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HOXD4 and regulation of the group 4 paralog genes

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SUMMARY

From an evolutionary perspective, it is important to understand the degree of conservation of *cis*-regulatory mechanisms between paralogous Hox genes. In this study, we have used transgenic analysis of the human *HOXD4* locus to identify one neural and two mesodermal 3′ enhancers that are capable of mediating the proper anterior limits of expression in the hindbrain and paraxial mesoderm (somites), respectively. In addition to directing expression in the central nervous system (CNS) up to the correct rhombomere 6/7 boundary in the hindbrain, the neural enhancer also mediates a three rhombomere anterior shift from this boundary in response to retinoic acid (RA), mimicking the endogenous *Hoxd4* response. We have extended the transgenic analysis to *Hoxa4* identifying mesodermal, neural and retinoid responsive components in the 3′ flanking region of that gene, which reflect aspects of endogenous *Hoxa4* expression. Comparative analysis of the retinoid responses of *Hoxd4*, *Hoxa4* and *Hoxb4* reveals that, while they can be rapidly induced by RA, there is a window of competence for this response, which is different to that of more 3′ Hox genes. Mesodermal regulation involves multiple regions with overlapping or related activity and is complex, but with respect to neural regulation and response to RA, *Hoxb4* and *Hoxd4* appear to be more closely related to each other than *Hoxa4*. These results illustrate that much of the general positioning of 5′ and 3′ flanking regulatory regions has been conserved between three of the group 4 paralogs during vertebrate evolution, which most likely reflects the original positioning of regulatory regions in the ancestral Hox complex.

Key words: Hox gene, gene regulation, hindbrain, retinoic acid, transgenic mice, HOXD4, somite, evolution

INTRODUCTION

The vertebrate Hox paralogy group 4 contains four genes highly related to the *Drosophila Deformed (Dfd)* gene. While their expression patterns are similar, there are differences in both the anterior boundaries and relative timing of expression during embryogenesis (Gaunt et al., 1989; Geada et al., 1992; Hunt et al., 1991). For example *Hoxa4*, *Hoxb4* and *Hoxd4* have anterior limits in the hindbrain that map to the junction between rhombomeric (r) segments r6 and r7 (Hunt et al., 1991; Keynes and Krumlauf, 1994), but their boundaries in paraxial mesoderm are all offset by a single somite (Gaunt et al., 1989; Hunt and Krumlauf, 1992). Mutation of these paralogous genes in mice reveals a partial functional redundancy or compensation illustrating that they can work synergistically to pattern individual vertebral components (Horan et al., 1994, 1995a,b; Kostic and Capecchi, 1994; Ramirez-Solis et al., 1993). More recently, it has been shown that this redundancy can operate at the level of individual target genes (Gould et al., 1997). *Hoxc4* is the most disparate member of the group with respect to both expression and function, as its anterior limit maps to the rostral spinal cord not the hindbrain (Geada et al., 1992) and loss-of-function mutations affect the patterning of more posterior vertebrae than the other group 4 genes (Boulet and Capecchi, 1996; Saegusa et al., 1996).

The vertebrate Hox clusters arose by the duplication of an ancestral complex (Duboule, 1994; Holland and Garcia-Fernandez, 1996; Krumlauf, 1992) and it is possible that each paralog may have conserved a subset or all of the *cis*-regulatory regions characteristic of their ancestral ortholog. These may have subsequently been modified in some of the paralogs to produce small variations in the timing or boundaries of expression. An alternative hypothesis is that, after duplication, each gene may have evolved control regions independently.

Analysis of the mouse *Hoxa4* and *Hoxb4* genes has previously shown that it is possible to reconstruct many aspects of their endogenous expression patterns using *lacZ* reporter genes in transgenic mice (Behringer et al., 1993; Whiting et al., 1991). Elements within the 5′ flanking region of *Hoxa4* can direct proper mesodermal and neural expression (Behringer et al., 1993). Conversely, in the case of *Hoxb4*, an enhancer in the intron mediates the proper mesodermal and posterior neural domains of expression, while a second enhancer 3′ of the gene controls anterior neural expression (Gould et al., 1997; Whiting et al., 1991). The position, sequence and general function of these *Hoxb4* enhancers is conserved between puffer fish, chick and mouse homologs (Aparicio et al., 1995; Morrison et al.,

1995). The differences between *Hoxb4* and *Hoxa4* in position and activities of regulatory regions suggested that, in general, control elements might not be conserved between paralogs.

Analysis of the 5′ flanking regions of a third group 4 member (*Hoxd4*) has identified sequences conserved between mouse and human that mediate autoregulatory (Popperl and Featherstone, 1992) and retinoic acid responses in tissue culture cells (Mavilio, 1993; Popperl and Featherstone, 1993). In transgenic mice, the conserved sequence block containing the human retinoic acid response element (RARE) functions in vivo as a retinoic acid (RA)-responsive enhancer that directs neural-specific expression of a basal promoter *lacZ* reporter, with a sharp boundary in the anterior spinal cord (Morrison et al., 1996). However, this is posterior to the r6/7 boundary of the endogenous *Hoxd4* gene (Hunt et al., 1991). In contrast, the human autoregulatory region, in the absence of the RARE, is unable to direct reporter expression in transgenic mice (Morrison et al., 1996). While both the autoregulatory and RA responsive elements may contribute to the full expression pattern of the *HOXD4* gene, they do not set the appropriate anterior boundaries in either hindbrain or paraxial mesoderm, suggesting other regulatory regions must be required.

In this study, we use transgenic analysis to identify *cis*-regulatory regions of the human *HOXD4* gene that generate the correct anterior boundaries of expression in mesoderm and neural ectoderm. We also evaluated the *Hoxa4* gene and compared the retinoid responses of *Hoxd4*, *Hoxb4* and *Hoxa4*. The results provide a basis for comparing and contrasting the activities and positions of the regulatory regions from these three group 4 paralogs.

MATERIALS AND METHODS

Transgene construction

Reporter constructs utilized the BGZ40 vector, containing the human β-globin promoter linked to the *lacZ* gene and SV40 polyadenylation signal (Yee and Rigby, 1993). Constructs #1-#15 contain fragments from the human *HOXD4* clone pG3-4.4cos3.1 (Cianetti et al., 1990). Construct #1 contains a 4.2 kb *Eco*RI fragment in the 5′-3′ orientation, blunt end cloned into the *Sal*I site. Constructs #2, #3 and #5 were made by digesting construct #1 with *Not*I and either *Stu*I or *Xba*I or *Bgl*II, respectively. Construct #4 was made in two steps, with inverse PCR (Clackson et al., 1991) generating the 95 bp deletion of CR1 sequences in a 720 bp subclone first, using the oligonucleotide primers: direct-5′-CAGAGGACCCAGGCTAACGGGCT-3′ and inverse-5′-CAGGTCAGGACCATGTGGCTGGC-3′. Construct #6 was generated by blunt ended cloning of the *HOXD4 Bgl*II-*Xba*I fragment in the 5′-3′ orientation. Constructs #7 and #8 were generated by digesting construct #6 with *Kpn*I and either *Msc*I or *Dra*II, respectively. Construct #9 was generated by digesting construct #1 with *Bgl*II and *Stu*I end filling, which removes the 1.65 kb internal fragment and self ligating. Construct #10 contained a 960 bp fragment of *HOXD4* 3‘ flanking sequence generated by PCR using the oligonucleotides (A) direct-5′-TTAGCTTCTAGAAGACTTAACAAAGG-3′and (B) inverse-5′-AATCTGTCTAGACATTGAACCTTCTC-3′. Construct #11 carried in the 5′-3′ orientation a 1.5 kb *Xba*I-*Apa*I *HOXD4* fragment. Constructs #12-#15 were generated by inserting *HOXD4* genomic fragments, generated by PCR into the *Xba*I site. The inserts for constructs #12-#14 were generated with the same 5′ primer (C) direct-5′-AAATTTAATTTCTAGATCCAGAAGGGGGG-3′ and the following 3′ primers respectively #12 (D) indirect-5′-CTTTC-CTCTAGAGCAGGGATGCAGTC-3′, #13 (E) indirect-5′-GAAG-GTTCAATGTCTAGACAGATTTGGG-3′, and #14 (F) indirect-5′-AAAGACGTCTAGACTATGTATGAACTCTG-3′. The insert for construct #15 was generated using primer E and the 5′ primer (G) direct-5′- TGGGGTCTAGACTTTGTTTCTCCTTGG-3′. Constructs #16 and #17 were generated using DNA fragments from the mouse *Hoxd4* genomic clone Cos E (Featherstone et al., 1988). Construct #16 contained a 1.6 kb *Xba*I 3′ fragment and construct #17 a 3.8 kb *Hin*dIII 3′ fragment in the 5′-3′ orientation. Constructs #18 and 19 were generated by inserting a 3.9 kb *Apa*I fragment, isolated from the 9 kb, mouse *Hoxa4* genomic clone 2195F (gift from D. Wolgemuth), into the *Apa*I site of BGZ40 in the 3′-5′ orientation.

Production of transgenic mice and β-galactosidase staining

In all cases, vector sequences were removed from fragments for injection by digestion with restriction enzymes followed by gel purification. Transgenic protocols and *lacZ* staining reactions were performed as previously described (Hogan et al., 1986; Whiting et al., 1991). Throughout this study, the allocation of expression to specific somite boundaries was based on careful counting of somite numbers and the position relative to the forelimb bud, assuming that the anterior margin of this structure at 9.5-10.5 dpc lies at the somite (s) 8/9 junction as previously described by (Burke et al., 1995). Under these criteria, our combined antibody and in situ analysis revealed that the expression of the endogenous *Hoxd4*, *Hoxb4* and *Hoxa4* genes were offset from each other by a single somite and mapped to the s5/6, s6/7 and s7/8 junctions, respectively. The assignment of absolute somite boundaries often varies between groups, depending upon: (1) whether the first somites formed which degenerate are taken into account; (2) whether the most anterior somite of expression is usually very weak and mosaic and could be missed and (3) which somite boundary is assigned to the anterior margin of the forelimb bud. In the case of non-expressing constructs, the total number of transgenic embryos was determined by PCR analysis of embryonic tissue. For timed pregnancies, noon on the day that the vaginal plug was observed was taken to be 0.5 dpc. RA treatment of embryos was by gavage, as described previously (Conlon and Rossant, 1992; Marshall et al., 1992; Morrison et al., 1996).

Mouse embryo culture

Mouse embryo culture was performed using 9.5 dpc embryos as described by Cockroft (1990) using 100% rat serum in rotating tubes in the dark and intermittent gassing with 40% O_2 and 5%CO_2 at 37°C. In RA treatments, RA (1.6 μM) was added and, after 4 hours, the culture media removed and the embryos washed extensively in RA-free media to remove exogenous RA. The embryos were then cultured for a further 12 hours before analysis.

Whole-mount in situ hybridisation and immunostaining

Mouse embryos were prepared using a modification of the method of Wilkinson (1992). The RNA probes were made using full-length *Hoxd4* and *Hoxa4* cDNAs (Featherstone et al., 1988; Wolgemuth et al., 1987). Immunostaining was performed using a mouse Hoxb4-specific monoclonal antibody as described (Gould et al., 1997).

RESULTS

Identification of *HOXD4* regulatory regions

In order to identify enhancer elements of the *HOXD4* gene responsible for generating the proper anterior limits of expression in the neural tube at the r6/7 junction and in paraxial mesoderm at the s5/6 boundary (Gaunt et al., 1989; Hunt et al., 1991), we tested regions from the human locus in transgenic mice. Previously, we showed that regions 5′ of the *HOXD4*

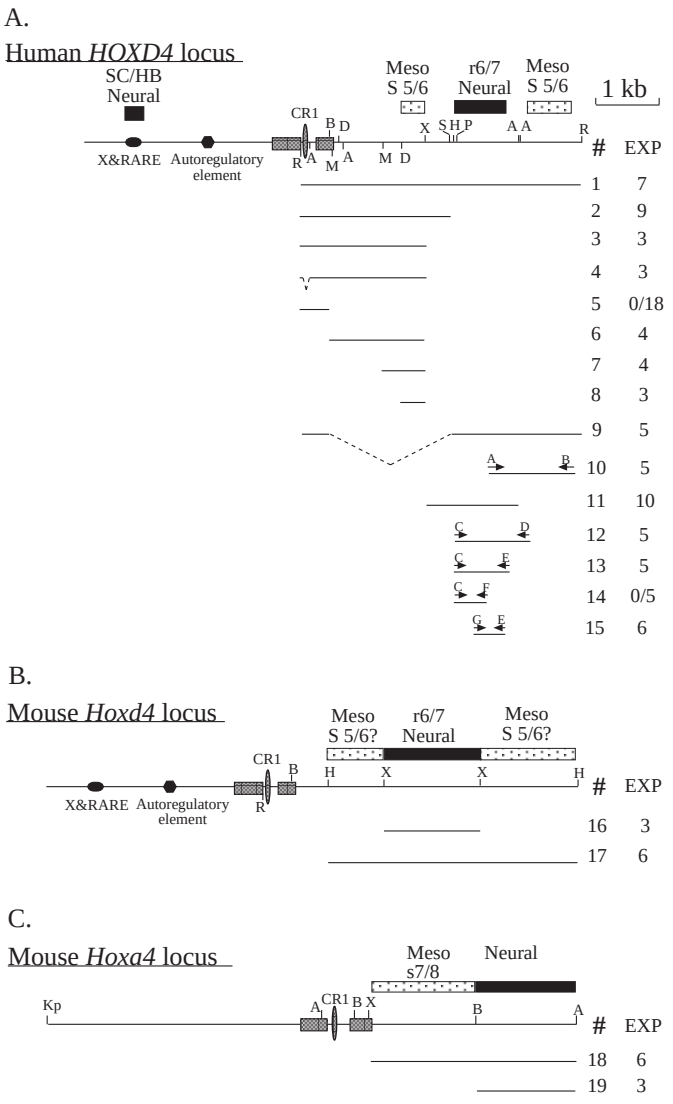

Fig. 1. Genomic organisation and transgenic constructs from three Hox group 4 genes. The human *HOXD4* (A), the mouse *Hoxd4* (B) and the mouse *Hoxa4* (C) loci are depicted by the partial restriction maps at the top of each panel, where the hatched rectangles mark the position of exons. The elongated hatched ellipse in the intron of each gene marks the position of a conserved (CR1) sequence element (Aparicio et al., 1995; Haerry and Gehring, 1996; Morrison et al., 1995). (A,B) The oval and hexagon in the 5′ flanking region of each gene marks the conserved positions of a complex RARE and a potential autoregulatory element, respectively. Above the loci are rectangles indicating the relative positions of neural (black) and mesodermal (stippled) enhancer regions identified in this and another (Morrison et al., 1996) transgenic study. Below each panel are the various genomic fragments used for transgenic reporter constructs and at the right of each is indicated the construct number (#) and the total number of transgenic mice producing the same consistent expression pattern (EXP). (A) Arrows over constructs 10-15 indicate the PCR primers (see methods) used to isolate the respective regions. Restriction enzyme sites, (A) *Apa*I, (B) *Bgl*II, (D)*Dra*II, (H) *Hin*dIII, (Kp) *Kpn*I, (P) *Pst*I, (R) *Eco*RI, (S) *Stu*I.

ATG mediated neural-specific expression with a boundary in the rostral spinal cord (Morrison et al., 1996). Since this neural limit was posterior to the boundary of the endogenous gene and mesodermal expression was missing, we examined regions 3′ of the ATG for additional enhancer activity.

First we tested a 4.2 kb *Eco*RI genomic fragment containing the *HOXD4* intron, exon two and 3.4 kb of sequence extending past the translational stop codon (construct #1; Fig. 1), linked to a *lacZ* reporter gene. In seven independent insertions at developmental time points from 9.0-11.0 days post coitum (dpc), this construct directed both neural and mesodermal reporter expression, which extended up to a sharp r6/7 anterior limit in the hindbrain and up to the level of the s5/6 boundary in the paraxial mesoderm (Fig. 2B,C,E). Typical of Hox genes, the expression of both the endogenous *Hoxd4* mRNA and the *lacZ* reporter in the most anterior domain of somitic expression (s6), was at a lower level than in more caudal somites. The anterior boundaries of expression directed by this 3′ regulatory region are the same as the endogenous *Hoxd4* gene (Fig. 2A,D) and, combined with the posterior neural enhancer located 5′ of *HOXD4* (Morrison et al., 1996), they reconstruct a major part of the *Hoxd4* pattern. However, these 5′ and 3′ regions do not completely reconstruct the endogenous *Hoxd4* pattern because there is an absence of reporter staining in lateral plate mesoderm and differences in limb expression. This suggests that other control regions may be missing or that the gene may need to be in the normal chromosomal context for proper regulation of these aspects of expression (compare Fig. 2A,B and D,E).

Mapping a proximal 3′ mesodermal enhancer

To further map the mesodermal and neural control regions, we generated a series of deletion constructs (#2-#15; Fig. 1A). First we tested a 2.36 kb *Eco*RI-*Stu*I fragment (construct #2) containing the *HOXD4* intron, exon 2 and 1.14 kb of 3′ sequence. In nine transgenic founders, neural expression was specifically lost, but we consistently observed the same s5/6 mesodermal expression pattern seen with construct #1 (data not shown). A shorter 2 kb *Eco*RI-*Xba*I fragment (construct #3) gave an identical mesodermal-restricted pattern in three transgenic embryos (Fig. 2F,J).

Initially, we focused on the intron within construct #3, because in previous work on the *Hoxb4* paralog we identified a mesodermal enhancer located in the equivalent region (Morrison et al., 1995; Whiting et al., 1991). Furthermore, within the *Hoxb4* intron, there is a highly conserved region (CR1) present not only in the mouse, chick and pufferfish homologs but also in the introns of the paralogous *Hoxd4* and *Hoxa4* genes from several species (Aparicio et al., 1995; Cianetti et al., 1990; Morrison et al., 1995). In addition, a conserved block related to CR1 is found within the introns of the *Hoxa7* (Haerry and Gehring, 1996) and *Hoxd9* (Zappavigna et al., 1991) genes. In all cases, the core of this block contains three consensus homeodomain binding sites, suggesting it may function as a Hox target in vertebrates (Haerry and Gehring, 1996). To examine the potential role of CR1 in the *HOXD4* enhancer (construct #3), we created a specific 95 bp deletion of CR1 (construct #4), but observed no change in the expression pattern (n=3; data not shown) compared with wild-type construct #3 or with other deletion variants (constructs #5-8; Fig. 2). Therefore, despite the extensive degree of evolutionary conservation, this result shows that CR1 is not necessary in this transgenic assay for the regulatory activity of the proximal *HOXD4* 3′ mesodermal enhancer. Intronic

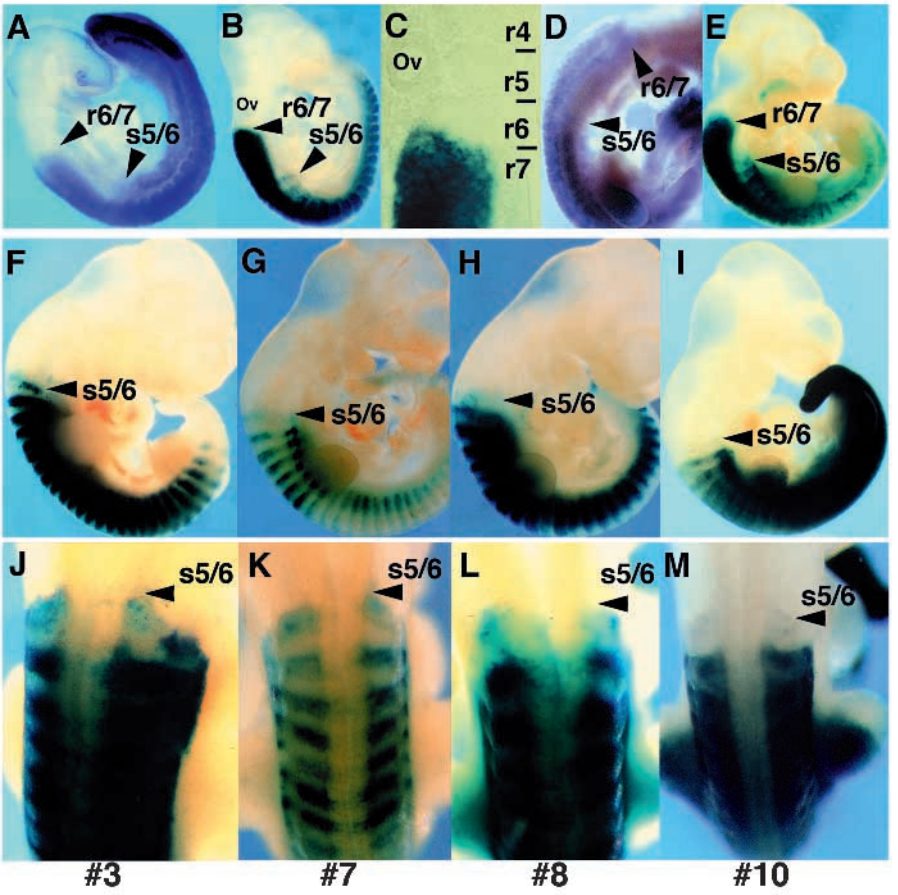

Fig. 2. Comparison of *Hoxd4* transgene and endogenous expression patterns and the mapping of two mesodermal enhancer activities. (A) Whole-mount in situ hybridisation of the mouse *Hoxd4* gene at 9.5 dpc. (B) Lateral view of ß-galactosidase expression in a 9.5 dpc transgenic embryo containing construct #1. The transgene has the correct anterior limits at the r6/7 hindbrain boundary (shown in flat mount in (C)) and the s5/6 junction in the paraxial mesoderm. (D) Whole-mount in situ hybridisation of the mouse *Hoxd4* gene at 10.5 dpc, note that expression in the anterior most somite is at a lower level than more caudally expressing somites. (E) β-galactosidase expression in a 10.5 dpc transgenic embryo containing construct #1. Expression of β-galactosidase in a transgenic embryos at 10.5 dpc carrying; (F,J) construct #3, (G,K) construct #7, (H,L) construct #8, (I,M) construct #10. In all cases, staining is restricted to the segmented mesoderm (F-M) with an anterior limit at the s5/6 junction but with lower levels of activity in somite 6. (Ov) Otic vesicle, (r) rhombomere, (s) somite.

sequences other than CR1 could be important for regulatory activity, so we tested a 710 bp *Eco*RI-*Bgl*II fragment containing only the *HOXD4* intron and 165 bp of exon 2 sequences (construct #5). However, in a total of 18 founder embryos, we detected no *lacZ* gene expression in mesoderm, demonstrating that the *HOXD4* intron alone is not sufficient for mesodermal regulation.

This suggested that the enhancer is located in the 1.2 kb *Bgl*II-*Xba*I fragment, which was confirmed by testing the activity of this region alone (construct #6). In four transgenic embryos, we detected mesodermal expression with a boundary at the somite 5/6 junction, identical to that obtained with constructs #2-#4 (data not shown). Transgenic embryos carrying two further deletions of the 1.2 kb enhancer (constructs #7 and #8) all had a s5/6 boundary of somitic expression identical to that of the full 1.2 kb enhancer and the endogenous *Hoxd4* gene (Fig. 2G,H,K,L). Thus elements capable of mediating the correct pattern of *HOXD4* expression in somites lie within a 3′ 375 bp *Dra*II-*Xba*I fragment.

A second distal mesodermal enhancer 3′ of *HOXD4*

We made an internal deletion of the 1.2 kb mesodermal enhancer in the context of the initial 4.2 kb fragment (construct #9) to determine whether the remaining regions were sufficient to mediate the proper r6/7 neural expression. Surprisingly, the patterns of transgene expression were identical in the neural tube and mesoderm to those observed with construct #1 (data not shown). This suggested that there is a second mesodermal/somite enhancer within these sequences capable of directing the proper anterior boundary of expression. Since we found no regulatory activity within the intron alone (construct #5), we examined the remaining 3′ region. A 960 bp fragment generated by PCR (construct #10), directed a consistent pattern of transgene expression in the paraxial mesoderm, with an anterior boundary at the s5/6 junction (Fig. 2I,M). However, we detected no expression in the hindbrain or anterior spinal cord of any of the embryos. This implies that somitic expression of *HOXD4* is generated by at least two independent regulatory regions each capable of directing mesodermal expression up to the s5/6 boundary. Sequence comparisons between these two enhancers revealed no extended blocks of identity and a search of the Eukaryotic Transcription Factor Database (TFD) (Ghosh, 1993) for potential known *cis*-acting binding sites was not informative with respect to candidate regulators.

Interestingly, in several of the embryos with construct #10, we observed staining in the limb bud, lateral plate mesoderm and tail bud (Fig. 2I) that were not seen with construct #1 (Fig. 2B,E), suggesting that negative regulatory influences may have been deleted in this smaller construct. Since the lateral palate and limb domains correspond to the specific sites where construct #1 transgene expression did not recreate the endogenous pattern, it is possible that in the normal chromosomal context these negative influences do not occur and we are not missing critical *cis*-elements needed for positive activation of the gene in these tissues.

Mapping the *HOXD4* neural enhancer

The results above implied that the components involved in regulating neural expression might be located in the 5′ portion of the 1.9 kb *Stu*I-*Eco*RI fragment (Fig. 1). This was confirmed using three different overlapping subfragments of this region (constructs #11-#13) (data not shown and Fig. 3A,E). In all cases, the anterior limit of expression extended up to a sharp r6/7 boundary with reporter staining gradually declining caudally. To further define the position of the r6/7 enhancer in the minimal 700 bp region (construct #13), we generated two partially overlapping subfragments. In five transgenic embryos carrying the 5′ 405 bp (construct #14), reporter activity was never detected (data not shown). In contrast a 3′ 468 bp fragment (construct #15) mediated a consistent pattern of neural-restricted expression (Fig. 3B,F) in six transgenic founders. However, the anterior limit of expression did not extend up to the proper r6/7 boundary, but mapped caudally to the hindbrain/spinal cord region. These results show that multiple elements in the *HOXD4* enhancer are required for setting the proper r6/7 neural expression domain. Elements in the 3′ part of the enhancer are able to direct neural-restricted expression, while elements in the 5′ region are required to set the correct anterior limit.

The *Hoxd4* regulatory elements are conserved between mouse and human

We wished to determine whether the position of 3′ regulatory regions were also present in the mouse gene. By sequencing in the 3′ flanking region of the mouse *Hoxd4* locus, we identified a region corresponding to the minimal 700 bp human neural enhancer within a 1.6 kb *Xba*I fragment. In three founder embryos, this fragment (construct #16) mediated neural-restricted staining up to the r6/7 boundary (Fig. 3C,G). This pattern was indistinguishable from that obtained with the human *HOXD4* neural enhancer demonstrating that the function and position of the regulatory region was conserved. We have tried to utilize sequence comparisons between these two elements to identify functionally conserved blocks, but the degree of identity over the entire 700 bp is so extensive (85%) that this approach has been uninformative.

The human *HOXD4* neural enhancer was flanked by two mesodermal control elements all contained in a 2.6 kb *Dra*II-*Eco*RI fragment (Fig. 1). Therefore, we used a 3.6 kb *Hin*dIII fragment (construct #17), which extended both 5′ and 3′ of the mouse neural enhancer, to assay for the presence of similar mesodermal control regions. In addition to the expected r6/7 neural domain, three of six transgenic embryos displayed strong mesodermal expression with the same s5/6 anterior limit as the endogenous *Hoxd4* gene (Fig. 3D). The three remaining embryos showed the r6/7 neural expression but had either weak or patchy mesodermal expression where it was hard to assign a distinct anterior boundary (Fig. 3H). We assume these differences are due to differential influences exerted by the sites of integration. Therefore, as in the human gene, this 3.6 kb fragment contains at least one regulatory region flanking the neural enhancer capable of mediating the proper pattern and boundary of somite expression, suggesting that the relative positions of the mesodermal and neural enhancers are conserved between these species.

The 3′ *HOXD4* neural enhancer responds to retinoids

The RARE located in the 5′ region of the *HOXD4* locus has been shown to mediate a response to ectopic retinoic acid (RA) in transgenic embryos (Morrison et al., 1996), and we wanted to determine if it is involved in the overall response of the *HOXD4* gene to RA through interaction with other 3′ control elements such as the neural enhancer defined here. As a basis for comparison, we first characterised the RA response of the endogenous *Hoxd4* gene in utero. Using conditions known to induce *Hoxb1* (Conlon and Rossant, 1992; Marshall et al., 1992), exposure to RA at 7.5-7.75 dpc fails to induce ectopic *Hoxd4* expression (Fig. 5I) even though the gene is expression at that time in the primitive streak. However, there is a rapid and dynamic induction of *Hoxd4* in the anterior hindbrain in response to RA treatments at 9.5 dpc, when *Hoxb1* is no longer induced by RA. The response can be seen within 6 hours, when ectopic expression is expanded anteriorly over three rhombomeres (r4-r6) but, after 12-24 hours, there is a gradual reduction in r5 (Fig. 5A, E). Under these conditions, we did not observe any change in the mesodermal pattern of *Hoxd4* gene expression following exposure to RA.

The RA response of the endogenous gene is significantly different to that seen with the 5′ RARE elements directing reporter expression (Morrison et al., 1996) and, since it occurs largely within the hindbrain, we wished to examine whether the 3′ neural enhancer was involved. We exposed transgenic founder embryos carrying either construct #16 (the mouse neural element) or construct #11 (the human neural element) to RA at 7.5-7.75 dpc or 9.5 dpc, and assayed for *lacZ* activity

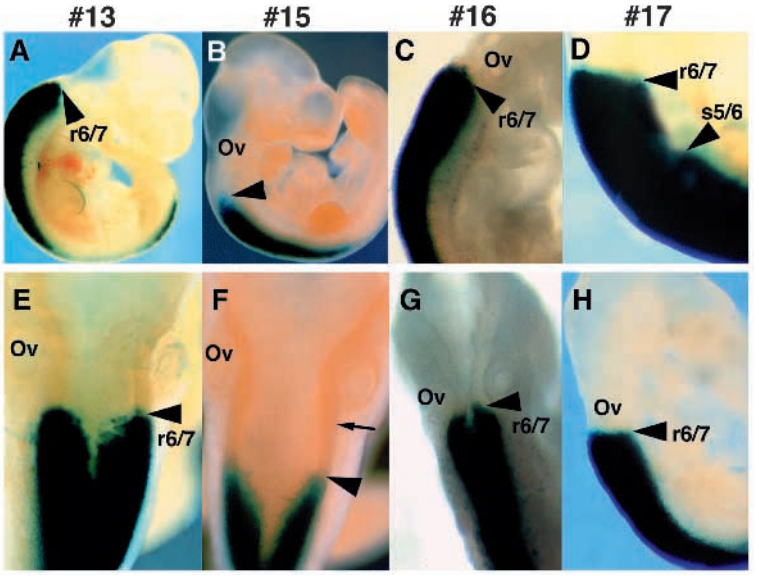

Fig. 3. Identification of the *HOXD4* /*Hoxd4* neural r6/7 enhancers. (A-D) Lateral and (E- H) dorsal views of neural-specific β-galactosidase expression in 10.5 dpc embryos containing constructs #13, #15, #16 and #17, respectively. For constructs #13, #16 and #17, the anterior limit to expression lies at the r6/7 junction in the hindbrain (arrowheads), posterior to the otic vesicle (Ov). (B,F) Note, however, that in the transgenic embryo carrying construct #15 the anterior limit to neural expression (arrowhead) is caudal to the r6/7 junction (small arrow in F).

at 10.5 dpc. Treatments at 7.75 dpc did not alter reporter expression. However, in both cases with treatments at 9.5 dpc, we detected an anterior shift in transgene expression virtually identical to that observed with the endogenous gene, where ectopic staining was seen in r4 and r6 after 24 hours (Fig. 5B,C,F,G). Weak r5 staining was also seen in some cases (data not shown). This demonstrates that the 3′ neural enhancer is itself capable of mediating a retinoid response, and is not strictly dependent upon the 5′ RARE elements. Within the 3′ enhancer, there are no typical consensus RAREs with a spacing of 2 or 5 base pairs between the direct repeats, but there are some atypical direct repeats with a spacing of 0 and 4 base pairs and two widely dispersed consensus half sites. Hence it is not clear if the RA response is direct. The nature of the RA response mediated by the 3′ enhancer and the endogenous gene are so similar, this element is likely to be a major component involved in regulating the ectopic response in the hindbrain. Thus the RA response of the *HOXD4* gene involves inputs from multiple control elements and they appear to have a common window of competence to respond, which opens between 8.5 and 9.5 dpc.

3′ regulatory elements in the mouse *Hoxa4* gene

Regulatory elements that can mediate *Hoxa4*-like patterns of expression lie in sequences 5′ to this gene (Behringer et al., 1993). However, we were interested in testing whether additional elements capable of directing *Hoxa4* expression lay in 3′ regions by analogy to *Hoxb4* (Whiting et al., 1991) and the *HOXD4/d4* results above. Therefore, a ~3.3 kb *Xba*I-*Apa*I 3′ fragment from *Hoxa4* (Fig. 1C; construct #18) was tested. In transgenic embryos, we consistently observed neural and mesodermal expression with sharp anterior boundaries (data not shown) and we generated a stable line (AL34) to investigate this pattern in more detail (Fig. 4A).

In transgenic embryos from the AL34 line and transient founders, there was staining in lateral plate and paraxial mesoderm, with the same anterior somite boundary at s7/8 observed with the endogenous *Hoxa4* gene (Fig. 4A,G). The mesodermal expression of this transgene (construct #18) is similar to that previously observed with 5′ regulatory elements from *Hoxa4* (Behringer et al., 1993). Therefore, like *HOXD4*, there are multiple overlapping mesodermal enhancer activities surrounding the *Hoxa4* gene some of which lie in its 3′ flanking region.

The anterior boundary of neural expression with this *Hoxa4* transgene appeared to be caudal to the r6/7 junction (Fig. 4A-D). To illustrate this more clearly, we bred the AL34 line with another line (ML19) that carries a *lacZ* reporter gene under the control of an r3/r5 enhancer from the *Hoxb-2* gene (Sham et al., 1993). This provides convenient internal reference points for identifying specific rhombomeric boundaries (Fig. 4B-D). From the lateral view of a 10.5 dpc embryo (Fig. 4B) and a flat mount of the hindbrain region (Fig. 4C), it is clear that the anterior limit of the *Hoxa4* transgene lies more than a single rhombomere's width from the r5 marker, in the vicinity of the r7/8 junction. Interestingly, in *Hoxa4* whole-mount in situ analysis at 9.5-10.5 dpc, we consistently found that the anterior expression limit of the endogenous gene was also caudal to the r6/7 boundary (Fig. 4G,H). Thus the endogenous *Hoxa4* neural pattern of expression at 10.5dpc closely resembles the transgene expression with construct #18.

At later stages between 11.5 and 12.5 dpc, endogenous *Hoxa4* expression extends more anteriorly up to the r6/7 junction, like *Hoxd4* and *Hoxb4*, indicating that there is a difference in the time at which these genes reach their respective anterior limits (Gaunt et al., 1989; Hunt et al., 1991). Hence we examined expression in the AL34 line at 11.5-12.5 dpc to determine if it would shift more anteriorly, but staining remained caudal to the r6/7 junction (Fig. 4D, and data not shown). This indicates that the 3′ *Hoxa4* enhancer does not mimic the endogenous pattern at later stages.

A deletion of the *Hoxa4* construct removing 1 kb of the DNA sequences that lie immediately adjacent to the gene (construct #19), also directed neural-restricted expression in a manner identical to that seen with the full enhancer (Fig. 4E,F). However, in these embryos, expression in mesoderm was specifically abolished. This demonstrates that separable neural and mesodermal control regions also operate in the 3′ flanking region of the *Hoxa4* gene, which may be a common property of the group 4 paralogs.

The response of *Hoxa4* to retinoids

To further our comparisons with *HOXD4/Hoxd4*, we have also analysed the RA response of *Hoxa4*. To check the endogenous RA response first, we performed whole-mount in situ hybridisation on 10.5 dpc embryos, which had been exposed to RA in utero at 9.5 dpc, as for *Hoxd4*. While we did observe an RA response for *Hoxa4* , this was quite different in nature to the *Hoxd4* response. There was an increase in the strength of the signal, suggesting higher levels of *Hoxa4* transcripts, and a small anterior shift in the expression boundary. This shift was from a region near the spinalcord/hindbrain boundary in control embryos (Fig. 4G,H), up to an anterior limit at the r6/7 junction (Fig. 5K,L), but with no expression in more anterior rhombomeres. The treatment at 9.5 dpc had no detectable effect upon the mesodermal pattern of expression and, like *Hoxd4*, RA treatments at earlier times also failed to induce *Hoxa4* expression (data not shown). Thus the temporal window in competence to respond to RA is conserved between *Hoxd4* and *Hoxa4* but the response itself is different.

Embryos carrying the *Hoxa4* 3′ neural enhancer (line AL34, construct #18) and the r3/r5 marker (ML19) were also treated with RA at 9.5 dpc and analysed for transgene induction after 24 hours. *lacZ* expression shifted more anteriorly to the r6/7 boundary, but only in ventral regions (Fig. 5D,H). Thus there is an RA response mediated by the *Hoxa4* neural element, but this enhancer was not sufficient to recreate the full induction observed for the endogenous gene.

Neural enhancers and the retinoid response of group 4 paralogs

The correlation between the 3′ neural enhancers and the ectopic response to RA for both the *Hoxd4* and *Hoxa4* genes suggests that this might be a general characteristic of group 4 paralogs. To explore this further, we examined the RA response of *Hoxb4* and of the 3′ r6/7 neural enhancer (region A), which we have previously identified in transgenic studies (Aparicio et al., 1995; Morrison et al., 1995; Whiting et al., 1991). We have used both whole-mount in situ hybridisation and immunohistochemistry to monitor the RA response of the endogenous *Hoxb4* gene and obtained identical results with both methods. In the neural ectoderm, there is a strong and rapid RA response

at both 8.5 dpc and 9.5 dpc, but no induction with treatments at 7.5-7.75 dpc (Fig. 6A-C and data not shown). At 9.5 dpc, there is initially a three rhombomere shift extending the anterior boundary from r6/7 up to the r3/4 junction (Fig. 6B), and this is followed by a downregulation in r5 (Fig. 6C). Again we detected no changes in mesodermal expression after RA treatment at 9.5 dpc. This response is identical to that observed with *Hoxd4*. Exposure to RA at 8.5 dpc resulted in an anterior shift of one rhombomere up to the r5/6 boundary (data not shown).

To examine the response of the 3′ *Hoxb4* neural enhancer, we applied the same RA treatment regime to transgenic embryos carrying the *lacZ* reporter under region A control (construct #9 in Whiting et al 1991). Like the endogenous gene, the transgene displayed the same temporal windows of response, giving a one rhombomere shift at 8.5 dpc and a three rhombomere anterior shift with dynamic downregulation in r5 at 9.5 dpc (Fig. 6D-I; and data not shown). Furthermore, to confirm that the transgene and endogenous patterns were coincident, in some cases we first performed Hoxb4 immunostaining (Fig. 6B), followed by β-galactosidase histochemistry on the same transgenic embryos (Fig. 6E) and found identical patterns.

We characterized the nature of the transgene RA response in two ways. Firstly, we exposed 9.5 dpc transgenic embryos to RA in culture and then assayed for reporter staining, observing a rapid three rhombomere anterior shift in the boundary of expression (Fig. 6H). Similarly, when embryos exposed to RA at 9.5 dpc in utero were harvested and stained for reporter activity 4-6 hours later we again observed the three rhombomere anterior shift in expression (Fig. 6I). Thus, the lack of response to early RA treatments (7.5-7.75 dpc) when both the transgene and endogenous gene are expressed in the neural ectoderm does not imply the *Hoxb4* gene itself is slow in responding or requires a higher dose of RA. Instead once the gene is competent to respond in later stages, the response to RA can be observed both in vitro and in utero within 4-6 hours of RA administration, suggesting that this is a rapid and direct result of RA exposure. Together these results demonstrate a conservation in the 3′ location of neural-specific enhancers from three group 4 paralogs, with a close association of regulatory elements capable of mediating responses to RA that closely resemble those seen with the respective endogenous genes.

DISCUSSION

In this study through transgenic analysis of the human *HOXD4* gene, we have identified control elements in the 3′ flanking region that direct expression of a reporter gene in a pattern similar to the endogenous *Hoxd4* mouse gene with respect to anterior boundaries. We found two different enhancers that independently direct mesodermal expression to the proper somite 5/6 boundary and a third regulatory element, conserved with the mouse gene, that mediates expression up to an r6/7 limit in the hindbrain. The neural enhancer involves the interaction of multiple components for r6/7 expression and also responds to ectopic doses of RA in a manner that mimics the RA response of the endogenous *Hoxd4* gene. In addition, our analysis of the *Hoxa4* gene identified mesodermal and neural RA-responsive regulatory elements in the 3′ flanking region. These findings, and previous work on *Hoxb4* and *Hoxa4*, have enabled us to examine the degree to which Hox group 4 regulatory elements have been conserved during duplication and divergence of the vertebrate clusters. The data show that the global organisation of *cis*-regulatory regions between *Hoxd4*, *Hoxb4* and *Hoxa4* is remarkably conserved, in support of the idea that they reflect the original positioning of regulatory regions in the ancestral vertebrate cluster (summarized in Fig. 7). Superimposed upon this organisation, in some cases the activity of individual elements appears to have diverged, which may account for some of the minor differences in anterior boundaries and timing of expression. In other cases activities have either been gained, lost or rearranged.

Neural regulation of *Hoxa4*, *Hoxb4* and *Hoxd4*

Enhancers capable of setting the r6/7 anterior limit of neural expression characteristic of both the endogenous *Hoxb4* and *Hoxd4* genes lie in the 3′ flanking sequences (Fig. 7). The timing, spatial restriction and RA response mediated by these two enhancers is very similar, suggesting that both genes may use common mechanisms to govern the pattern of neural expression. Although we used sequence comparisons to search for conservation between the *Hoxb4* and *Hoxd4* r6/7 enhancers, we detected no obvious block of similarity shared between them. Hence if the similar functional activity of these enhancers is derived from common ancestral regulatory elements located in this position, it would appear these activities are mediated by small dispersed motifs.

Although *Hoxa4* regulation differs from that of *Hoxb4* and *Hoxd4*, there are still important similarities. One difference is that the anterior limit of endogenous *Hoxa4* expression only reaches the r6/7 junction at later time points (after 11.5 dpc) (Gaunt et al., 1989; Hunt et al., 1991) while, in earlier stages, the anterior boundary lies caudal to r6/7 (Fig. 4G,H). The 3′ enhancer from *Hoxa4* identified here, directed neural expression with an anterior boundary in the caudal hindbrain/spinal cord region, but the boundary never mapped to the r6/7 junction at any stage (Fig. 4A-F). This pattern closely resembles only the early phase of endogenous *Hoxa4* expression. The activity of the *Hoxa4* enhancer is very similar to that of the *HOXD4* enhancer containing only the neural-specific component (compare Fig. 3F and Fig. 4F). Our transgenic analysis revealed that the human *HOXD4* neural enhancer could be divided into two separate components; one that mediated neural specificity and a second required for setting the proper anterior boundary at r6/7 (Fig. 3E,F). This suggests that the *Hoxa4* gene may differ from its paralogs in having lost the 3′ component(s) required to set the r6/7 anterior limit at 10.5 dpc. On the basis of these data, we conclude that the three group 4 paralogs, *Hoxa4*, *Hoxd4* and *Hoxb4*, have maintained the position of a 3′ neural enhancer (see Fig. 7), presumably derived from a common ancestral regulatory element, during duplication and divergence of Hox complexes in vertebrate evolution.

Regulation of the 3′ neural enhancers by retinoids

One of hallmarks of the Hox gene complexes is the colinear nature of their response to retinoids (Conlon and Rossant, 1992; Kessel and Gruss, 1991; Papalopulu et al., 1991; Simeone et al., 1990, 1991). However, this response is poorly

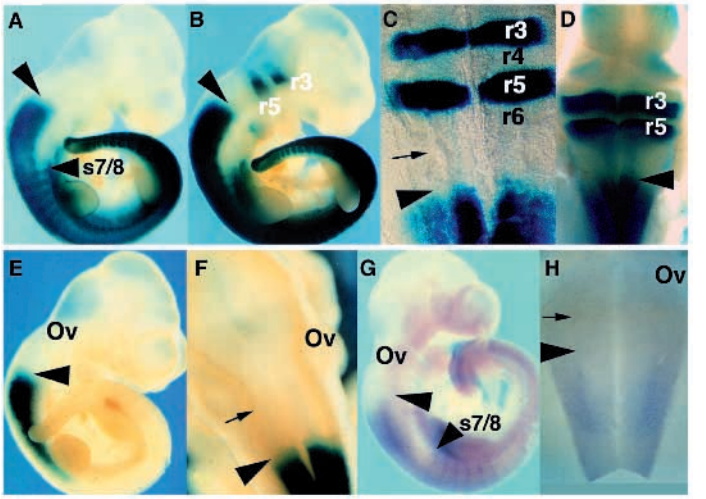

Fig. 4. The mouse *Hoxa4* gene contains 3′ neural and mesodermal enhancer activities. (A) A 10.5 dpc transgenic embryo from the *Hoxa4* AL34 line containing construct #18. Expression is seen in the lateral and somitic mesoderm, the anterior limit of which lies at the somite 7/8 junction. (B-D) Double transgenic embryos from a mating of AL34 with another transgenic line (ML19) carrying an r3/r5 enhancer directing lacZ expression (Sham et al., 1993). The anterior limit of expression at 10.5 dpc (B,C) and 12.5 dpc (D) lies caudal to the r6/7 junction. A hindbrain flat mount (C) of the embryo in B shows that expression limit (arrowhead) does not correspond to r6/7 (arrow) and maps more than a single rhombomere width from the r5/6 junction. (E,F) A lateral (E) and dorsal (F) view of a 10.5 dpc embryo carrying construct #19, again the anterior limit of neural expression (arrowhead) lies caudal to the r6/7 junction (arrow). (G,H) *Hoxa4* in situ hybridisation in a 10.5 dpc embryo (H), with a flat-mount preparation of the embryo (G) illustrating that, at this stage of development, the anterior limit of *Hoxa4* neural expression is caudal to the r6/7 junction. (Ov) otic vesicle. The small arrow in C,F,H indicates the position of the r6/7 junction.

understood and it is not clear whether the RA-induced changes in expression are direct or indirect and whether they involve graded sensitivities to RA concentrations. In support of a direct role for RA and its nuclear receptors, RAREs have been found near some genes and several are implicated in the normal control of Hox expression (Dupé et al., 1997; Frasch et al., 1995; Langston and Gudas, 1992; Marshall et al., 1994; Moroni et al., 1993; Morrison et al., 1996; Popperl and Featherstone, 1993; Studer et al., 1994). The RA response of the most 3′ genes has previously been examined in the greatest detail, but our analysis of three group 4 Hox genes has revealed that they respond in a different manner. RA treatment at times (7.25-7.75 dpc) known to affect *Hoxb1* expression (Conlon and Rossant, 1992; Marshall et al., 1992) had no effect upon the endogenous expression pattern of *Hoxa4*, *Hoxb4* or *Hoxd4* even though these genes are being expressed in the early neural ectoderm and establishing their respective anterior boundaries closely following *Hoxb1*. Conversely, RA treatment at later times (8.5-9.5 dpc) rapidly induced anterior shifts in expression of the group 4 genes (Figs 5, 6), when *Hoxb1* is no longer responsive even though it is still expressed in the hindbrain.

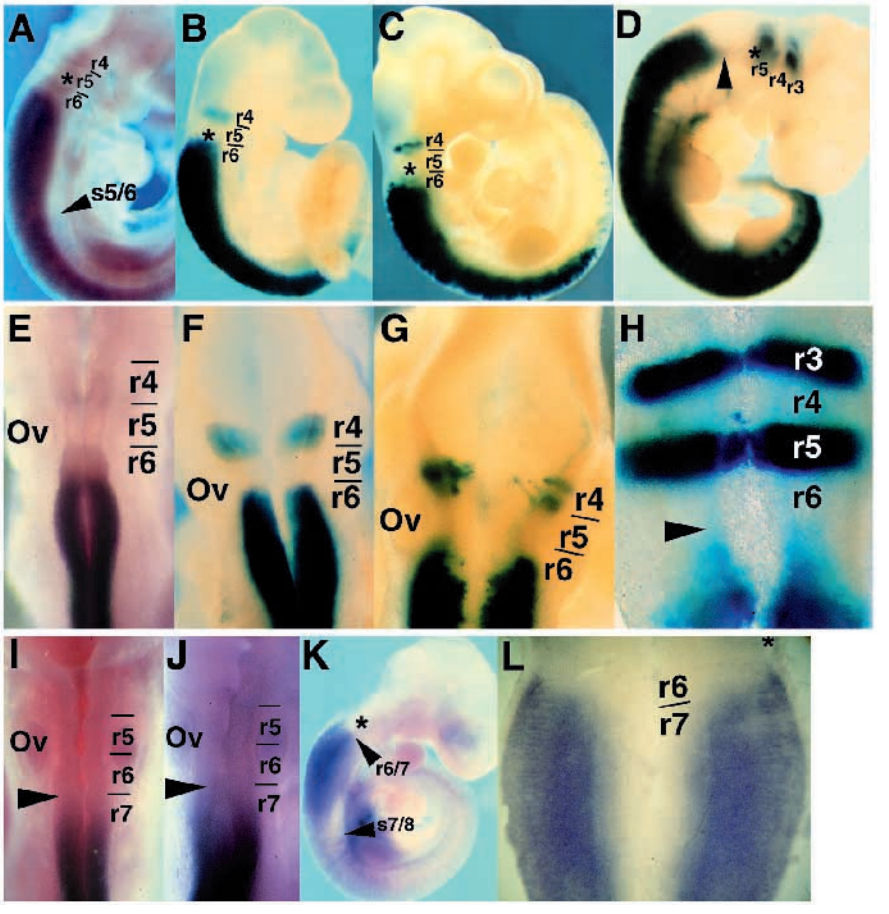

Fig. 5. The RA response of the endogenous *Hoxd4* and *Hoxa4* genes and transgenic reporters under control of their 3′ neural enhancers. (A-C) The endogenous *Hoxd4* gene (A), the mouse *Hoxd4* transgene (B) and human HOXD4 transgene show a similar response to RA administered in utero at 9.5 dpc. In all cases ectopic expression is induced in r4, r5 and r6, with down regulation in r5 after 12-24 hours. The embryos shown here were assayed either 12 hours (A) or 24 hours (B,C) after treatment. (E-G) Dorsal views of the same embryos shown in A-C, respectively. (I) A 9.5 dpc embryo treated with RA at 7.5 dpc; the endogenous *Hoxd4* neural expression is unchanged. (J) A 10.5 dpc control untreated embryo. (D,H) A lateral view of a whole 10.5 dpc embryo (D) and its dorsal flat mounted hindbrain (H) from the *Hoxa4* AL34 line (construct #18), exposed to RA at 9.5 dpc. A *Hoxb2/lacZ* transgene (Sham et al., 1993) was again used to mark r3 and r5. There is a rostral shift in expression up to the r6/7 boundary; however, this response is limited to the ventral regions of the neural tube (arrowhead). (K,L) *Hoxa4* in situ hybridisation in a 10.5 dpc embryo (K) treated with RA at 9.5 dpc and the flat-mounted hindbrain of this embryo (L) shows neural expression shifts up to the r6/7 junction. The * in A-D and K, L represents the position of the otic vesicle.

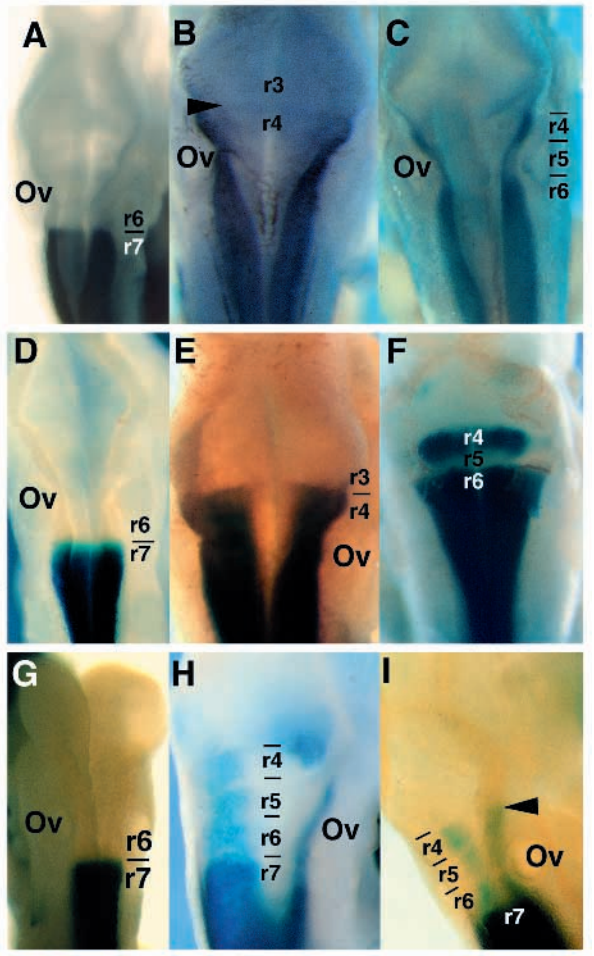

Fig. 6. Analysis of the RA response of endogenous *Hoxb4* and a *Hoxb4/lacZ* transgene under control of the 3′ region A neural enhancer. (A-C) The distribution of the Hoxb4 protein is detected using a monoclonal antibody in a control untreated 10.5 dpc embryo (A) and a 10.5 dpc embryo (B) and a 11.0 dpc embryo (C) each exposed to RA at 9.5 dpc. Ectopic expression (B) is induced in r4, r5 and r6 with an anterior limit to expression at the r3/r4 boundary. (C) Ectopic expression in r5 is reduced at later times after RA treatment while that in r4 and r6 is maintained. (D-F) Transgene expression in a control 10.5 dpc transgenic embryo containing the *Hoxb4* Region A *lacZ* reporter (D) and in same 10.5 dpc embryo shown in B, which was also stained for reporter activity after Hoxb4 immunostaining (E) showing that the expression is coincident. At 12.5 dpc ectopic expression of the transgene is down regulated in r5 (F). (G) A 9.5 dpc transgenic embryo exposed to RA at 7.5 dpc. The *Hoxb4* transgene, like the endogenous gene (not shown), fails to respond to RA at this time. (H) An ~10.5 dpc *Hoxb4* transgenic embryo exposed transiently to RA in embryo culture, again a three rhombomere anterior shift of expression is observed. (I) The same rapid RA response is seen in a 9.75 dpc transgenic embryo exposed to RA at 9.5 dpc, illustrating that the response occurs within 4-6 hours of exposure.

Both *Hoxb4* and *Hoxd4* displayed identical rapid rostral shifts in expression of one rhombomere at 8.5 dpc. Similarly, at 9.5 dpc, they had an anterior shift of three rhombomeres (r6-r4) in the limits of expression, but the induction is dynamic and followed by a specific down regulation in r5. This suggests that there are differences in the molecular environment of r5 which are incompatible with sustained expression of the *Hoxb4* and *Hoxd4* genes, and might reflect a basic difference between odd- and even-numbered rhombomeres. The ability of the 3′ enhancers to mediate a response to RA and r6/7 neural expression that resembles the endogenous *Hoxb4* and *Hoxd4* genes, provides further support for the idea that both genes use common mechanisms in directing neural expression in the hindbrain (Gould et al., 1997). With respect to the RA response, it remains unclear if this is directly mediated by retinoid receptors, because there are no typical consensus RARE direct repeats (DR2 or DR5) in the enhancers, but there are several dispersed half sites.

The temporal window of the RA response of *Hoxa4* was similar to that of *Hoxb4* and *Hoxd4*, but the nature of the response was very different. While normal *Hoxa4* expression does not extend up to the r6/7 junction prior to 10.5 dpc, RA treatment prematurely resulted in r6/7 expression but there was no induction in more anterior rhombomeres. The neural enhancer that we identified 3′ to the *Hoxa4* also responds to RA in a similar manner to the endogenous gene, but the response was restricted only to the ventral regions of the neural tube. Hence, the *Hoxa4* 3′ enhancer is only capable of mediating part of the overall endogenous neural expression and RA response.

Thus, three members of the Hox group 4 paralogs and their 3′ neural enhancers have a conserved temporal window of sensitivity to exogenous retinoids that opens around 8.5-9.0 dpc and may be related to their positions in the Hox complexes. This is also similar to the RA response that we observed with a 5′ HOXD4 neural enhancer containing an RARE (Morrison et al., 1996). Once these genes are competent, their response to retinoids is rapid (within 4-6 hours in all cases). Indeed it would appear that, once a group 4 gene is able to respond to RA, it is as sensitive as the most 3′ genes, *Hoxb1* or *Hoxa1*. These results suggest that the colinear response of Hox genes to retinoids is not a simple function of variable thresholds or graded concentrations along the A-P axis as previously suggested (Dekker et al., 1992; Gaunt and Strachan, 1994, 1996; Simeone et al., 1991). Other cofactors or inhibitors appear to be involved in limiting the ability of the genes to respond directly or indirectly to RA. The molecular basis for these temporal windows of RA sensitivity is not known but it has been suggested that vertebrate members of the trithorax and polycomb groups might be involved in controlling the nature of the RA response and patterns of Hox genes (Coré et al., 1997).

Mesodermal regulation of *Hoxa4*, *Hoxb4* and *Hoxd4*

Despite their coincident expression up to the r6/7 junction in the hindbrain, the three group 4 paralogs, *Hoxa4*, *Hoxb4* and *Hoxd4*, have anterior boundaries of expression in somitic mesoderm that are offset from each other by a single somite. Previous analysis of *Hoxb4* indicated that the intron contained an enhancer that directed expression up to s6/7 (Whiting et al., 1991). Here we have shown that the 3′ flanking regions of the *Hoxd4* and *Hoxa4* genes contain mesodermal enhancers able to direct somitic expression which correspond to the endogenous genes (Fig. 7). In the case of *HOXD4*, there are two separate mesodermal enhancers flanking the neural element, each capable of mediating expression up to the proper s5/6 junction (Fig. 2F-M). For *Hoxa4*, we have mapped a single

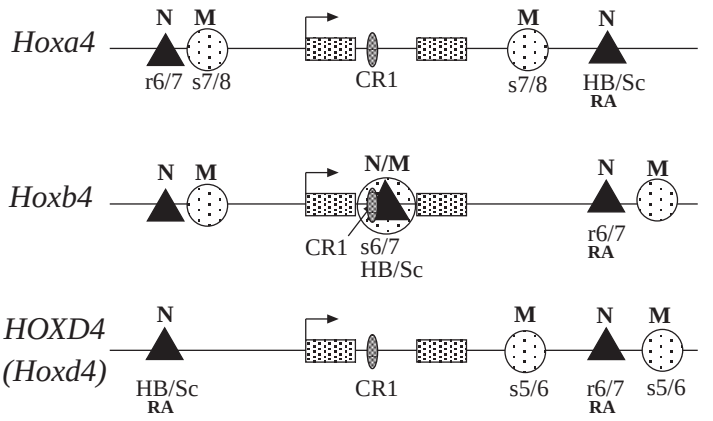

Fig. 7. Alignment of three Hox group 4 paralogous genes and their respective regulatory regions. The two exons and proximal promoters for each gene are indicated by the shaded rectangles and arrows. The black triangles indicate neural (N) regulatory elements, the stippled circles mesodermal (M) regulatory components and the hatched ellipse in the intron conserved region 1 (CR1). Below each is indicated the activity or anterior boundary of expression set by the control regions. s, somite; r, rhombomere; HB, hindbrain; Sc, spinal cord; RA, response to ectopic retinoids. Diagram also summarises data from previous studies on *Hoxb4* (Aparicio et al., 1995; Gould et al., 1997; Morrison et al., 1995; Whiting et al., 1991), *HOXD4* (Morrison et al., 1996) and *Hoxa4* (Behringer et al., 1993).

mesodermal enhancer 5′ to the neural element which directs expression up to the s7/8 junction. Regions 3′ to the neural element were not examined, so by analogy to *HOXD4* it remains possible that there are additional mesodermal control regions. The similar organisation between 3′ mesodermal control regions of *HOXD4* and *Hoxa4* led us to re-examine *Hoxb4*, and we also detected posterior somitic expression in a line containing the 3′ region A neural enhancer and have mapped a separate mesodermal control region flanking the minimal neural enhancer element CR3 (Fig. 7; unpublished observations). Therefore, these three group 4 paralogs have similarly positioned 3′ mesodermal enhancers, but each with a slightly different anterior boundary in somites. Sequence comparisons between the different mesodermal enhancers have not identified any extended blocks of sequence conservation but, if they are evolutionarily related, they may depend on a number of small dispersed motifs for activity.

Extended paralog comparisons

Given the apparent similarity in organisation of 3′ regulatory regions described here to what extent are additional control elements in the *Hoxa4*, *Hoxb4* and *Hoxd4* loci also conserved? Transgenic studies have mapped mesodermal and neural components 5′ of *Hoxa4* (Behringer et al., 1993) that are involved in regulating its expression. A 5′ neural region is required for late phase expression of *Hoxa4*, which reaches the r6/7 junction, whereas the 3′ region identified here mediates the earlier pattern. In contrast, the 5′ and 3′ mesodermal regions appear to mediate similar somite expression up to s7/8. Therefore, there is considerable overlap in the regulatory activities of the *Hoxa4* 5′ and 3′ enhancers (Fig. 7).

In the case of HOXD4, we previously characterised a 5′ enhancer containing an RARE that directed neural restricted expression and a response to RA (Morrison et al., 1996). Recently, in a parallel study of the mouse *Hoxd4* 5′ flanking regions, neural activity similar to that seen with the human *HOXD4* gene, and a mesodermal enhancer activity associated with the 5′ RARE was identified (Zhang et al., 1997). In further support of a general positioning of 5′ control elements in the group 4 genes, we have begun to analyse the 5′ flanking regions of the *Hoxb4* gene and also found neural and mesodermal enhancer activities that direct expression with anterior boundaries caudal to the endogenous gene (Fig. 7; S. Nonchev, J. Sharpe and R. K., unpublished). In addition to the 5′ and 3′ control regions, the intron of *Hoxb4* contains an enhancer (region C) with both neural and mesodermal activities (Whiting et al., 1991). This enhancer is capable of mediating mesodermal expression directed up to the s6/7 junction observed with the endogenous gene, neural domains with an anterior limit in the caudal hindbrain/spinal cord region. The presence of a highly conserved region (CR1) within the introns of *Hoxb4*, *Hoxd4* and *Hoxa4* in broad range of vertebrate species (Aparicio et al., 1995; Cianetti et al., 1990; Morrison et al., 1995) suggested that there might also be conserved regulatory activities in the introns of all the group 4 paralogs (Fig. 7). However, our analysis here on the *HOXD4* intron and CR1, and previous analysis on *Hoxa4* (Behringer et al., 1993), indicates that in contrast to *Hoxb4* neither of these introns have independent enhancer activities. Therefore, if CR1 has a general role in the group 4 paralogs, it may be through interaction/cooperation with additional 5′ or 3′ regulatory regions.

Fig. 7 summarises the relative positioning of regulatory activities in three group 4 paralogs. On the basis of the similar 5′ and 3′ distribution of neural and mesodermal enhancers and retinoid-responsive regions, it appears that there has been considerable conservation in the position of regulatory components of the group 4 paralogs during the duplication and divergence of the ancestral vertebrate Hox complex. Mesodermal regulation appears to be complex and variable between paralogs. The identification of multiple mesodermal-specific enhancers, each with the ability to regulate or impose the same or slightly different anterior limits, provides a future basis for examining the mechanisms that set anterior somite boundaries of Hox genes. With respect to neural control, the timing of segmental expression and organisation of neural regulatory components of *Hoxb4* and *Hoxd4* are very similar. Furthermore, as both the endogenous *Hoxb4* and *Hoxd4* genes are required for boundary maintenance of the *Hoxb4* r6/7 neural enhancer (Gould et al., 1997), this para-regulation (i.e. cross-regulation between paralogs) indicates the segmental regulatory mechanisms of these two genes are also linked. Since the expression of *Hoxa4* overlaps with *Hoxb4* and *Hoxd4* in regions posterior to the r6/7 boundary, cross talk involving all three of these paralogs may be critical for maintaining neural expression.

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