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Retinoids regulate the anterior expression boundaries of 5′ *Hoxb* genes in posterior hindbrain

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We describe the regulatory interactions that cause anterior extension of the mouse 5′ *Hoxb* expression domains from spinal cord levels to their definitive boundaries in the posterior hindbrain between embryonic day E10 and E11.5. This anterior expansion is retinoid dependent since it does not occur in mouse embryos deficient for the retinoic acid-synthesizing enzyme retinaldehyde dehydrogenase 2. A retinoic acid response element (RARE) was identified downstream of *Hoxb5* and shown to be essential for expression of *Hoxb5* and *Hoxb8* reporter transgenes in the anterior neural tube. The spatio-temporal activity of this element overlaps with rostral extension of the expression domain of endogenous *Hoxb5*, *Hoxb6* and *Hoxb8* into the posterior hindbrain. The RARE and surrounding sequences are found at homologous positions in the human, mouse and zebrafish genome, which supports an evolutionarily conserved regulatory function.

Keywords: mouse *Hox* genes/posterior hindbrain patterning/retinoic acid response/transcriptional regulation

Introduction

In mammals, 39 *Hox* genes are arranged in four genomic clusters. Their sequential activation results in spatially and temporally restricted expression patterns along the antero–posterior (AP) axis of the embryo (reviewed by Krumlauf, 1994; Deschamps *et al*., 1999). Gain-of-function and loss-of-function studies have shown that *Hox* genes are involved in patterning structures along this main body axis (Krumlauf, 1994). The positioning of the definitive anterior expression boundaries is of decisive importance for proper patterning of axial and paraxial structures. Transient alteration of the early timing and level of expression of these genes also can lead to defects in AP patterning (van der Hoeven *et al*., 1996; Zákány *et al*., 1997; Greer *et al*., 2000). Therefore, tight spatio-temporal regulation of *Hox* genes is essential for correct patterning of target tissues. This regulation seems to depend on a hierarchy of molecular controls (van der Hoeven *et al*., 1996). At the highest level, the progressive accessibility of *Hox* genes for transcription is thought to be controlled by the release of a repression acting on the whole cluster during early development (Kondo and Duboule, 1999). Subsequently, differentially expressed transcriptional activators would bind to *cis*-acting elements to induce expression of individual genes.

Reporter transgenes containing proximal regulatory elements of 3′ *Hoxb* genes (*Hoxb1*–*Hoxb4*) recapitulate their correct anterior expression boundaries (Marshall *et al*., 1994; Gould *et al*., 1998). In contrast, expression patterns of the 5′ *Hoxb* genes such as *Hoxb8* were not reproduced faithfully using only surrounding genomic sequences (Charité *et al*., 1995). The anterior extent of the expression domain of these genes in the neural tube was never recapitulated using reporter transgenes, despite extensive reporter scanning between *Hoxb5* and *Hoxb9* (Eid *et al*., 1993; Valarché *et al*., 1997; R.Vogels and J.Deschamps, unpublished data). At a location >30 kb 3′ from the *Hoxb8* promoter, we identified a 550 bp element (called the distal element, DE) between *Hoxb4* and *Hoxb5*, which is able to mediate *Hoxb8* expression with the correct rostral expression boundary in the neural tube, when combined on a transgene with the *Hoxb8* proximal regulatory sequences (Valarché *et al*., 1997). Interestingly, the *Hoxb8* DE maps within the 3.5 kb sequence found by Sharpe *et al*. (1998) to drive the expression of *Hoxb5* in the neural tube. This suggested that the same *cis*-acting element and transcriptional activator(s) may control some aspects of the expression of both *Hoxb5* and *Hoxb8*.

Retinoid signalling has been shown to play a crucial role in setting the anterior boundary of 3′ *Hox* genes at the level of the first to the fifth inter-rhombomeric boundaries in the hindbrain (reviewed by Gavalas and Krumlauf, 2000). Retinoic acid (RA) response elements (RAREs) present in the 3′ part of the *Hoxb* cluster have been implicated in the regulation of *Hoxb1*, *Hoxb2* and *Hoxb4 in vivo* (Studer *et al*., 1994; Gavalas *et al*., 1998; Gould *et al*., 1998; Huang *et al*., 1998; Gavalas and Krumlauf, 2000; see Manzanares *et al*., 2001). In contrast, no RARE identified so far has been found to control *Hox* genes more 5′ than paralogy group 4, in spite of the fact that RA was shown to induce sequential 3′ to 5′ activation of *Hoxb* genes in human embryonal carcinoma cells (Simeone *et al*., 1990). Moreover, experiments using chicken embryos suggested that *Hoxb8* is a direct target of RA in limb bud tissues (Lu *et al*., 1997), but it had remained unclear which mechanism underlies this RA sensitivity.

In this study, we show that the late rostral extension of 5′ *Hoxb* gene expression in the neural tube depends on endogenous retinoids, and that it is abolished in

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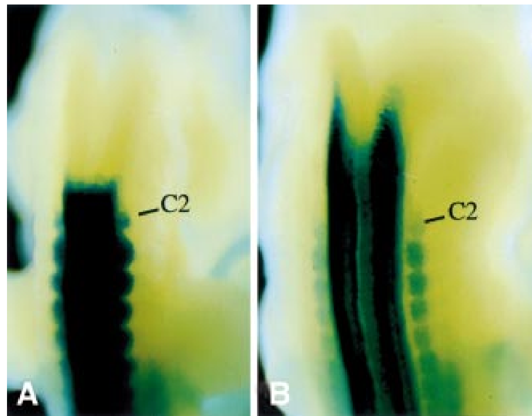

Fig. 1. Expression of *Hoxb8* expands anteriorly into the posterior hindbrain between E10.5 and E11.5. Expression pattern of the *Hoxb8lacZ* knockin (van den Akker *et al.*, 1999) in an E10.5 (**A**) and an E11.5 (**B**) embryo. C2 is the second spinal ganglion, the most anterior persistent dorsal root ganglion.

Raldh2-null (Niederreither *et al.*, 1999) mutant embryos. We identify a novel RARE, which provides transcriptional stimulatory activity to the DE regulatory element, and which is sufficient to induce rostral expression of *Hoxb8/lacZ* reporters in the neural tube. In addition, this RARE induces anterior neural expression of a *Hoxb5/lacZ* transgene. The spatio-temporal window of RA sensitivity of this element matches the windows of activation of the 5′ *Hoxb* genes in the most anterior part of their neural expression domains. This RARE has been well conserved between mammals and fish, which suggests that it may play an important role in mediating RA-dependent patterning of the caudal hindbrain through 5′ *Hoxb* genes.

Results

5′ genes of cluster Hoxb undergo a late anterior extension of their expression domain under control of a previously unknown mechanism

The domain of transcript accumulation of *Hoxb8* in the neural tube undergoes a late phase of rostral extension between E10.5 and E11.5. The mechanism responsible for this change was not understood. The anterior expression boundary of *Hoxb8* in the central nervous system (CNS) was at the level of the upper spinal cord (first dorsal root ganglion, level of somite 5–6 boundary) at E10.5, but shifted to an AP level located well within the hindbrain by E11.5 (Figure 1), whereas the boundaries in mesoderm remained unchanged. We postulated the existence of a regulatory interaction specifically responsible for the induction of this *Hox* gene between upper spinal cord and hindbrain levels. This assumption was based on the fact that none of the reporter transgenes containing *Hoxb8* genomic sequences covering the interval between *Hoxb9* and *Hoxb5* reproduced this rostral extension of the transcription domain in the neural tube (Charité *et al.*, 1995; Valarché *et al.*, 1997). In addition, the expression domain of *Hoxb6* and *Hoxb5* also spread rostrally in the neural tube during these advanced developmental stages (T.Oosterveen, F.Meijlink and J.Deschamps, in preparation).

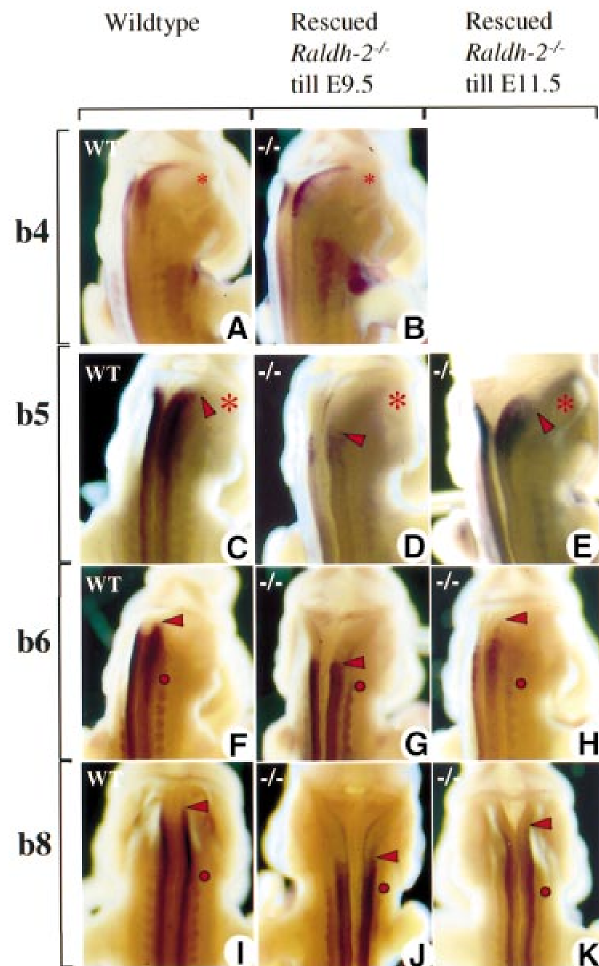

Fig. 2. Alteration of 5′ *Hoxb* hindbrain expression in *Raldh2*$^{-/-}$ embryos transiently rescued from E7.5 to E9.5, or completely rescued up to E11.5. *Hoxb4* (**A** and **B**) has already reached its rostralmost expression boundary at E9.5, and is therefore not affected in transiently rescued E11.5 *Raldh2*-null embryos. The neural expression boundary of *Hoxb5* (**C**–**E**), *Hoxb6* (**F**–**H**) and *Hoxb8* (**I**–**K**) in transiently rescued E11.5 *Raldh2*-null embryos is more posterior than in the wild-types and completely rescued mutants. (A), (C), (F) and (I), untreated wild-type embryo; (B), (D), (G) and (J), *Hox* gene expression in early and transiently rescued *Raldh2*-null embryos; (E), (H) and (K), *Hox* gene expression in completely rescued *Raldh2*-null embryos, serving as controls. Asterisks give the position of the otic vesicle, the red dot the position of dorsal root ganglion C2, and the red arrowheads the anterior boundary of gene expression.

The rostral extension of the expression domain of 5′ Hoxb genes in the neural tube depends on retinoid signalling

We analysed the evolution of the expression patterns of the 5′ *Hoxb* genes from E9.5 to 11.5 in mutants severely impaired in their biosynthesis of RA following inactivation of the enzyme retinaldehyde dehydrogenase 2 (Raldh2) (Niederreither *et al.*, 2000). In order to overcome the early lethality of this mutation due to cardiac defects, subteratogenic doses of RA were administered from E7.5 to E9.0, i.e. prior to the window of *Hox* gene regulation under investigation. Such transiently RA-supplemented *Raldh2* mutant and wild-type littermate embryos developed normally, as documented by marker gene expression (see Figure 2A and B; Materials and methods). The expression boundaries of *Hoxb8*, *Hoxb6* and *Hoxb5* were

A

DR5	: A/GGT/GTCA nnnnn A/GGT/GTCA
Mouse	: **GGATCA** cgcag **AGGTCA**
Human	: **GGATCA** tgcag **AGGTCA**
Zebrafish	: **GGATCA** tccaa **GGGTCA**
mut	: **GGCCCA** cgcag **AGTACT**

B

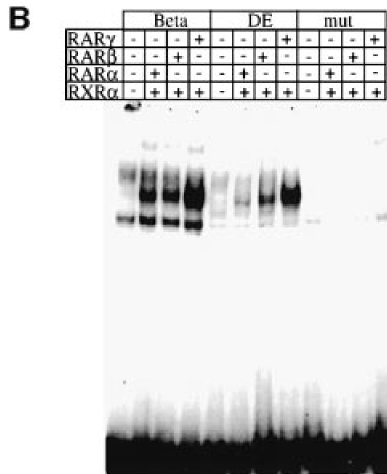

Fig. 3. (**A**) Sequence of the RARE consensus (Mader *et al*., 1993), and of the DE RARE (DR5) in the mouse, and in the human and zebrafish genome. The mutations experimentally introduced in the mouse DE RARE are also indicated. (**B**) EMSA: RAR–RXR heterodimers bind to the wild-type but not to the mutated DE RARE. The top column gives the three different labelled probes tested: β, the well-characterized DR5 RARE derived from the RARβ2 promoter; DE, the DR5 RARE of the DE; and DEmut, a mutated DE RARE (see A). The probes were tested with different combinations of RARs and RXRα proteins (indicated by + or – in the lower columns) present in whole-cell extracts (WCEs) of transfected COS-1 cells. In the lanes where no RA receptors were added, probes were incubated with WCE of COS-1 cells transfected with the empty expression vector. Note that the specific complexes formed on the RARβ2 RARE run at the identical position to those formed on the DE RARE.

positioned correctly in the upper spinal cord at time points earlier than the anterior expansion of the expression domains described above for these genes (data not shown). At E11.5, the expected anterior expansion of neural expression had not occurred for any of these 5′ *Hoxb* genes in *Raldh2* mutant embryos (Figure 2D, G and J), whereas it had occurred normally in wild-type controls (Figure 2C, F and I). In wild-type embryos, there was no difference between the *Hox* expression domains of early RA-supplemented and non-treated controls (data not shown). Furthermore, supplementation of *Raldh2* mutant embryos with RA until E11.5 fully rescued the anterior expansion of 5′ *Hoxb* gene expression into the posterior hindbrain (Figure 2E, H and K).

A conserved RARE confers expression of Hoxb8DE/lacZ and Hoxb5DE/lacZ transgenes in the posterior hindbrain

In a distal genomic region potentially participating in the regulation of *Hoxb8* (called DE), we discovered a putative RARE element matching the consensus A/GGT/GTCA-nnnnnA/GGT/GTCA (Figure 3A; Mader *et al*., 1993). This sequence is highly conserved between mouse, human and zebrafish (Figure 3A), as it was found in all three genomes at a corresponding position, between the paralogues of *Hoxb4* and *Hoxb5*. Moreover, sequences flanking the consensus on both sides reveal a 74% conservation of identity over 160 nucleotides. The consensus behaved as a functional RARE by binding retinoic acid receptor (RAR)–retinoid X receptor (RXR) heterodimers *in vitro* (Figure 3B) and by activating a reporter gene in an RA-dependent manner in cultured cells (data not shown). In E11.5 transgenic embryos, the DE did provide a *lacZ* reporter driven by a minimum *Hoxb8* promoter (Figure 4A, construct 1) with expression localized where RA signalling is known to act (Figure 4C; Shen *et al*., 1992). The DE was also inserted into a transgene controlled by the promoter and proximal regulatory sequences of *Hoxb8* or *Hoxb5*, which normally is expressed in a posterior region at E11.5 (Figure 4A, constructs 3 and 5, respectively). The resulting constructs were expressed in the rostral neural tube up to the posterior hindbrain region (Figure 4D and F). The combination of the DE with the *Hoxb5* promoter and this proximal regulatory element gave rise to a weaker *lacZ* activity than with the *Hoxb8* promoter and proximal element. This was observed for three independent integration sites of both transgenes (data not shown), and may therefore result from the features of the respective promoter–enhancer combination. Both transgenes, however, depend on the DE RARE to be expressed in the rostral neural tube, since nucleotide substitutions abolishing DNA binding of the DE RARE *in vitro* (Figure 3A) prevented their anterior neural expression (Figure 4E and G, constructs 4 and 6, respectively). When transgenic mice containing construct 3 (the *Hoxb8* promoter/*lacZ*, DE and posterior enhancer BH1100; Figure 4A and H) were examined in a *Raldh2*-null background, the anterior neural expression domain was completely abolished, apparently due to RA deficiency (Figure 4I compared with H).

In summary, these results show that the DE element contains a functional RARE that is necessary and sufficient to activate expression of the *Hoxb8/lacZ* and *Hoxb5/lacZ* transgenes in the posterior hindbrain.

The DE RARE is active in a spatio-temporal window compatible with late rostral expansion of 5′ Hoxb gene expression in the neural tube

β-galactosidase activity in the neural tube of *Hoxb8/lacZ*DE transgenic mice (Figure 4A, construct 1) appeared between the 27 and 32 somite stages (E10.0) at the level of the forelimb bud (Figure 5A). Expression at the level of the rostral spinal cord then intensified (E10.5, Figure 5B), to label the caudal hindbrain strongly by E11.5 (Figure 5C) and, more weakly, the spinal cord at the level of the hindlimb buds. These two regions of transgene expression driven by the DE in the neural tube are reminiscent of the expression driven by the RARβ2 promoter (Shen *et al*., 1992), and correspond to the areas of most intense *Hoxb8* expression (Figure 5E). The anteriormost of the two regions spans the most rostral part of the *Hoxb8* expression domain in the posterior hindbrain, which is generated between E10.5 and E11.5. It overlaps both spatially and temporally with the late anterior extension of the neural expression domain of *Hoxb5* and *Hoxb6* described above. The DE also mediates a response of construct 1

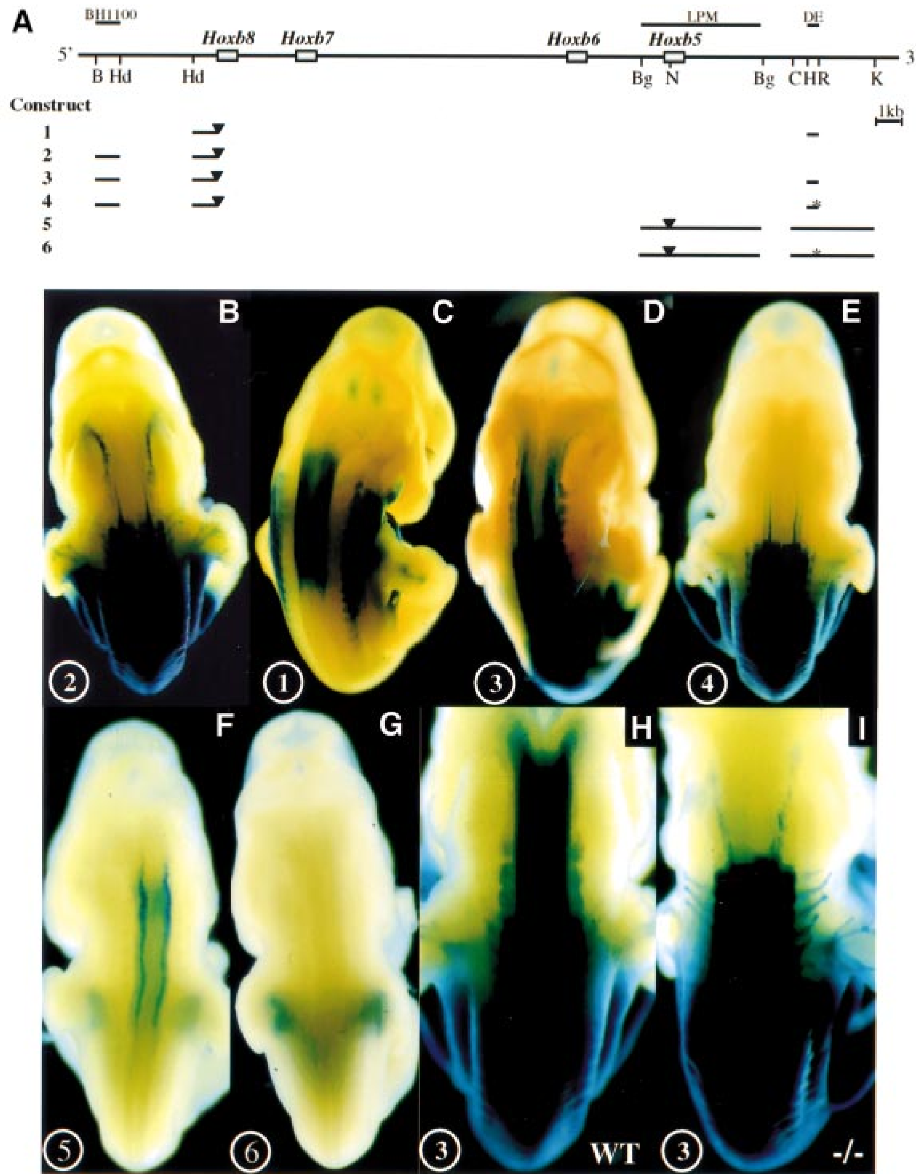

Fig. 4. The DE RARE mediates an RA-dependent rostral shift of 5′ *Hoxb* gene expression into the hindbrain. (**A**) Reporter constructs. The physical map of the *Hoxb* cluster with the *Hoxb5–Hoxb8* genes is shown at the top. Regulatory sequences relevant for this study are shown as lines above the cluster. Below: lines depict the regions that are included in the reporter constructs. Black triangles represent the insertion site of the *lacZ* gene. The mutations in the two half-sites of the RARE are indicated by an asterisk. DE, distal element; LPM, lateral plate mesoderm; B, *Bam*HI; Hd, *Hin*dIII; Bg, *Bgl*II; N, *Nco*I; C, *Cla*I; H, *Hin*cII; R, *Eco*RI; K, *Kpn*I. (B–E) *In vivo* properties of the DE combined with the *Hoxb8* promoter and proximal regulatory elements. Construct numbers are indicated on the lower left of the panels. (**B**) E11.5 embryos expressing construct 2 (*Hoxb8*BH1100); (**C**) construct 1 (*Hoxb8*DE); (**D**) construct 3 (*Hoxb8*BH1100 + DE); and (**E**) construct 4 (*Hoxb8*BH1100 + DE with mutated RARE). (F and G) *In vivo* properties of the DE combined with the *Hoxb5* promoter and a proximal mesoderm-specific element (Sharpe *et al*., 1998). (**F**) E11.5 embryos expressing construct 5 (*Hoxb5*LPM + DE); and (**G**) E11.5 embryos expressing construct 6 (*Hoxb5*LPM + DE with mutated RARE). (H and I) RA dependence of the DE activity. E11.5 embryo expressing construct 3 in the presence (**I**) or absence (**H**) of the *Raldh2*-null mutation.

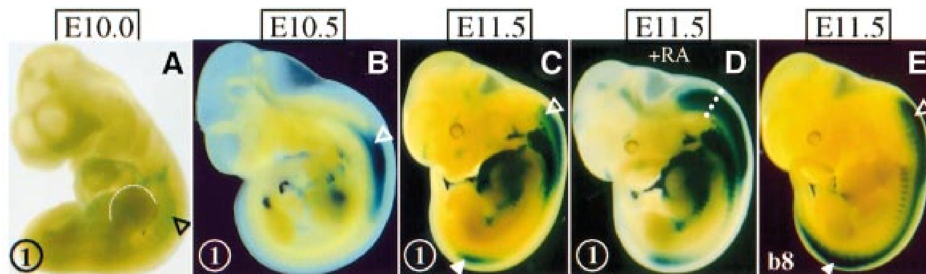

Fig. 5. Expression dynamics of a *Hoxb8/lacZ* reporter construct driven by the DE (construct 1). (**A–C**) E10.0, E10.5 and E11.5 embryos, respectively. (**D**) Expression pattern of the *Hoxb8/lacZ* DE after *in utero* RA exposure from E10.5 on. (**E**) Endogenous *Hoxb8* expression as revealed by *lacZ* expression of a *Hoxb8/lacZ* knockin allele (van den Akker *et al*., 1999). Open triangles, anterior domain of stronger expression; filled triangles, posterior domain of stronger expression.

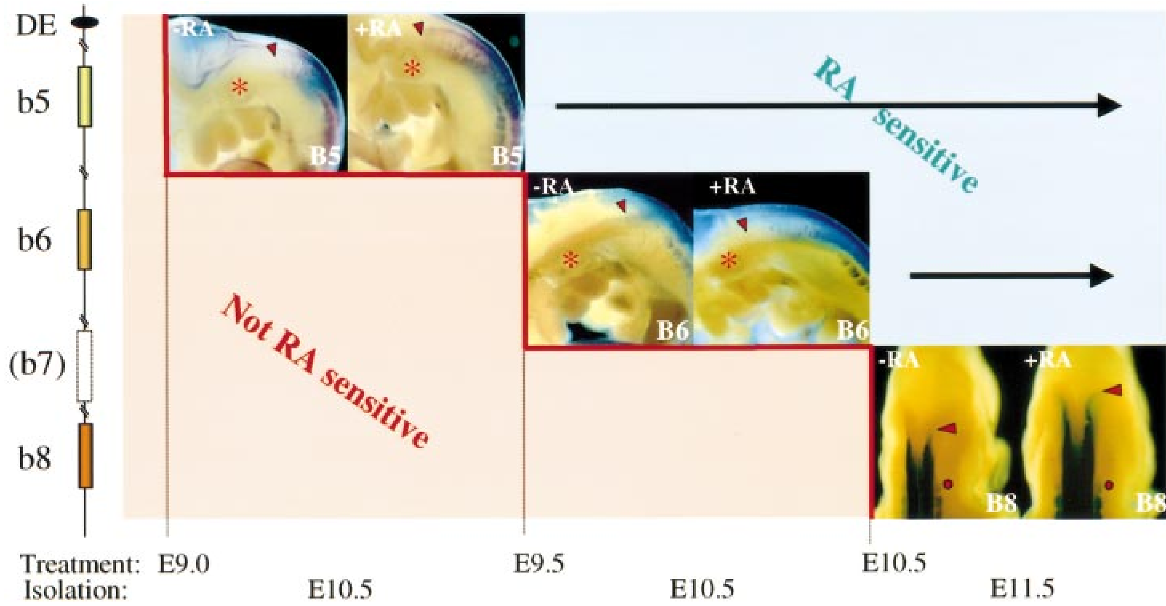

Fig. 6. Sequential windows of RA sensitivity of *Hoxb5* (B5), *Hoxb6* (B6) and *Hoxb8* (B8). Depicted on the left is a schematic representation of the 5′ part of the *Hoxb* cluster between *Hoxb8* and the DE 3′ of *Hoxb5*. Time points of RA treatment and of embryo collection are indicated below. For each gene, the left panel represents the endogenous expression pattern in normal conditions (with solvent treatment), and the right panel shows the expression after RA treatment. The expression patterns shown correspond to the beginning of the window of RA sensitivity.

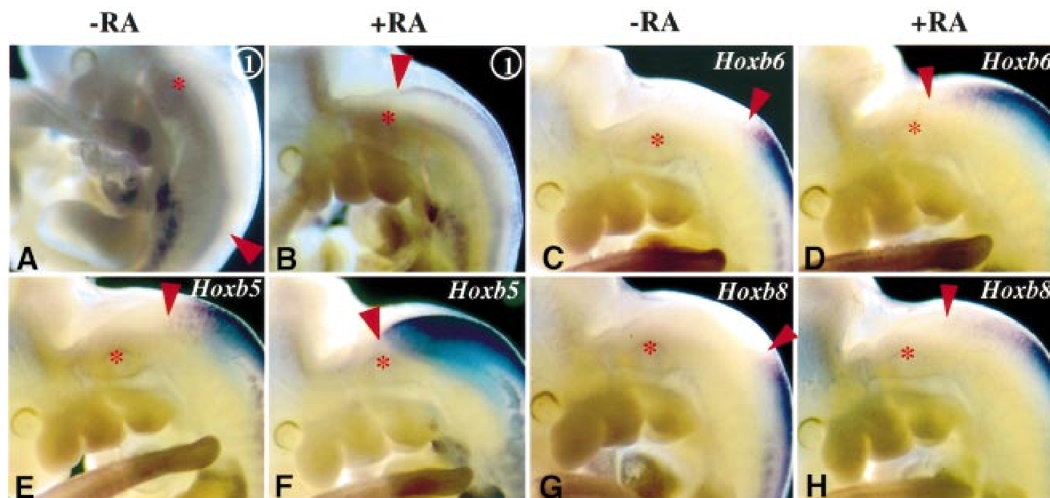

Fig. 7. RA treatments result in a rapid sequential activation of the *Hoxb5*, *Hoxb6* and *Hoxb8* genes. (A–H) RA treatment from E10.5 to E10.75 (6 h) results in mRNA induction of *Hoxb8/lacZ* driven by the DE (construct 1, **A** and **B**), *Hoxb6* (**C** and **D**), *Hoxb5* (**E** and **F**) and *Hoxb8* (**G** and **H**). The asterisk gives the position of the otic vesicle, the red dot the position of DRG C2, and the red arrowheads the anterior boundary of gene expression.

to exogenous RA, resulting in an anterior shift of its expression domain (Figure 5D compared with C).

In summary, the DE regulatory element activates transcription of reporter genes with temporal and spatial kinetics matching the late anterior expansion of 5′ *Hoxb* gene expression in the posterior hindbrain in wild-type embryos.

Neural expression of Hoxb5–Hoxb8 becomes sequentially sensitive to exogenous RA within the activity period of the DE RARE

We analysed and compared the timing of RA sensitivity of the DE RARE coupled to the *Hoxb8* promoter, with the RA sensitivity of endogenous *Hoxb4–Hoxb8*.

Transgenic embryos carrying a *lacZ* reporter driven by the *Hoxb8* promoter and the DE (construct 1, Figure 4A) are RA sensitive upon treatment starting at E9.5 or E10.5 (Figure 5C and D). The expression domain of *Hoxb4*, known to depend on a more 3′ RARE-containing early neural enhancer (Gould *et al*., 1998), expanded rostrally after *in utero* exposure of the embryos to RA at E8.5, thus before the DE RARE is active (Gould *et al*., 1998; data not shown). *Hoxb5–Hoxb8* did not respond to such an early treatment, but only to RA applied at successively later time points. *Hoxb5* and *Hoxb6* exhibited their earlier response to RA treatment at E9.0 and E9.5, respectively (Figure 6). No alteration in endogenous *Hoxb8* expression occurred during E8.5–9.5 RA treatments. Only at E10.5 were RA-induced rostral shifts in *Hoxb8* expression first

observed (Figure 6). These responses fit within the window of sensitivity of the DE RARE.

Hoxb5, Hoxb6 and Hoxb8 respond rapidly to exogenous RA

A prerequisite for a possible direct involvement of the DE RARE in the regulation of 5′ *Hoxb* genes would be rapid responsiveness to exogenous RA exposure. RA treatment of *Hoxb8/lac*ZDE (construct 1, Figure 4A) transgenic embryos at E10.5 resulted in transcriptional induction within 6 h (Figure 7A and B). Under these conditions, the expression domains of endogenous *Hoxb5*, *Hoxb6* and *Hoxb8* also clearly extended rostrally in RA-treated embryos (Figure 7C–H). This is in agreement with previous studies showing that RA can activate *Hoxb8* in the absence of protein synthesis in chicken limb bud cells (Lu *et al*., 1997).

Discussion

The late regulatory phase of 5′ Hoxb genes and posterior hindbrain patterning

The expression domains of the 3′ *Hoxb* genes, *Hoxb1–Hoxb4*, reach their rostralmost boundaries before rhombomeric segmentation (Wilkinson *et al*., 1989; Gould *et al*., 1998). Several regulatory elements containing binding sites for RAR/RXR have been identified and characterized for these genes, and shown to contribute to the establishment of their early expression domains prior to and during segment formation in the rhombencephalon (Studer *et al*., 1994; Gavalas *et al*., 1998; Huang *et al*., 1998; see Gould *et al*., 1998; Gavalas and Krumlauf, 2000). In some cases, a later maintenance of *Hox* expression in specific rhombomeres is ensured by a different element receiving auto- and cross-regulatory Hox signals (Ogura and Evans, 1995; Pöpperl *et al*., 1995; Gould *et al*., 1998). The restricted reporter gene expression directed by the early neural enhancers of *Hoxb1* and *Hoxb4* is an integral part of the complete expression domain of these genes, and has a rostral boundary identical to the initial boundary of the endogenous gene [*Hoxb1* (Marshall *et al*., 1994) and *Hoxb4* (Gould *et al*., 1998)]. The 5′ *Hoxb* genes *Hoxb5*, *Hoxb6* and *Hoxb8* are expressed in domains that do not reach their definitive rostral boundaries in the posterior hindbrain until after rhombomeric segmentation. By E9.5, their anterior boundary of expression in the neural tube is at the level of somite 4 (*Hoxb5*), somite 5 (*Hoxb6*) and the S5–S6 boundary (*Hoxb8*). Thereafter, expression spreads rostrally in the neural tube, to reach successively their gene-specific anteriormost boundaries in the posterior hindbrain. Their definitive anterior boundaries are specified in rhombomeres 7/8 (r7/r8) in the hindbrain by E11.5, long after pre-otic rhombomere identity has been determined. Ontogenesis of the late rostral expression domains of these 5′ *Hoxb* genes between the upper spinal cord and the caudal hindbrain therefore occurs relatively late. It may correspond to a late function of 5′ *Hoxb* genes in the CNS, possibly contributing to specifying the identity of r7 and 8 (much less obvious morphologically than r1–r6).

It is interesting to put these observations side by side with the recent data on the graded role of RA signalling in hindbrain patterning in chick (Dupé and Lumsden, 2001). These studies showed that RA dependency of the specification of rhombomere identity is lost progressively in an anterior to posterior sequence between the definitive streak stage and the 16 somite stage, and that the post-otic neural tube between r5/r6 and the sixth somite belongs to the hindbrain and is anteriorized by abrogating RA signalling.

Comparison between the kinetics of the DE RARE and the sequential acquisition of definitive expression boundaries of 5′ Hoxb genes

The present study demonstrates that the late rostral extension of the expression of *Hoxb8* and other 5′ *Hoxb* genes in the CNS depends on endogenous RA. A regulatory sequence containing a functional RARE was identified 3′ of *Hoxb5*. Our previous transgenic analyses (Charité *et al*., 1995; Valarché *et al*., 1997; our unpublished data) showed that the DE is the only sequence between *Hoxb9* and *Hoxb4* that can extend the expression of a *Hoxb8* transgene rostrally into the posterior hindbrain. We now show that the RARE located within the DE is required for expression of *Hoxb8/lacZ* transgenes in the posterior hindbrain. This RARE, analysed using *Hoxb8* and *Hoxb5* transgenes, exhibits a window of spatio-temporal RA sensitivity encompassing that of the endogenous *Hoxb8–Hoxb5*.

A search in the Celera database for sites matching the RARE DR(1–5) consensus, as defined by Mader *et al.* (1993) (see also Figure 3A), did not reveal any such RARE between mouse *Hoxb4* and 77 kb 5′ to *Hoxb9*. This sequence analysis revealed that the DE RARE, which we identified close to *Hoxb5*, was unique in the entire mouse *Hoxb* cluster. Although this information makes the DE RARE a very good candidate to account for RA sensitivity of *Hoxb5–Hoxb8*, we cannot rule out that other, divergent and unsuspected consensuses have escaped our analysis and are functionally involved in the RA-stimulated induction of the most rostral expression of these *Hox* genes. It will be necessary to inactivate the DE RARE in the mouse genome to understand its function definitely. The existence of a shared RARE regulatory element, which would act sequentially on successive 5′ *Hoxb* genes, might facilitate coordinated regulation of the establishment of their anteriormost expression boundaries in the neural tube, independently of their expression in the paraxial mesoderm. This mechanism would differ significantly from the regulation of 3′ *Hoxb* genes, which are controlled by several RAREs [*Hoxb1* (Marshall *et al*., 1994; Ogura and Evans, 1995; Huang *et al*., 1998) and *Hoxb4* (Gould *et al*., 1998)].

The newly identified RARE is evolutionarily conserved

Few binding sites for *trans*-acting factors have been characterized in the 5′ half of the *Hox* clusters so far, whereas binding sites for several *trans*-acting factors have been identified that control the expression of paralogous genes 1–4 (reviewed by Deschamps *et al*., 1999). The only sites that were found to bind *trans*-acting factors modulating the expression of 5′ *Hoxb* genes during their establishment were Cdx-binding sites (Charité *et al*., 1998; van den Akker *et al*., 2001). While Cdx proteins play a role during the ontogenesis of the *Hox* expression patterns during early embryogenesis (van den Akker *et al*.,

2002), the RA-mediated control described in this work occurs later, between E9 and E11.5. The finding of strong conservation of the RARE sequence at corresponding positions of the mammalian and zebrafish genomes suggests a functional importance for this regulatory element. The relatively high conservation of the sequences flanking the RARE on both sides further suggests that essential co-activators may bind at positions flanking the RAR/RXR-binding site.

Endogenous retinoid signalling and 5′ Hoxb gene expression

RA distribution in developing embryos has been found to be restricted to posterior paraxial mesoderm, with a sharp anterior boundary at the level of the first somite (corresponding to r6/r7 in the hindbrain; Maden *et al*., 1998; Berggren *et al*., 1999; Swindell *et al*., 1999). Accordingly, *Raldh2* expression is detected in cervical somites at E8.5, and in cervical mesenchyme by E9.5 (Niederreither *et al*., 1997). The endogenous RA regulating 5′ *Hoxb* genes in the posterior hindbrain between somite 6 and the r6/r7 region must therefore originate from that source between E9.5 and E11.5, and diffuse over a rather long distance (see also Dupé and Lumsden, 2001). The resulting RA gradient, together with a combination of other factors, including the *Hox* promoter-specific features of the RARE enhancer element, and differential availability of *Hox* genes for transcription (Kondo and Duboule, 1999; Kmita *et al*., 2000), might contribute to the sequential establishment of the definitive expression boundaries of the 5′ *Hoxb* genes.

Materials and methods

DNA constructs

Constructs 1 and 2 have been described previously (Charité *et al*., 1995; Valarché *et al*., 1997). Construct 3 was generated by cloning of a *Bam*HI–*Bam*HI fragment, containing BH1100 and *Hoxb8/lacZ*, from construct 2 into a plasmid containing the *Hin*cII–*Eco*RI distal element. Construct 4 was generated in an identical way with the DE containing a mutated RARE, GGCCCACGCAGAGTACT instead of the GGATCACGCAGAGGTCA motif. The *Hoxb5* reporter gene, in constructs 5 and 6, was generated by the in-frame fusion of the *lacZ* gene to the *Hoxb5* gene, by making use of the *Nco*I site that includes the ATG start codon of the *lacZ* gene in PSDK*lacZ*pA (a gift of J.Rossant) and the *Nco*I site in the first exon of *Hoxb5*. Constructs 5 and 6 include *Hoxb5* upstream sequences up to the *Bgl*II site and, downstream from *lacZ*, the sequence from *Eag*I in the *Hoxb5* gene up to the *Bgl*II site downstream from *Hoxb5*, fused to the 3.5 kb *Cla*I–*Kpn*I neural element (region E; Sharpe *et al*., 1998). Construct 6 contained the same mutation in the RARE as construct 4. All construct were linearized prior to microinjection of zygotes (Vogels *et al*., 1993).

DNA sequence analysis

The nucleotide sequence of the mouse DE (exactly 533 nucleotides) containing the RARE was determined and compared with its human equivalent using the human genome public database (web site: www.ensembl.org). The most conserved block of sequences spanning ~160 nucleotides around the RARE was then used to screen the zebrafish genome database (web site: www.sanger.ac.uk), revealing 74% overall conservation along a 130 bp area surrounding the perfectly conserved RARE consensus, in the zebrafish *Hoxba* cluster, at a homologous position between *Hoxb4* and *Hoxb5*. Mouse genomic sequences encompassing the *Hoxb* cluster were obtained from the Celera database and analysed using McDraw from DNAstar, Laser Gene.

Protein–DNA binding assays

Electropheretic mobility shift assays (EMSAs) were performed as described previously in Folkers *et al*. (1998). The oligonucleotides, labelled by filling in a 5′-AGG overhang, were as follows (without the overhang): β, AG**GGTTCA**CCGAAA**AGTTCA**CTCGCA; DE, CG**GG**ATCACGCAG**AGGTCA**GCAGAC; and DEmut, CG**GGCCCA**CGCAG**AGTACT**GCAGAC. The RARE sequences are shown in bold, and the mutations in DEmut are underlined.

X-gal staining and in situ hybridization

Whole-mount *lacZ* staining of embryos was performed as described previously (Hogan *et al*., 1994). Whole-mount *in situ* hybridization was performed according to Wilkinson (1992), with modifications in the post-hybridization washes (see Haramis *et al*., 1995), using the following probes: *Hoxb1* (Conlon and Rossant, 1992), *Krox20* (Conlon and Rossant, 1992; Seitanidou *et al*., 1997), *Hoxb4* (Conlon and Rossant, 1992), *Hoxb5* (Krumlauf *et al*., 1987), *Hoxb6* (Schughart *et al*., 1988), *Hoxb8* (SS420; Charité *et al*., 1998) and *lacZ* (Valarché *et al*., 1997).

In utero RA exposure of wild-type embryos

RA administration to pregnant mothers by oral gavage was performed as described previously by Conlon and Rossant (1992).

Early partial rescue of Raldh2-null embryos

Raldh2-null embryos die at E10.5 due to severe heart malformations (Niederreither *et al*., 1999), preventing the examination of later developmental events such as *Hox* gene regulation studied here. Whereas RA administration to *Raldh2*$^{+/-}$ pregnant mice by oral gavage resulted in teratogenic effects and a lower percentage of mutant survival (Nierderreither *et al*., 1999), RA supplementation in the food resulted in near normal development of *Raldh2* mutant embryos (Nierderreither *et al*., 2001). Under food rescue conditions, the hindbrain of *Raldh2*-null embryos was morphologically normal, as indicated by the wild-type expression of 3′ *Hoxb* genes and *Krox20* (Seitanidou *et al*., 1997; data not shown). These data exclude the possibility that early patterning defects in the hindbrain could have interfered with *Hox* gene expression at later stages of development. The landmarks for determining the position of the neural expression boundary of 5′ *Hoxb* genes, the dorsal root ganglia, were shown to be unaltered by histological inspection (data not shown). Finally, we ruled out that the *Raldh2* mutation caused a loss of competence of the caudal hindbrain cells to express the 5′ *Hoxb* genes. Homozygous *Raldh2* mutant embryos were completely rescued by supplementing their food with RA until the day of isolation. These experiments show that it is the late depletion of RA in *Raldh2*$^{-/-}$ embryos that causes the failure of 5′ *Hoxb* gene expression in the caudal hindbrain.

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