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In vivo functional analysis of the *Hoxa-1* 3′ retinoic acid response element (3′RARE)

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SUMMARY

Retinoids are essential for normal development and both deficiency and excess of retinoic acid (RA) are teratogenic. Retinoic acid response elements (RAREs) have been identified in *Hox* gene promoters suggesting that endogenous retinoids may be involved in the direct control of *Hox* gene patterning functions. In order to test this hypothesis, we have mutated the *Hoxa-1* 3′RARE using the Cre-loxP targeting strategy, and studied its functional role during mouse development. We find that this enhancer plays an important role in the early establishment of the *Hoxa-1* anterior expression boundary in the neural plate. This early disturbance in *Hoxa-1* activation results in rhombomere and cranial nerve abnormalities reminiscent of those obtained in the *Hoxa-1* total knockout, although their severity and penetrance are lower, thus providing strong evidence for direct control of *Hox* gene function by retinoids during normal development. Interestingly, we also find that the *Hoxa-1* expression response to RA treatment is not entirely controlled by the RARE, suggesting the existence of other retinoid-induced factors mediating the *Hoxa-1* response to RA and/or the presence of additional RAREs. Interestingly, although the RARE is not required for the spatiotemporal control of colinear expression of the *Hoxa* genes, it is absolutely required for correct *Hoxa-2* expression in rhombomere 5.

Key words: hindbrain, segmentation, rhombomeres, retinoids, *Hox*, gene targeting, mouse

INTRODUCTION

The hindbrain of the early mouse embryo is divided into seven compartments, or rhombomeres, which appear to be lineage restriction units (Fraser et al., 1990), since only a limited amount of cell mixing is allowed after the establishment of boundaries between rhombomeres (Birgbauer and Fraser, 1994). Later in development, this early metameric organisation is reflected in rhombomere-specific patterns of neuronal differentiation and distribution of neuronal types (Clarke and Lumsden, 1993), suggesting that rhombomeres are not just reiterated units of a uniform developmental plan, but that each of them displays a unique identity. Hindbrain neural crest cells contributing to cranial ganglia and branchial arches also display a segmental pattern of migration which correlates to their rhombomeric origin (Lumsden et al., 1991; Birgbauer et al., 1995).

Our understanding of how segmental specification is achieved along the rostrocaudal axis of the hindbrain is still in an early phase. Several classes of genes, including transcription factors such as the *Krox-20* gene and members of the *Hox* family, as well as several receptor tyrosine kinase (RTK) genes, have been found to be expressed in a segmental fashion in the vertebrate hindbrain (reviewed by Wilkinson, 1995), thus providing clues to the genetic basis of hindbrain segmentation and patterning. The *Hox* genes, in particular, are likely to be prime candidates for a role of selectors of rhombomeric identities, given their evolutionary conservation with the *Drosophila* homeotic genes, which specify segment identity in the fly. In the 8.5-9.5 day post-coitum (dpc) mouse embryo, *Hox* genes at the 3′ ends of the *Hoxa* and *Hoxb* clusters show overlapping expression domains from the caudal end of the neural tube up to sharp anterior boundaries mapping to distinct rhombomeric junctions. In addition, they are expressed in migrating neural crest cells and branchial arches, (reviewed by Krumlauf, 1993). These ordered expression patterns led to the suggestion that a '*Hox* code' might be used to specify segment phenotype and to pattern neural crest-derived craniofacial structures (Hunt et al., 1991).

Hox loss-of-function studies in the mouse have supported this prediction (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992; Rijli et al., 1993; Gendron-Maguire et al., 1993). For instance, targeted disruption of *Hoxa-1* resulted in defects of hindbrain rhombomeres 3 to 7, cranial nerve, and inner ear abnormalities (Mark et al., 1993; Carpenter et al., 1993). Detailed analysis of the mutant phenotypes by dye injection and use of other genes as molecular markers of hindbrain structures has revealed that r3 was apparently enlarged, r5 was either absent or reduced, r4 was reduced as well, and the organization of cranial nerves and neural crest derivatives was affected (Mark et al., 1993; Dollé et al., 1993; Carpenter et al., 1993).

But how is the code established? There are several lines of evidence pointing to retinoic acid (RA) as one of the molecules which might regulate the graded and colinear expression patterns of *Hox* genes within the plane of the hindbrain neuroepithelium. RA has been detected endogenously at gastrulation in zebrafish, *Xenopus*, chick, and mouse embryos, suggesting that it may be involved in the establishment of the anteroposterior axis (reviewed by Conlon, 1995). A quantitative balance of retinoids must be maintained during development, since deficiency or excess is teratogenic. The finding that RA could induce colinear *Hox* gene expression in cell lines (with 3′ *Hox* genes being more sensitive to RA than increasingly 5′ genes) (Simeone et al., 1990) suggested that some of the teratogenic effects of RA on antero-posterior (A-P) patterning in the embryo might be mediated by abnormal expression of 3′ *Hox* genes. Indeed, treatment of mouse embryos with RA leads to a more anterior expression of 3′ *Hox* genes of the paralogue group 1 and 2 (Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Marshall et al., 1992; Kessel, 1993; Wood et al., 1994), which is associated with homeotic transformation of the segmental identities of r2 and r3 into r4 and r5 (Marshall et al., 1992; Kessel, 1993). This phenotype can be partially reproduced by ubiquitous misexpression of *Hoxa-1* (Zhang et al., 1994).

Although the above results support a functional correlation between RA, *Hox* gene function, and neuronal organization in the developing hindbrain, a direct link has yet to be conclusively proved, and interpretation of the mechanism underlying the effects of RA treatment on segmentation/specification of hindbrain structures is difficult. The discovery of retinoic acid responsive elements (RAREs) in close proximity to the *Hoxa-1* (Langston and Gudas, 1992; Frash et al., 1995), *Hoxb-1* (Marshall et al., 1994; Studer et al., 1994), and *Hoxd-4* (Pöpperl and Featherstone, 1993; Morrison et al., 1996) promoters suggests that at least some of the teratogenic effects of RA might be mediated by the direct binding of retinoic acid receptors (RARs) to these enhancers. The presence of RAREs also suggests that endogenous retinoids may play a role in the direct control of *Hox* gene function during normal development.

In order to test this hypothesis, we decided to study the functional role of the *Hoxa-1* RARE in vivo. To this purpose, we used the Cre-loxP targeting strategy (Gu et al., 1993) to generate a mouse line carrying a targeted deletion of this enhancer. We find that the RARE is an important component of the regulation of *Hoxa-1*, particularly for the early establishment of its rostral expression boundary in the neural plate. The early disturbance of *Hoxa-1* activation in the anterior portion of its domain results in rhombomere and cranial nerve abnormalities reminiscent of those observed in *Hoxa-1* total knockout (*Hoxa-1*^{tot}) mutants (Mark et al., 1993; Carpenter et al., 1993), although they are much less severe and penetrant. These results provide the first evidence supporting a role of endogenous retinoids in the direct control of *Hox* gene function during normal development. Furthermore, we also demonstrate that the *Hoxa-1* response to RA administration is not entirely mediated by the RARE, suggesting the existence of retinoid-induced regulatory factors which might also be important for *Hoxa-1* expression during normal development, and/or the presence of alternative RA-responsive elements. Interestingly, the RARE mutation affects the normal expression of *Hoxa-2* in rhombomere 5, but not that of neighbouring genes of the *Hoxa* cluster.

MATERIALS AND METHODS

Targeted mutation of the *Hoxa-1* 3′RARE, Cre/loxP-mediated in vivo excision of the TKneo cassette, and generation of ΔRARE mutant mice

A deletion of just the 18 bp *Hoxa-1* 3′ RARE was initially obtained by performing site-directed mutagenesis using single-stranded DNA derived from p879, kindly provided by Dr T. Lufkin (see also Frash et al., 1995), using the oligonucleotide 5′-CCCCTGTTTAATCTCG-GTACCAGACGAGATGAATGC-3′ which introduces a *Kpn*I site at the place of the RARE. From this construct, a 3.5 kb *Bgl*II genomic fragment was subcloned into the *Bam*HI site of a pBluescript II SK+ (Stratagene) in which the *Kpn*I and *Sal*I sites had been previously destroyed (*Kpn*$^{-}$, *Sal*I^{-}). This construct, designated 551KS, was digested with Asp718 (a *Kpn*I isoschizomer) and the oligonucleotide 5′-GTACTGTCGACA-3′ was introduced, so that the *Kpn*I site was destroyed, a new *Sal*I site was created, and the original 18 bp spacing was reproduced in the *Hoxa-1* 3′ genomic region. This construct was designated ΔRARE construct (Fig. 1A). An *Xho*I fragment containing a fusion of the herpes simplex virus thymidine kinase (TK) and bacterial neomycin phosphotransferase (neo) gene driven by the phosphoglycerate promoter, and flanked by loxP sites (L-TKNEO-L), a kind gift of Dr P. Kastner, was inserted into the *Sal*I site of the ΔRARE construct, to obtain a targeting construct (Fig. 1A). Electroporation of ES cells with this targeting vector and selection of neomycin resistant clones was done as described Lufkin et al. (1991). DNA from individual G418-resistant colonies was analyzed by genomic Southern blotting using 5′ (Probe 1) and 3′ (Probe 2) external probes (Fig. 1A), as well as a neo probe (not shown). Probe 1 and Probe 2 correspond to a *Bam*HI-*Bgl*II 0.5 kb fragment and a *Bgl*II-*Kpn*I 1.0 kb fragment, respectively, derived from p879 (Frash et al., 1995). Out of several independent neo-resistant colonies carrying the targeted mutation, one (LW97) was injected into C57BL/6 blastocysts to derive chimeric mice, as described by Lufkin et al. (1991). Male founder chimeras were crossed to C57BL/6 females and germ line transmission of the mutation was obtained (Fig. 1A,B, HR(I) or ΔRARE$^{+/-(LNL)}$ allele).

A 640 bp *Eco*RI fragment containing the CMV promoter, derived from the pCMV-neo plasmid (Boshart et al., 1985), was inserted into the *Eco*RI site of pBluescript II SK– to obtain pJB7. A 1.5 kb *Pst*I fragment containing the Cre gene (Metzger et al., 1995) equipped with a polyadenylation site, was inserted into the *Pst*I site of the pJB7 polylinker to yield the CMV-Cre construct designated pJB8. The *Xho*I-*Sma*I fragment of pJB8 was injected into 1-cell mouse embryos isolated from superovulated B6/SJL females, and the injected embryos were reimplanted into the oviducts of pseudopregnant Swiss Webster females. 2 transgenic mice were identified by Southern blotting analysis of DNA from 2-week-old offspring tail biopsies, using a 1.3 kb *Bam*HI probe derived from pJB8, 1 stable line was established which transmitted the transgene through the germline, and homozygotes for the CMV-Cre transgene (Cre$^{+/+}$) were generated.

A Cre$^{+/+}$ male was crossed with 2 *Hoxa-1*$^{\Delta RARE+/-(LNL)}$ females and a total of 14 (*Hoxa-1*$^{\Delta RARE+/-(L)}$, Cre$^{+/-}$) viable littermates were obtained which were analysed by genomic Southern blotting on tail-biopsy DNA using Probe 1, Probe 2, and a neo probe (not shown). In all of 14 siblings (100%) the selectable marker was excised. Genomic PCR confirmed the expected recombination event and the presence of a single loxP site in place of the L-TKneo-L cassette. DNAs from different tissues, dissected from a randomly chosen adult animal, were analysed and no mosaicism of the recombination event was detected. 4 double heterozygous males were crossed with wild type 129/Sv females and *Hoxa-1*$^{\Delta RARE+/-(L)}$ mice devoid of the Cre transgene were derived, thereafter designated as ΔRARE$^{+/-}$.

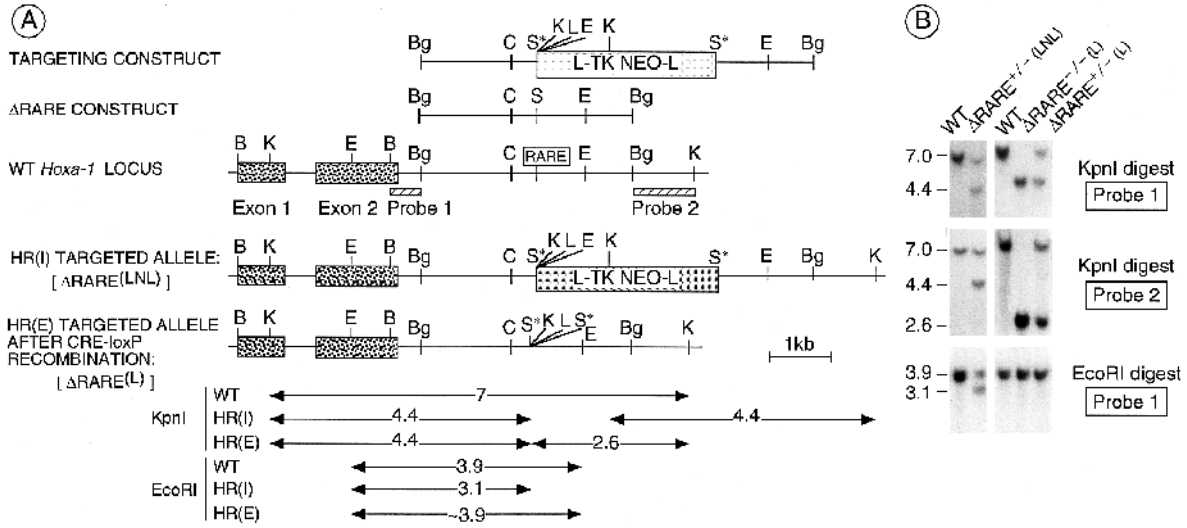

Fig. 1. Targeted disruption of the *Hoxa-1* 3′ RARE by homologous recombination using the Cre-*loxP* system. (A) Diagram showing the wild-type (WT) *Hoxa-1* locus, the targeting construct, and the mutated locus after first recombination event in ES cells leading to integration [HR(I)], and subsequent CRE-mediated in vivo excision [HR(E)] of the loxP-flanked TKneo cassette. The sizes of the restriction fragments that are required to distinguish between the two recombination events by Southern blot analysis using probes 1 or 2, are indicated below (in kilobases). See Materials and Methods for details. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; L, LoxP site; S, *Sal*I. (B) Genomic Southern blots of tail DNA from mice of various ΔRARE genotypes (indicated above) using Probes 1 or 2 shown in A and digested as indicated.

Genotyping of the ΔRARE mice and embryos at different developmental stages was either performed by Southern blotting (Fig. 1A,B), or by PCR (not shown). The oligonucleotides 5′-GGTCTTCT-GTTCTGGTCTCTGAGGGGCAGA-3′ and 5′-ACCTGAGT-GAATGGGCAGAAAAACTCGAGG-3′ were used in a standard 30 cycle PCR reaction. The amplified mutant fragment is about 60 bp longer than the wild-type fragment (about 700 bp), due to the presence of a few restriction sites surrounding the loxP site, derived from the polylinker of the L-TKneo-L cassette. DNA for PCR reaction was extracted by overnight digestion at 60°C of tail-biopsies, yolk sacs, or whole embryos in a buffer containing 100 μg/ml proteinase K (PK) (Boehringer), 0.45% NP-40, and 0.45% Tween-20. After PK heat inactivation, and DNA denaturation, 1/10 of the sample volume was used directly for PCR reactions.

RNA whole-mount and tissue section hybridisation and immunohistochemistry

Whole-mount and tissue section in situ RNA hybridisation procedures were carried out as described by Conlon and Rossant (1992) and Dollé et al. (1993), respectively. The various probes used in this study have been described (*Hoxa-1*, Duboule and Dollé, 1989; *Krox-20*, Wilkinson et al., 1989; *Hoxa-3*, *Hoxb-3*, *Hoxa-2*, *Hoxb-2*, *Hoxb-1*, Hunt et al., 1991; *Otx2*, Ang et al., 1994, a kind gift from Dr S. L. Ang). Whole-mount immunohistochemistry using the neurofilament-specific monoclonal antibody 2H3 was performed as described by Mark et al. (1993).

Retinoic acid treatments of ΔRARE mice

Homozygous $\Delta RARE^{-/-}$ males were crossed to heterozygous mutant females, and pregnant females were administered all-*trans* retinoic acid (RA) essentially as described by Conlon and Rossant (1992). Briefly, a 50 mg/ml RA stock solution in DMSO was diluted in vegetable oil to a final concentration of 2 mg/ml, and 0.3 ml delivered by oral gavage (final dose approximately 20 mg/kg of body weight). 12 am on the day of plug observation was taken as day 0.5. Mice were treated either for 5 hours or 7 hours at 7.25 dpc (7 am on embryonic day 7) and 7.5 dpc (12 am on embryonic day 7).

RESULTS

Targeted mutation of the *Hoxa-1* 3′RARE and generation of mutant mice

A 3.5 kb genomic fragment containing the *Hoxa-1* 3′RARE (RARE) was used to construct a targeting vector (Fig. 1A). After deletion by site-directed mutagenesis of the RARE and replacement by a SalI restriction site, a neomycin-thymidine kinase fusion gene driven by the phosphoglycerate kinase promoter (PGK), equipped with a polyadenylation site, and flanked by two loxP sites (loxP-neoTK-loxP, LNL) was cloned into the SalI site to obtain the targeting vector (Fig. 1A). The polylinker of the LNL cassette contained a *Kpn*I site external to the 5′ loxP site and a *Eco*RI site was present in the neo gene (Fig. 1A). These sites have been used as diagnostic sites in Southern blot analysis (Fig. 1B). Upon electroporation in 129Sv ES cells several targeted clones were obtained (data not shown), one of which was injected into C57BL/6 recipient blastocysts. Male founder chimeras were crossed to C57BL/6 females and the *Hoxa-1*$^{\Delta RARE(LNL)}$ mutation [HR(I)] was passed through the germline (Fig.1A,B). In order to obtain in vivo Cre/loxP-mediated excision of the neoTK cassette, we generated a transgenic mouse line constitutively expressing the Cre gene under the control of the cytomegalovirus promoter (CMV) (see Materials and Methods). Upon crossing of *Hoxa-1*$^{\Delta RARE+/-(LNL)}$ females with homozygous males for the Cre-expressing transgene ($Cre^{+/+}$), viable progeny were obtained (*Hoxa-1*$^{\Delta RARE+/-(L)}$, $Cre^{+/-}$) in which the selectable marker was replaced by a single loxP site in place of the RARE at the *Hoxa-1* locus (Fig. 1A). Southern blot analysis with suitable probes (Fig. 1B, and data not shown) and genomic PCR sequencing (data not shown) from tail DNA biopsies confirmed the expected recombination event for all the siblings (see Methods). Double heterozygous males were crossed with wild-

type 129/Sv females to obtain germline transmission of the mutation (see Methods) and to derive *Hoxa-1*$^{\Delta RARE+/-(L)}$ ($\Delta RARE^{+/-}$) heterozygotes devoid of the Cre transgene.

Unlike the *Hoxa-1* total knockout (*Hoxa-1*^{tot}; Lufkin et al., 1991; Chisaka et al., 1992) in which homozygotes die at birth, $\Delta RARE^{-/-}$ were viable and externally normal, indicating that in these homozygous embryos the overall *Hoxa-1* expression levels remain sufficient for survival and, therefore, that *Hoxa-1* expression might be controlled by other regulatory regions in addition to the RARE. Both homozygous males and females were fertile and had a life span comparable to that of wild-type mice.

Hoxa-1 expression pattern in ΔRARE mutants

The main effect of a RARE mutation in the context of a 12 kb *Hoxa-1*/*lacZ* transgene construct, which recapitulated the entire *Hoxa-1* expression pattern in 9.5 days post-coitum (dpc) transgenic embryos, was the loss of *lacZ* expression in the neural tube caudal to rhombomere 4 and a quantitative down-regulation of β-galactosidase activity in paraxial mesoderm (Frash et al., 1995). However, the above analysis did not provide insights as to whether the RARE would be required for the activation of *Hoxa-1* at early gastrulation and/or for the establishment of its anterior expression boundary in the developing hindbrain, where *Hoxa-1* performs its main function (Mark et al., 1993; Carpenter et al., 1993).

To investigate the role played by the RARE on the spatio-temporal control of expression of *Hoxa-1* within its normal genomic environment, we performed whole-mount in situ hybridization analysis of 7.5 to 8.5 dpc ΔRARE mutant and wild-type (WT) embryos (Fig. 2, and data not shown). The normal *Hoxa-1* expression pattern has been described in detail (Baron et al., 1987; Sundin et al., 1990; Murphy and Hill, 1991). At an early gastrulation stage, the gene is first activated at the posterior end of the embryo within the primitive streak, and subsequently its expression spreads anteriorly both in mesoderm and overlying neurectoderm. By headfold stage (7.5-7.75 dpc), *Hoxa-1* expression has reached a sharp anterior boundary coinciding with the presumptive r3/r4 border. At about 8.5 dpc, *Hoxa-1* expression starts retreating caudally and, in the neurectoderm, it is no longer detected in the hindbrain but only in the spinal cord. Dynamic stage-specific alterations of the *Hoxa-1* expression pattern were found in the homozygous mutants (Fig. 2). In 7.5 dpc mutants, abnormalities in the antero-posterior extent of the *Hoxa-1* expression domain were observed, particularly at its anterior boundary. In addition, the overall *Hoxa-1* expression levels were lower in mutants than in WT embryos both in the neurectoderm and mesoderm, as confirmed by transverse sections (not shown). For example, in the mutant shown in Fig. 2B, the *Hoxa-1* expression domain extended from the posterior end of the embryo towards the node region (no), but without reaching the normal (WT) anterior boundary (compare arrows in Fig. 2A,B). However, in slightly older embryos the *Hoxa-1* expression domain in the mutant neurectoderm extended anteriorly to the node, although the expression level was lower in the mutants and the boundary less defined than in WT embryos (compare Fig. 2C,D; and data not shown). In order to assess whether the *Hoxa-1* anterior expression boundary in the mutants was comparable to that of WT embryos a double

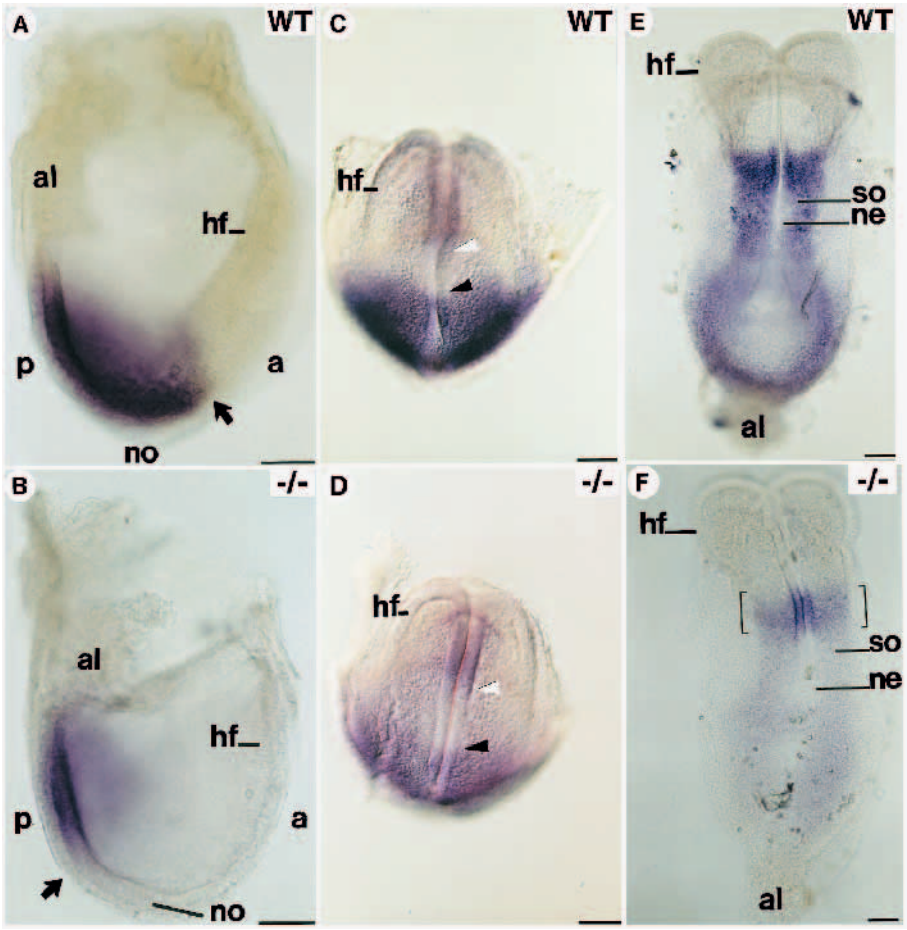

Fig. 2. *Hoxa-1* expression pattern in ΔRARE mutant embryos.Whole-mount in situ analysis of *Hoxa-1* expression patterns in WT embryos (A,C,E) and $\Delta RARE^{-/-}$ embryos (B,D,F) at 7.5 dpc (A,B, lateral views), 7.75 dpc (C,D, views from the ventral side of the headfolds) and 8.0 dpc (E,F, dorsal views). (A,B) *Hoxa-1* transcripts have reached their definitive anterior boundary in the WT (arrow in A), but not in the mutant embryo (arrow in B). (C, D) Embryos double-stained by *Hoxa-1* and *Otx-2* probes. The posterior limit of *Otx-2* expression at the level of the presumptive midbrain-hindbrain boundary is indicated by a white arrowhead, and the *Hoxa-1* anterior expression boundary is indicated by a black arrowhead in WT (C) and mutant (D) embryos. Note that the gap between *Otx2* and *Hoxa-1* expression boundaries (i.e. the distance between white and black arrowhead) is wider in the mutant (D) than in the WT (C). Note also that *Hoxa-1* expression levels are lower in the mutant (D). (E,F) Overall *Hoxa-1* expression levels appear lower in mutant (F) than in WT (E); note, however, that stronger expression is detected in the presumptive r4-r6 region of the mutant (brackets in F) compared to more posterior regions. a, anterior; al, allantois; hf, headfold; ne, neurectoderm; no, node; p, posterior; so, somitic mesoderm. Scale bars, 100 μm.

hybridisation was carried out, using a *Hoxa-1* and an *Otx2* probe on the same embryo (Fig. 2C,D). At this developmental stage, the posterior limit of *Otx2* expression is at the level of the presumptive midbrain/hindbrain boundary (Simeone et al., 1993; Ang et al., 1994), thus providing a landmark point to compare the *Hoxa-1* border between the mutant and WT embryos. The gap between the anterior *Hoxa-1* (black arrowhead) and posterior *Otx2* (white arrowhead) expression boundaries was wider in homozygous mutant than in WT embryos (compare Fig. 2C,D). At 7.75 dpc (headfold-presomite stage), the *Hoxa-1* anterior expression boundary was better defined and the mutant embryo transcript levels more similar to those observed in WT embryos (data not shown). Thus, at this early embryonic stages the main effects of the RARE deletion appear to be a reduction in the overall *Hoxa-1* expression levels, and a delay in the establishment of its anterior boundary in the presumptive hindbrain.

In 3-4 somite (8.0 dpc) homozygous mutant embryos (4 out of 4 tested), significant down-regulation of the *Hoxa-1* transcript signal was observed in the somitic and pre-somitic mesoderm, as compared to WT embryos (compare Fig. 2E,F ; and data not shown). Interestingly, in the developing neural tube of these mutants, *Hoxa-1* expression was restricted to a stripe (brackets, Fig. 2F), with a rostral limit roughly coinciding with the *Hoxa-1* anterior expression boundary of WT embryos (i.e. the presumptive r3/r4 junction; Murphy and Hill, 1991) (compare Fig. 2E,F), and a less defined posterior boundary slightly rostral to the axial level of the first somite (so; Fig. 2F), which is in register with the presumptive r7 (Grapin-Botton et al., 1995). Therefore, this domain of *Hoxa-1* expression most likely encompasses the presumptive r4-r6 region, which corresponds to the *Hoxa-1* functional domain (Mark et al., 1993; Carpenter et al., 1993). Posteriorly to this stripe, substantial down-regulation of *Hoxa-1* expression was observed in the mutant neurectoderm (ne; compare Fig. 2E,F). It is interesting to note that in WT embryos at early somite stages the *Hoxa-1* transcript signal was already found slightly higher in the presumptive r4-r6 region than posteriorly (Fig.

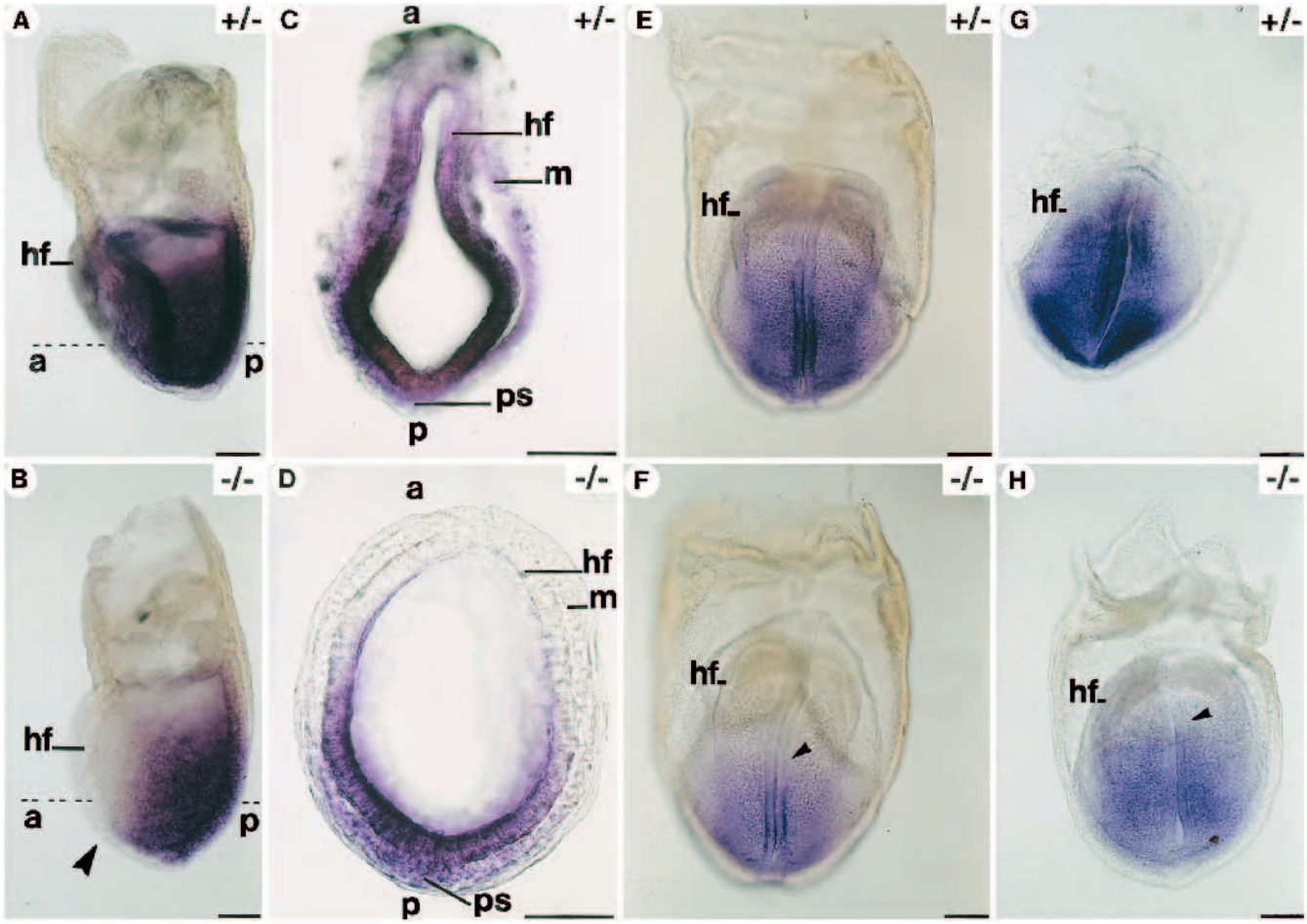

Fig. 3. Response of ΔRARE mutants to exogenous retinoic acid. Whole-mount in situ analysis of *Hoxa-1* transcript distribution in heterozygous (A,C,E,G) and homozygous ΔRARE mutant embryos (B,D,F,H) treated with RA for 5 hours at 7.25 dpc (A-D) and 7.5 dpc (E,F), or for 7 hours at 7.25 dpc (G,H). A,B are lateral views, and E-H are views from the ventral side of the headfolds. (C,D) Cross-sections of the embryos shown in A and B, respectively, at the level indicated by the dotted lines. Note that the RA-induced shift of the *Hoxa-1* expression boundary is less anterior in homozygous (arrowheads in B,F,H) than in heterozygous (A,E,G) mutants. However, the *Hoxa-1* expression boundary in RA-treated homozygotes appear more rostral than that of untreated $\Delta RARE^{-/-}$ mutants (e.g. compare F, H with Fig 2D), indicating a partial *Hoxa-1* expression response to RA administration in the homozygotes. a, anterior; hf, headfold; m, mesoderm; p, posterior; ps, primitive streak. Scale bars, 100 μm.

2E; and data not shown), but in the mutants this difference was greatly exacerbated (Fig. 2F). In 8.25-8.5 dpc WT embryos, the *Hoxa-1* expression domain progressively retreats caudally (Murphy and Hill, 1991; and data not shown). At these developmental stages, both mutant and WT embryos showed similar *Hoxa-1* expression patterns, although overall transcript levels were lower in the mutants (data not shown).

A partial *Hoxa-1* expression response to exogenous retinoic acid is observed in ΔRARE mutants

The RARE had been previously shown to confere retinoic acid (RA) inducibility to the *Hoxa-1* gene in cell culture studies (Langston and Gudas, 1992). In addition, Frash et al. (1995) have shown that the *Hoxa-1* enhancer III, containing the RARE, responds to exogenous RA treatment in *lacZ* transgenic embryos. However, transgenic constructs in which only the RARE had been mutated in the context of the *Hoxa-1* promoter were not tested for their responsiveness to RA, raising the question whether the RARE is actually mediating the *Hoxa-1* expression response to exogenous RA in vivo.

We examined the *Hoxa-1* expression pattern in ΔRARE heterozygous and homozygous mutant embryos treated with RA for 5 hours at 7.25 dpc (Fig. 3A-D) and 7.5 dpc (Fig. 3E,F) and for 7 hours at 7.25 dpc (Fig. 3G,H). In heterozygous mutants (as in WT embryos, not shown), there was an anterior shift in expression boundaries within both the neural fold and mesoderm, and an increase in overall expression levels (Fig. 3A,C,E,G). In their homozygous littermates, the expression boundary was less anteriorly shifted (arrowheads) and *Hoxa-1* expression levels were lower (Fig. 3B,D,F,H). Nevertheless, the *Hoxa-1* expression boundary in RA-treated homozygotes was more rostral than in untreated homozygous mutants at comparable developmental stages (compare, for instance, Figs 3F,H with 2D), indicating the occurrence of a partial *Hoxa-1* expression response to RA treatment in $\Delta RARE^{-/-}$ homozygotes.

Analysis of ΔRARE mutant hindbrain with molecular markers reveals rhombomere abnormalities

The *Hoxa-1* targeted disruption (*Hoxa-1*^{tot}) resulted in an altered hindbrain segmentation pattern at 9.5 dpc (Mark et al., 1993; Carpenter et al., 1993). Whole-mount in situ hybridisation analysis on 8.5-8.75 dpc *Hoxa-1*^{tot} homozygous embryos using a *Krox-20* antisense probe as an r3/r5 molecular marker, revealed that the pre-r3 hybridisation signal was less intense and wider than in the controls (Carpenter et al., 1993; our unpublished data). In addition, the pre-r5 band of *Krox-20* expression was reported to be either missing (Carpenter et al., 1993) or drastically reduced to a few patches of *Krox-20*-expressing cells (Dollé et al., 1993; our unpublished data). Rhombomere 4 was also altered in the *Hoxa-1*^{tot} mutants, as homozygous 9.5 dpc embryos showed reduced levels of *Hoxb-1* expression with less sharp expression boundaries (Dollé et al., 1993; Carpenter et al., 1993). Alteration of the position of *Hoxb-1*-expressing cells was also observed in the *Hoxa-1*^{tot} mutants, and patches of cells not expressing *Hoxb-1* were interspersed with labeled cells (Dollé et al., 1993; Carpenter et al., 1993).

We therefore investigated whether the spatiotemporally restricted alterations of the *Hoxa-1* expression pattern observed in the neurectoderm of $\Delta RARE^{-/-}$ mutants, particularly at the anterior part of its expression domain, might result in hindbrain malformations and correlate with altered expression patterns of *Krox-20* and/or *Hoxb-1* (Fig. 4, and data not shown). Whole-mount in situ hybridizations were carried out with the *Krox-20* probe at the 9-12 somite stage on WT (or heterozygous embryos, which were identical to WT) and homozygous embryos. As expected, in WT embryos *Krox-20* expression in pre-r3 and pre-r5 exhibited sharp boundaries, with expression levels in pre-r3 lower than in pre-r5, and the two segments being approximately the same size (Fig. 4A,C; see also Irving et al., 1996). In 5 out of 15 mutant embryos (30%) both the *Krox-20* expression boundaries and the size of pre-r3 and pre-r5 were altered (Fig. 4B,D; and data not shown). The pre-r3 band was wider and transcript levels lower than in WT (compare Fig. 4A,B). The width of the pre-r5 band appeared to be reduced, and both anterior and posterior boundaries to be less sharp than in WT embryos (compare Fig. 4A,B and C,D). Interestingly, when the experiment was performed at a slightly earlier stage (7-8 somites) *Krox-20* expression was found to be already altered in 3 out of 5 homozygotes (data not shown), suggesting that the penetrance of the pre-r3/r5 alterations may be stage-dependent. On the other hand, the gap between pre-r3 and pre-r5 always appeared unaffected (compare Fig. 4C and D; and data not shown). Furthermore, hybridisation with a *Hoxb-1* probe at 6-10 and 20-25 somite stages showed an extent of r4 staining in homozygotes similar to that of WT or heterozygous embryos (data not shown).

Cranial nerve defects in ΔRARE mutants

The *Hoxa-1*^{tot} targeted disruption resulted in abnormalities of hindbrain cranial nerves, including the glossopharyngeal nerve (cranial nerve IX) (Mark et al., 1993; Carpenter et al., 1993). To investigate cranial nerves and ganglia in ΔRARE mutants, 10.5 dpc homozygote, heterozygote, and wild-type embryos were assayed by whole-mount immunohistochemistry with an antineurofilament antibody. In 3 out of 23 (13%) homozygous mutants, the proximal portion of the glossopharyngeal nerve (n9) was unilaterally absent (bracket, Fig. 5B). This agenesis was also observed in 1 out of 58 (1.7%) heterozygous mutants, but never in wild-type embryos (out of a total of 31 embryos). The same defect has been observed in the *Hoxa-1*^{tot} homozygote mutants (Mark et al., 1993), although with a higher penetrance. Thus, at least some aspects of *Hoxa-1* function in cranial nerve formation during normal development appear to require the integrity of the RARE, although its presence is clearly not crucial.

Hoxa-2 expression is altered in the hindbrain of ΔRARE homozygotes

Hox genes show ordered expression domains in the hindbrain of a 9.0-9.5 dpc embryo, with anterior expression boundaries mapping at the junction between distinctive rhombomeres (reviewed by Krumlauf, 1993). For instance, *Hoxa-2* rostral boundary maps at the r1/r2 boundary (Krumlauf, 1993; Dollé et al., 1993; Prince and Lumsden, 1994), *Hoxa-3* at the r4/r5, and *Hoxa-4* at the r6/r7 boundaries (Krumlauf, 1993 and refs. therein). Paralogous genes from the *Hoxb* cluster display the same anterior expression cutoffs, with the exception of *Hoxb-2* whose boundary is at the r2/r3 junction (Hunt et al., 1991). Interestingly, variations in the expression levels in the hindbrain are seen between paralogous genes: for instance,

Hoxb-2 expression is strong in r3, r4, and r5, while *Hoxa-2* expression is maximal only in r3 and r5 at 9.0 dpc (Hunt et al., 1991).

We investigated whether the mutation of the *Hoxa-1* RARE might influence the expression pattern of neighbor genes of the *Hoxa* complex (*Hoxa-2* and *Hoxa-3*) as compared to that of paralogous genes of the *Hoxb* cluster (*Hoxb-2* and *Hoxb-3*) in the hindbrain of 9.0 dpc $\Delta RARE^{-/-}$ mutant embryos (Fig. 6C; and data not shown). *Hoxb-2*, *Hoxb-3*, and *Hoxa-3* expression patterns and levels were similar to those of WT and $\Delta RARE^{+/-}$ embryos (compare Fig. 6C with A,B; and data not shown). However, in 5 out of 5 homozygous mutants *Hoxa-2* transcripts levels in r5 (arrowhead) were lower than in r3 (arrow in C), and comparable to those of r4 and r6. The lower *Hoxa-2* expression levels in r5 did not appear to be due to a developmental delay, since in the same homozygotes *Hoxb-2* expression levels were as strong in r3, r4, and r5 as in WT and heterozygous embryos (compare Fig. 6C with A,B; and data not shown).

DISCUSSION

Understanding how *Hox* genes establish their coordinated and spatially restricted expression patterns along the antero-posterior axis of the embryo is a major challenge. Several lines of evidence indicate that retinoic acid (RA) could be involved in regulating the graded and colinear expression patterns of *Hox* genes within the plane of the hindbrain neuroepithelium (see Introduction). We have used here the Cre-LoxP targeting strategy to study the functional role of the *Hoxa-1* RARE enhancer (Langston and Gudas, 1992). Although the RARE deletion does not abolish *Hoxa-1* expression, our results show that this enhancer plays an important role in the control of *Hoxa-1* expression. We also find that the *Hoxa-1* expression response to RA administration is decreased but not abolished by the RARE deletion, suggesting that other retinoid-induced regulatory factors might also be involved in *Hoxa-1* expression during normal development and/or the presence of additional RA-responsive element(s) within the *Hoxa-1* locus. Furthermore, the rhombomere and cranial nerve abnormalities observed in the mutants provide the first evidence in support of a role of endogenous retinoids in the direct control of *Hox* gene function in patterning. Interestingly, within the developing hindbrain, the *Hoxa-1* $\Delta RARE^{-/-}$ mutation affects the expression of *Hoxa-2*, but not of other neighboring genes of the *Hoxa* cluster.

The establishment of the *Hoxa-1* rostral expression boundary is delayed in ΔRARE mutants

Hox gene expression is first turned on in the posterior part of the embryo, at the late primitive streak stage (Frohman et al., 1990; Murphy and Hill, 1991; Sundin and Eichele, 1992; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994). Each *Hox* gene expression domain is then established by a caudal-to-rostral spreading along and lateral to the primitive streak until the definitive anterior boundary is reached. This forward spreading of expression occurs independently of cell lineage and of cell migration, both in mesoderm and ectoderm (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994). Once the anterior limit of expression is reached (rostrally to the node), its relative position along the antero-posterior (A-P) axis is maintained through subsequent embryo elongation and growth (e.g. Murphy and Hill, 1991), and *Hox* expression is clonally transmitted from one cell generation to the next. The existence of a morphogen as the primary activator of *Hox* expression has been postulated (Gaunt and Strachan, 1994; Grapin-Botton et al., 1995), but there is still very little known about the nature of the signal(s) involved in the establishment of the *Hox* expression domains and of their anterior boundaries. It has been suggested that retinoic acid could play this role (Conlon, 1995) since it is enriched at gastrulation in the mouse and chick Hensen's node and present in a posterior-to-anterior concentration gradient in the early *Xenopus* embryo (Hogan et al., 1992; Chen et al., 1992, 1994). In addition, *Hox* genes are responsive to RA treatment of the embryo (Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Marshall et al., 1992; Kessel, 1993; Woods et al., 1994). The discovery of enhancers containing retinoic acid responsive elements (RAREs) in the context of the *Hoxa-1* (Langston and Gudas, 1992; Frash et al., 1995), *Hoxb-1* (Marshall et al., 1994; Studer et al., 1994), and *Hoxd-4* (Pöpperl and Featherstone, 1993; Morrison et al., 1996) promoters further supported the idea of a direct link between RA and the establishment of *Hox* gene expression pattern along the rostrocaudal axis during normal embryo development.

The results of our analysis of the *Hoxa-1* expression pattern between 7.5 and 8.5 dpc in *Hoxa-1* $\Delta RARE^{-/-}$ embryos are summarised in Fig. 7. At the earliest stage analysed (headfold stage), we find that *Hoxa-1* is properly activated in the posterior-most part of the embryo, although at lower levels, but its expression domain does not spread as rostrally as in the WT embryo (Figs 2B,D and 7). At later stages, the anterior limit of the expression domain is eventually reached (late headfold-presomite stage), but *Hoxa-1* expression levels remain lower in the homozygous mutants than in WT embryos (Fig 7, and data not shown). We propose that the RARE mutation results in a delay in the posterior-to-anterior spreading of the *Hoxa-1* expression domain and in the setting of its anterior boundary in the neural plate of the early embryo. Although this finding is entirely consistent with the RARE being required for *Hoxa-1* expression along the axis, it does not provide any information, at the present time, to be able to discriminate among proposed models of RA action on axial patterning (Hogan et al., 1992; Chen et al., 1994; Conlon, 1995).

At early somite stage, the lower *Hoxa-1* expression in the presumptive r4-r6 domain of the mutants indicates that the RARE is not critically required for the maintenance of expression in this region, although this enhancer appears to be absolutely required for efficient *Hoxa-1* expression mostly in more posterior regions of the hindbrain and in the spinal cord (Figs 2F, 7). These results are in keeping with those obtained by Frash et al. (1995) who analyzed the *Hoxa-1* regulatory regions in *lacZ* transgenic constructs. Mutations of the 3′RARE in the context of a 12 kb *Hoxa-1*/*lacZ* construct resulted in loss of β-gal staining in the posterior hindbrain and in the spinal cord of transgenic embryos, whereas expression in r4 was shown to be independently mediated by an independent enhancer located in the *Hoxa-2* locus (Frash et al., 1995). The finding that the *Hoxa-1* response to exogenous RA is only

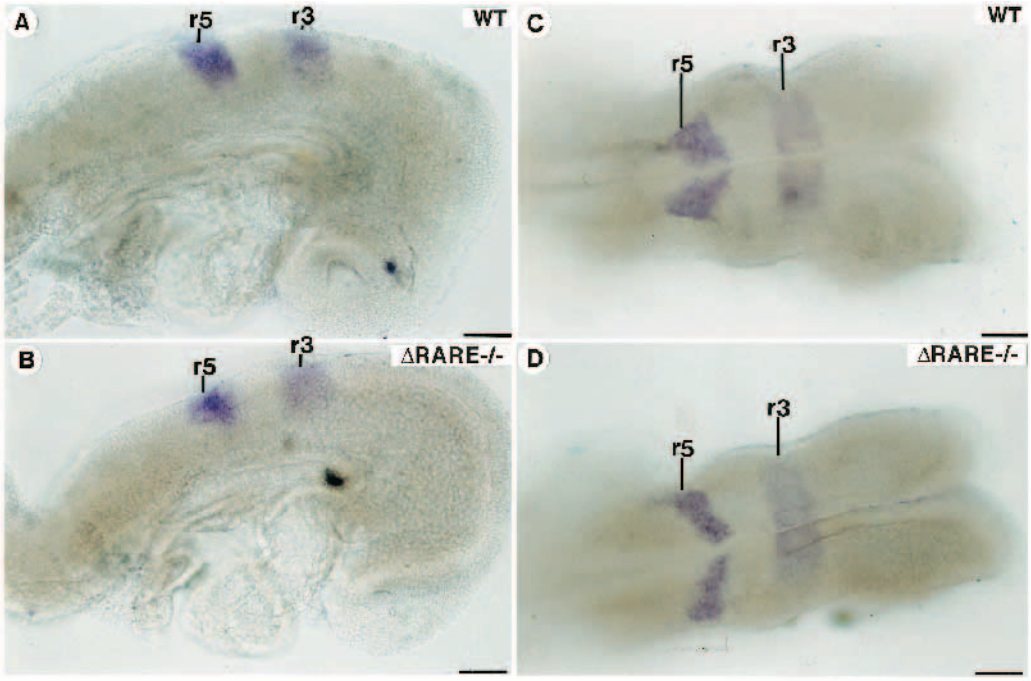

Fig. 4. Hindbrain abnormalities in ΔRARE mutants. Whole-mount in situ analysis of *Krox-20* expression in WT embryos (A,C), ΔRARE$^{-/-}$ embryos (B,D) at 8.75 dpc. (A,B) Lateral and (C,D) dorsal views of the same embryos showing an enlarged, although weaker, expression domain in pre-r3 (r3) and a reduced expression domain in pre-r5 (r5) of mutant (B,D) versus the WT (A,C) embryo. Scale bars, 100 μm.

partially impaired in ΔRARE$^{-/-}$ mutants (Fig. 3), raises the possibility that the *Hoxa-1* expression observed in untreated homozygous mutants might still be under the control of endogenous retinoids, and that RA-induced transcription factor(s) (see below) might bind to the additional *Hoxa-1* enhancer(s) identified in the study of Frash et al. (1995). In this respect, it will be interesting to analyse the *Hoxa-1* expression pattern in RA-deficient mouse embryos.

The *Hoxa-1* expression response to (exogenous) RA is decreased but not abolished in ΔRARE mutants

An interesting outcome of the present study is that residual RA-mediated *Hoxa-1* ectopic activation is observed upon RA treatment of homozygous embryos (Fig. 3). Only one DNAse I hypersensitive site, in which the RARE was identified, was found in about 20 kb of genomic DNA surrounding the *Hoxa-1* locus, upon RA treatment of F9 cells (Langston and Gudas, 1992). Thus, the presence of additional RAREs appears unlikely, although this possibility cannot be excluded.

Other RA-induced regulatory factors may account for the *Hoxa-1* expression response to RA observed in the mutants. For example, *Hoxa-1* shares several features of its early expression pattern with its paralogue *Hoxb-1*, both spatially and temporally (Frohman et al., 1990; Murphy and Hill, 1991). Furthermore, *Hoxb-1* ectopic expression in the rostral headfold is observed upon RA administration at gastrulation, concomitant to that of *Hoxa-1* as well as of other 3′*Hox* genes (Marshall et al., 1992; Kessel, 1993), and evidence for cross-regulation between the paralogous genes *Hoxa-1* and *Hoxb-1* has been provided in transgenic mice where *Hoxa-1* was ectopically expressed (Zhang et al., 1994). Other RA-inducible transcription factors such as AP-2 (Mitchell et al., 1991) and AP-2.2 (Chazaud et al., 1996) may also mediate the induction of *Hoxa-1* gene expression in response to retinoids, since their expression patterns in the hindbrain and cranial neural crest

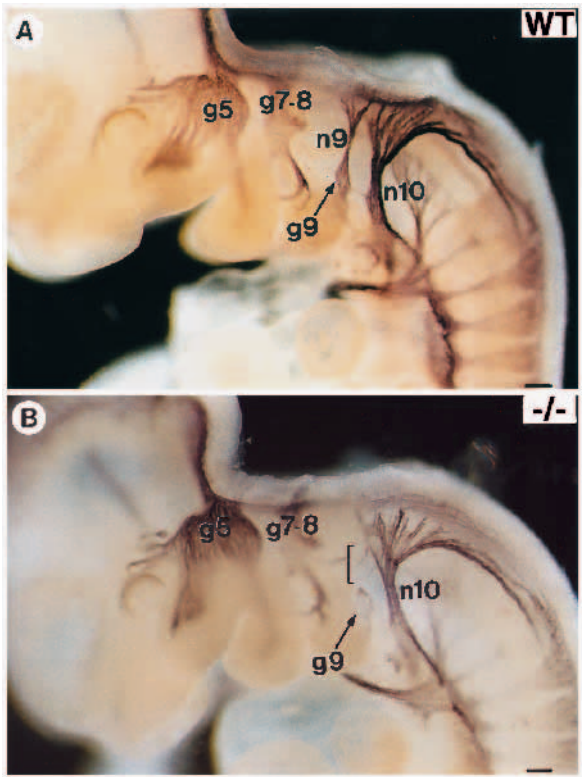

Fig. 5. Cranial nerve defects in ΔRARE$^{-/-}$ embryos. Lateral views of 10.5 dpc WT (A) and ΔRARE$^{-/-}$ (B) embryos stained with an anti-neurofilament antibody. Note that the proximal portion of the glossopharyngeal nerve (n9) is lacking in the mutant embryo (bracket in B). g5, trigeminal (cranial nerve V) ganglion; g7-8, facial-acoustic (cranial nerve VII-VIII) ganglion; g9, lower ganglion of the glossopharyngeal nerve (cranial nerve IX); n10, vagus nerve (cranial nerve X). Scale bars, 100 μm.

partially overlap with those of the 3′ *Hox* genes and knockout experiments have revealed an important role for AP-2 in craniofacial development (Zhang et al., 1996; Schorle et al., 1996).

The phenotypical alterations observed in the ΔRARE mutants provide evidence that some aspects of *Hoxa-1* function are directly controlled by endogenous retinoids

Exogenous RA causes specific alterations in the development of midbrain and hindbrain, which are invariably associated to ectopic activation of *Hox* genes (Morriss-Kay et al., 1991, Conlon and Rossant, 1992; Marshall, et al., 1992; Kessel, 1993; Woods et al., 1994). Furthermore, *Hoxa-1* ectopic expression in the rostral hindbrain results in partial homeotic transformation of the segmental identity of r2 into r4 (Zhang et al., 1994), a phenotype also obtained upon RA treatment of mouse embryos (Marshall et al., 1992). Although these results suggest a link between RA-induced teratogenesis and *Hox* gene function, they cannot be taken as evidence that endogenous retinoids play a role in the direct control of *Hox* genes under physiological conditions. Targeted disruptions of retinoic acid receptors (RARs) (Lohnes et al., 1993, 1994; reviewed by Kastner et al., 1995) resulted in phenotypes reminiscent of those of some *Hox* loss-of-function mutations (reviewed by Krumlauf, 1994), thus supporting the idea that *Hox* genes are retinoid targets in the normal embryo.

The *Hoxa-1* RARE mutation provides conclusive evidence that some aspects of *Hoxa-1* function are under the direct control of endogenous retinoids, during normal development. In a model proposed by Conlon (1995) of the role of RA in axial patterning, RA would promote *Hox* paralog 1 (i.e. *Hoxa-1*, *Hoxb-1*) expression in the posterior part of the gastrulating embryo up to an anterior limit corresponding to a 'primary' boundary (the presumptive r3/r4 boundary), resulting in early rostrocaudal regionalisation of the neural plate. It is hypothesised that further generation, by local signals, of gene expression boundaries in the same vicinity would be dependent on the establishment of the RA-induced primary boundary, and perturbations in the establishment of the primary boundary (e.g. disturbance of RA signalling) would affect patterning

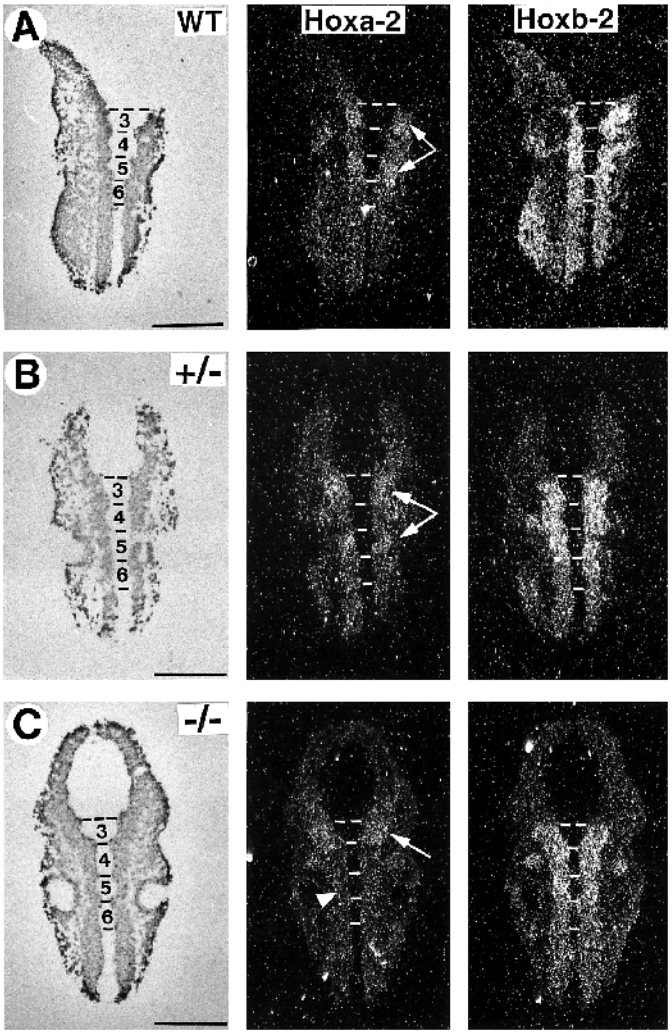

Fig. 6. *Hoxa-2* expression is altered in the hindbrain of $\Delta RARE^{-/-}$ mutants. Successive coronal sections of 9.0 dpc WT (A), $\Delta RARE^{+/-}$ (B), and $\Delta RARE^{-/-}$ (C) embryos hybridized to *Hoxa-2* and *Hoxb-2* ^{35}S-labelled riboprobes and viewed under dark-field illumination. Representative bright-field images are displayed on the left. *Hoxb-2* transcript patterns are comparable in the WT, $\Delta RARE^{+/-}$, and $\Delta RARE^{-/-}$ mutant embryos, with sharp cutoffs at the r2-r3 boundary. *Hoxb-2* is expressed at higher levels in r3, r4, and r5 in WT (A), heterozygous (B), and homozygous (C) embryos. *Hoxa-2* transcripts are up-regulated in r3 and r5 of WT and heterozygous embryos (arrows in A, B; see also Hunt et al., 1991). In homozygous mutant embryos, however, *Hoxa-2* transcripts levels in r5 (arrowhead) are clearly lower than in r3 (arrow in C) and are comparable to those of r4 and r6. Note that the WT embryo in A is slightly younger than the mutants in B and C, yet *Hoxa-2* up-regulation in r3 and r5 is already evident. 3-5, rhombomeres 3-5. Dotted lines indicate rhombomere boundaries. Scale bars, 100 μm.

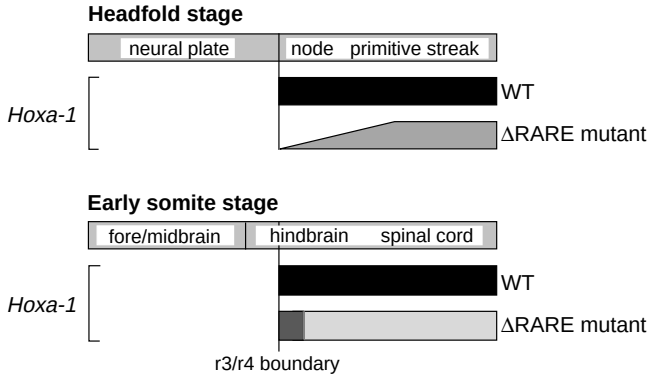

Fig. 7. Summary diagram and comparison of *Hoxa-1* expression pattern in wild-type (WT) and $\Delta RARE^{-/-}$ mutant embryos. At headfold stage, *Hoxa-1* expression domain in the WT extends from the posterior end of the embryo to a sharp limit anterior to the node, corresponding to the presumptive r3/r4 boundary (Murphy and Hill, 1991) (black rectangle). In the $\Delta RARE^{-/-}$ mutants, overall *Hoxa-1* expression levels are lower (grey-shaded rectangle), and the slope indicates a delay in the establishment of the anterior *Hoxa-1* expression boundary in the neural plate. The anterior limit of the expression domain is eventually reached (late headfold-presomite stage), and at early somite stage the lower *Hoxa-1* expression in the presumptive r4-r6 domain of the $\Delta RARE^{-/-}$ mutants (grey-shaded insert) indicates that the RARE is not critically required for the maintenance of expression in this region, although this enhancer appears to be absolutely required for efficient *Hoxa-1* expression in more posterior regions of the hindbrain and in the spinal cord (light grey-shaded rectangle).

(and gene expression domains) in adjacent regions. Strikingly, this is what is observed in the pre-r3 and pre-r5 regions of the ΔRARE mutants, as a possible consequence of a small disturbance in the early establishment of the *Hoxa-1* r3/r4 anterior boundary. This interpretation might also explain why, for instance, we observe an alteration of the pre-r3 domain of *Krox-20* expression in the ΔRARE mutants, even though *Hoxa-1* has not been reported to be expressed in pre-r3.

However, the mildness of the alterations in hindbrain segmentation and cranial nerve formation observed in the mutants suggests that the RARE loss-of-function can be mostly compensated for in the embryo. Continous assessment of rhombomeric identities through changes in gene expression has been suggested by recent transplantation studies (Grapin-Botton et al., 1995; Itasaki et al., 1996). *Hoxa-1* activation in the presumptive r4-r6 region of 7.75-8.0 dpc $\Delta RARE^{-/-}$ embryos (Fig. 2F, and data not shown), although delayed, may still rescue early patterning defects in most of the mutants, and, therefore, explain both the low penetrance of the r3/r5 phenotype and lack of nerve IX, as well as the apparent absence of r4 alterations.

The RARE is not required for the spatiotemporal control of colinear expression of the *Hoxa* genes, but controls some aspects of *Hoxa-2* expression

The study of *Hox* gene expression patterns and of their genomic organization has revealed a strict correlation between the 3′ to 5′ order of the genes in each complex and that of their expression domains along the antero-posterior (A/P) axis of the embryo. This phenomenon, termed colinearity, first observed for the homeotic genes of *Drosophila* (Lewis, 1978), remains a puzzle. In the mouse embryo, the sequential activation of the *Hox* genes follows both a spatial (anterior to posterior) and a temporal (3′ early – 5′ late) colinearity (Duboule and Dollé, 1989; Graham et al., 1989). Ordered activation of *Hox* genes (3′ to 5′) can be also obtained in mouse embryonal carcinoma (EC) cells upon induction with RA in a dose-dependent manner (Simeone et al., 1990; Simeone et al., 1991). In *Xenopus* embryos, the response of *Hoxb* genes to exogenous RA was both spatially and temporally colinear with the organization of the cluster (Dekker et al., 1993), suggesting that the colinear response to RA might not be specific just to the EC cell system, but might have a relevance for the sequential activation of *Hox* genes during embryo development.

One of the proposed models of *Hox* gene cluster activation by RA (Langston and Gudas, 1994) supposes a single RARE at the 3′ end of the cluster being necessary and sufficient to sequentially activate the entire cluster in a 3′ to 5′ direction. The present mutation of the *Hoxa-1* 3′RARE in the mouse provides an opportunity to test this hypothesis. Our results do not support such a role for the *Hoxa-1* 3′RARE, mainly because its targeted inactivation resulted in mild phenotypical alterations constituting only a subset of the phenotypes obtained with the $Hoxa\text{-}1^{tot}$ mutation, and not reminiscent, for instance, of the malformations obtained with the *Hoxa-2* (Rijli et al., 1993; Gendron-Maguire et al., 1993) and/or *Hoxa-3* (Chisaka and Capecchi, 1991) knockouts. In addition, the results of the expression pattern analysis of the *Hoxa-1*, *Hoxa-2*, and *Hoxa-3* genes in mutant embryos are not compatible with the hypothesis that transcriptional activation of the whole *Hoxa* cluster is controlled by the *Hoxa-1* RARE (Figs 2, 6).

Although the RARE does not play a major role in the sequential activation of the the *Hoxa* cluster genes, the analysis of *Hoxa-2* expression pattern in the hindbrain of 9.0 dpc mutant embryos revealed that it is required to provide the correct *Hoxa-2* expression level in r5. At this stage, *Hoxa-2* is upregulated in r3 and r5, where its expression levels are higher than in even numbered rhombomeres (Fig. 6A,B; Hunt et al., 1991; Krumlauf, 1993). It has been recently shown that *Hoxa-2* expression in r5 may be dependent on Krox-20 direct regulation through a specific enhancer (Nonchev et al., 1996). Interestingly, we find that the presence of the RARE is absolutely required for normal levels of *Hoxa-2* expression in r5, demonstrating that Krox-20 is not the only regulator of *Hoxa-2* in this rhombomere. Finally, we cannot conclude from these results whether *Hoxa-2* regulation in r5 is directly mediated through the RARE or indirectly through cross-regulation by the *Hoxa-1* product. Although *Hoxa-2* overall expression appeared unchanged in the hindbrain of the $Hoxa\text{-}1^{tot}$ mutants (Dollé et al., 1993), the absence of r5 in these mutants does not allow any firm conclusion as to whether *Hoxa-1* might be required for proper *Hoxa-2* expression in this rhombomere. However, *cis*-regulation of *Hoxa-2* through the RARE is suggested by the fact that, in $\Delta RARE^{-/-}$embryos, *Hoxa-1* is still present in pre-r5 (Fig. 2F) yet *Hoxa-2* expression is decreased, although the possibility remains that an early temporal delay in *Hoxa-1* expression and/or reduced levels would affect *Hoxa-2* expression.

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