA4 is directly regulated by Krox-20, EphA7 lies at least one step further downstream.

**Potential relationships between EphA4 regulation and function**

Studies of Krox-20 and EphA4 gene expression have shown that each are initially upregulated in fuzzy domains that then sharpen and become precisely restricted to r3 and r5 (Irving et al., 1996). This restriction presumably reflects the requirement for each rhombomere to establish a distinct, homogenous identity. Clonal analyses indicate that cell interactions are important to establish sharp domains, since these reveal intermingling of cells between presumptive rhombomeres, whereas after segmentation there is a restriction of cell movement between rhombomeres (Fraser et al., 1990; Birgbauer and Fraser, 1994). Based on these data, two mechanisms are proposed to underlie the segmental restriction of gene expression (Irving et al., 1996): first, those cells that cross presumptive rhombomere boundaries switch identity to that of their new neighbours, and second, restriction of the movement of cells between rhombomeres by establishment of alternating cellular properties (Guthrie et al., 1993). Function-blocking experiments suggest that the complementary expression of EphA4 in r3/r5 and interacting ephrins in r2/r4/r6 might underlie the restriction of cell intermingling between odd- and even-numbered rhombomeres (Xu et al., 1995).

Taken together, these data indicate that Krox-20 is a direct upstream regulator of a receptor that restricts intermingling of cells between rhombomeres. Since Krox-20 also regulates Hox gene expression in r3/r5 (Sham et al., 1993; Nonchev et al., 1996), it couples processes that restrict the movement and specify the A-P identity of r3/r5 cells (Fig. 6). As a consequence, cells with a specific A-P identity are restricted from intermingling into adjacent domains, such that sharp, homogenously specified segments are established. It is intriguing that in Krox-20 null mutants there is a progressive narrowing and loss of presumptive r3/r5, since an increased intermingling and/or loss of A-P identity of r3/r5 cells could contribute to this phenotype. Finally, this raises an interesting possibility regarding the overlap of Krox-20 and EphA4 expression: neural crest cells that migrate from r5 only into the third branchial arch (Nieto et al., 1995; Xu et al., 1995). Inhibition of EphA4 function leads to these neural crest cells entering adjacent territories (Smith et al., 1997). Similarly, in Krox-20 null mutants some of these neural crest cells are observed migrating towards the second branchial arch (Schneider-Maunoury et al., 1993), and EphA4 expression is absent in r5 neural crest indicating that Krox-20 is upstream in the regulatory hierarchy (Seitanidou et al., 1997). Thus, by analogy with the situation in the hindbrain described here, it will be interesting to determine whether Krox-20 may also couple the restricted movement of third arch neural crest cells to other aspects of their identity.

**General perspectives**

The generation of segmental units that form related but distinct derivatives is a crucial aspect of patterning in vertebrates and invertebrates, and involves both the compartmentalisation of cell populations and specification of their A-P identity. The coupling of Eph gene and Hox gene regulation in the hindbrain may restrict the movement of cells with a specific A-P identity, thus establishing sharply delimited segmental domains. In contrast, little is known of mechanisms that underlie the formation of compartments in other systems. In view of emerging evidence that Eph receptors and ephrins may have general roles in restricting cell movement, it will therefore be interesting to ascertain whether in other tissues there is a similar coupling of their expression to that of genes that regulate A-P identity.

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