Shifting boundaries of retinoic acid activity control hindbrain segmental gene expression

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Summary

Retinoic acid (RA) generated by *Raldh2* in paraxial mesoderm is required for specification of the posterior hindbrain, including restriction of *Hoxb1* expression to presumptive rhombomere 4 (r4). *Hoxb1* expression requires 3′ and 5′ RA response elements for widespread induction up to r4 and for r3/r5 repression, but RA has previously been detected only from r5-r8, and *vHnf1* is required for repression of *Hoxb1* posterior to r4 in zebrafish. We demonstrate in mouse embryos that an RA signal initially travels from the paraxial mesoderm to r3, forming a boundary next to the r2 expression domain of *Cyp26a1* (which encodes an RA-degrading enzyme). After *Hoxb1* induction, the RA boundary quickly shifts to r4/r5, coincident with induction of *Cyp26c1* in r4. A functional role for *Cyp26c1* in RA degradation was established through examination of RA-treated embryos. Analysis of *Raldh2*$^{-/-}$ and *vHnf1*$^{-/-}$ embryos supports a direct role for RA in *Hoxb1* induction up to r4 and repression in r3/r5, as well as an indirect role for RA in *Hoxb1* repression posterior to r4 via RA induction of *vHnf1* up to the r4/r5 boundary. Our findings suggest that *Raldh2* and *Cyp26* generate shifting boundaries of RA activity, such that r3-r4 receives a short pulse of RA and r5-r8 receives a long pulse of RA. These two pulses of RA activity function to establish expression of *Hoxb1* and *vHnf1* on opposite sides of the r4/r5 boundary.

Key words: Retinoic acid, Hindbrain, Segmentation, *Raldh2* (*Aldh1a2*), *Cyp26*, *Hoxb1*, *Hnf1* (*Tcf2*), Mouse

Introduction

Retinoic acid (RA) is generated from vitamin A (retinol) in a metabolic process of fundamental importance for development of mammalian embryos as well as other chordate embryos (Duester, 2000; Clagett-Dame and DeLuca, 2002). RA serves as a ligand that controls the action of nuclear RA receptors (Mangelsdorf et al., 1994; Kastner et al., 1995) either in cells where it is synthesized or in neighboring cells via paracrine signaling (Mic et al., 2002). RA plays an important role in development of the posterior central nervous system, including rhombomeric pattern formation in the hindbrain, as previously reviewed (Gavalas and Krumlauf, 2000; Gavalas, 2002; Maden, 2002). Hox homeobox genes exhibit segmental expression in the developing hindbrain and are intimately involved in the formation and identity of the eight rhombomeres that constitute the mouse hindbrain (Krumlauf, 1993; Wilkinson, 1993). Several members of the mammalian Hox gene family are direct targets of RA signaling during hindbrain development, including *Hoxb1* (Simeone et al., 1990), and studies in amphioxus indicate that RA regulation of Hox genes is conserved in chordates (Holland and Holland, 1996). Prior to rhombomeric boundary formation, *Hoxb1* is initially expressed throughout the posterior hindbrain up to the boundary between presumptive rhombomeres 3 and 4 (r3/r4), but soon becomes restricted to r4 (Wilkinson et al., 1989). Gene targeting in mice has revealed that *Hoxb1* expression in r4 is required for facial motor neuron differentiation in this segment (Goddard et al., 1996; Studer et al., 1996).

The transcriptional regulation of Hox genes has been examined extensively as previously reviewed (Lufkin, 1996). It has been discovered that the mouse *Hoxb1* gene is regulated by a retinoic acid response element (RARE) located 3′ to the promoter that is required for early widespread induction in the posterior hindbrain up to the presumptive r3/4 boundary (Marshall et al., 1994). This is consistent with the more recent demonstration that *Hoxb1* expression anterior to the node requires cell to cell signaling and does not rely on proliferative expansion of *Hoxb1*-expressing cells at the level of the node (Forlani et al., 2003). Interestingly, another RARE located 5′ to the promoter has been demonstrated to be required for repression of *Hoxb1* in r3 and r5 to provide restricted expression in r4 (Studer et al., 1994). In addition, an autoregulatory element has been found in the *Hoxb1* promoter that is important for maintenance of r4 expression (Pöpperl et al., 1995). However, RA activity has not previously been detected anterior to r5 (see below) and other factors have been found to regulate *Hoxb1* r4 restriction. In zebrafish, repression of *Hoxb1* in the posterior hindbrain up to r5 has been demonstrated to depend upon the homeodomain protein encoded by *vHnf1* (variant of Hepatocyte nuclear factor 1; *Tcf2* – Mouse Genome Informatics), which is expressed in the posterior hindbrain up to the r4/r5 boundary (Wiellette and

Sive, 2003). Recent studies also indicate that RA is required for expression of zebrafish *vHnf1* in the posterior hindbrain (Hernandez et al., 2004). In addition, zebrafish *iro7* encodes an iroquois homeodomain protein (homologous to mouse *Irx3*) expressed in the anterior hindbrain down to r4, and mutual repression of *iro7* and *vHnf1* positions the r4/r5 boundary (Lecaudey et al., 2004). Thus, it is unclear if mouse *Hoxb1* induction and r4 restriction involves direct effects of RA signaling on the *Hoxb1* promoter as RA has not been detected anterior to r5, plus it is unknown if RA may have indirect effects on mouse *Hoxb1* r4 restriction through regulation of *vHnf1* or *Irx3* to set the r4/r5 boundary.

In the mouse, RA is first generated at embryonic day 7.5 (E7.5) just prior to the onset of hindbrain development (Rossant et al., 1991; Ang et al., 1996). RA synthesis for mouse development between E7.5-E8.25 is controlled by retinaldehyde dehydrogenase 2, encoded by *Raldh2* (*Aldh1a2* – Mouse Genome Informatics), expressed in the trunk paraxial mesoderm destined to become somites (Niederreither et al., 1999; Mic et al., 2002). The timing and location of the initial expression of *Raldh2* coincides with the onset of posterior neural development, and RA as well as *Raldh2* are indeed required for posterior hindbrain development and *Hoxb1* expression in amniote embryos (Maden et al., 1996; Dickman et al., 1997; White et al., 1998; Niederreither et al., 2000; Dupé and Lumsden, 2001) as well as *Xenopus* (Blumberg et al., 1997; Kolm et al., 1997; Van der Wees et al., 1998; Chen et al., 2001) and zebrafish embryos (Begemann et al., 2001; Grandel et al., 2002; Kudoh et al., 2002). This suggests that RA generated in the trunk paraxial mesoderm by *Raldh2* travels anteriorly into the hindbrain. It has been further suggested that a gradient of RA may exist across the hindbrain with the high point located posteriorly where the paraxial mesoderm lies adjacent to the posterior hindbrain. Examination of RA activity in mouse embryos carrying an RA-reporter transgene (*RARE-lacZ*) has revealed that RA activity is limited to the posterior portion of the embryo at the late primitive streak stage (E7.5) (Rossant et al., 1991), but the exact location of its anterior limit was not determined. At E8.25-E9.25, the *RARE-lacZ* RA signal has been shown to be present in the posterior hindbrain up to r5 (Sakai et al., 2001; Mic et al., 2002), but there has been no clear indication that RA activity is ever present further anterior in mouse embryos. This issue has not been resolved in other vertebrate embryos because of the lack of an appropriate RA reporter. As RA activity has not been convincingly demonstrated in r3 and r4, it remains unclear whether the *Hoxb1* RARE DNA control elements described above require RA to function in those segments. A failure to observe RA activity in r3 and r4 may be due to expression of RA-degrading P450s in the hindbrain. Indeed, three RA-degrading P450s encoded by *Cyp26a1* (Fujii et al., 1997; Hollemann et al., 1998; Swindell et al., 1999; Kudoh et al., 2002), *Cyp26b1* (MacLean et al., 2001; Reijntjes et al., 2003) and *Cyp26c1* (Tahayato et al., 2003; Reijntjes et al., 2004) are expressed in dynamic patterns during hindbrain development in several vertebrate embryos.

We now provide further insight into the mechanism of RA action during establishment of r3/r4/r5 gene expression boundaries through analysis of *RARE-lacZ*, *Raldh2*$^{-/-}$ and *vHnf1*$^{-/-}$ mouse embryos. We demonstrate the existence of dynamic shifting boundaries of hindbrain RA activity during *Hoxb1* induction/repression that correspond to the *Cyp26a1* and *Cyp26c1* expression patterns. We show that RA is transiently present throughout the posterior hindbrain up to the r2/r3 boundary (abutting the anterior *Cyp26a1* expression domain) and that this RA is needed to induce *Hoxb1* expression throughout the posterior hindbrain up to the presumptive r3/r4 boundary and to induce *vHnf1* (a repressor of *Hoxb1*) up to the presumptive r4/r5 boundary. However, subsequent to induction of *Hoxb1* and *vHnf1*, the boundary of RA activity is quickly shifted to r4/r5, coincident with initiation of *Cyp26c1* expression in r4, and this coincides with strict limitation of *Irx3* and *vHnf1* expression to opposite sides of the r4/r5 boundary, plus restriction of *Hoxb1* expression to r4. Studies on *Raldh2*$^{-/-}$ and *vHnf1*$^{-/-}$ embryos indicate that RA is required for induction of *Hoxb1* and *vHnf1*, and that *vHnf1* is required to repress *Hoxb1* posterior to r4. Analysis of RA-treated embryos supports a functional role for *Cyp26c1* in RA degradation. Our findings thus provide evidence that RA activity exists in the appropriate location to directly induce *Hoxb1* throughout the posterior hindbrain and to directly repress *Hoxb1* in r3 and r5 through previously described 3′ and 5′ RAREs (Marshall et al., 1994; Studer et al., 1994). We also demonstrate that RA-mediated repression of *Hoxb1* posterior to r4 also functions indirectly through RA induction of its repressor *vHnf1*.

Materials and methods

Mouse strains

As the *Raldh2*$^{-/-}$ genotype is embryonic lethal by E11.5, this line was maintained as a heterozygous *Raldh2*$^{-/+}$ line as previously described (Mic et al., 2002). A line of *Raldh2*$^{-/+}$ mice carrying the RA-reporter transgene *RAREhsplacZ* (*RARE-lacZ*) (Rossant et al., 1991) on one chromosome has also been described (Mic et al., 2002). This strain was mated to generate an *Raldh2*$^{-/+}$ line carrying *RARE-lacZ* on both homologous chromosomes (homozygous). For analysis of *RARE-lacZ* expression in *Raldh2*$^{-/-}$ embryos, matings were performed between male mice homozygous for *RARE-lacZ* with female mice lacking *RARE-lacZ*, resulting in embryos that were all heterozygous for *RARE-lacZ*. We also generated a wild-type line homozygous for the *RARE-lacZ* transgene in order to increase the signal strength of the transgene. For enhanced analysis of *RARE-lacZ* expression in wild-type embryos, matings were performed between males and females homozygous for *RARE-lacZ* to generate embryos homozygous for *RARE-lacZ*.

In order to examine *vHnf1*$^{-/-}$ embryos during hindbrain development, we used a conditional knockout of *vHnf1* described previously (Coffinier et al., 2002). We also used the *Mox2-Cre* (MORE) transgene, which expresses Cre throughout the epiblast following implantation but not in extra-embryonic tissues (Tallquist and Soriano, 2000). Conditional *vHnf1*$^{-/-}$ embryos were generated from matings between mice homozygous for a conditional *vHnf1* allele flanked by *loxP* sites with mice that were heterozygous for the null allele of *vHnf1* (Coffinier et al., 1999) and also carried a *Mox2-Cre* allele.

Embryo genotyping and staging

Embryos from timed matings were genotyped by PCR analysis of yolk sac DNA. Embryos were staged according to morphology as previously described (Downs and Davies, 1993; Forlani et al., 2003) and were assigned the following embryonic day numbers with noon on the day of vaginal plug detection being considered embryonic day 0.5 (E0.5): early headfold (E7.4), headfold (E7.5-E7.75), late headfold (E7.8-7.9), one to three somites (E8.0), four to six somites (E8.25), seven to ten somites (E8.5) and 11-14 somites (E8.75).

Rescue of mutant embryos with a physiological dose of RA

Rescue of *Raldh2*$^{-/-}$ embryos by maternal dietary RA supplementation was performed as previously described with an RA dose that has previously been demonstrated to be in the normal physiological range (Mic et al., 2003). Briefly, all-*trans*-RA (Sigma) was dissolved in corn oil and mixed with powdered mouse chow to provide a final concentration of 0.1 mg/g for treatment from E6.75-E8.25 or from E6.75 to the point of analysis for embryos analyzed prior to E8.25. Such food was prepared fresh twice each day (morning and evening) and provided ad libitum. For mice analyzed after E8.25, mice were returned to standard mouse chow at E8.25 until the point of analysis.

Treatment of wild-type embryos with excess RA

Pregnant wild-type mice were treated with exogenous RA similar to a previous description (Conlon and Rossant, 1992). Following timed matings, female mice were orally administered all-*trans*-RA (Sigma) at a dose of 20 mg/kg body weight dissolved in 0.2 ml corn oil at 12 pm on day 7 (E7.5). Embryos were analyzed 18 hours after dosing (E8.25).

Whole-mount in situ hybridization

Embryonic mRNAs were detected by whole-mount in situ hybridization using the alkaline phosphatase substrate NBT-BCIP as described (Wilkinson, 1992). Antisense RNA probes were generated from mouse cDNAs encoding *Raldh2* (Haselbeck et al., 1999), *Hoxb1* (Hunt et al., 1991), *vHnf1* (Coffinier et al., 1999), *Irx3* (Cohen et al., 2000), *Cyp26a1* and *Cyp26c1* (Tahayato et al., 2003), *Krox20* (Wilkinson, 1993), and *Epha2* (Becker et al., 1994).

Detection of retinoic acid activity

Detection of RA activity was performed in embryos carrying the *RARE-lacZ* RA-reporter transgene, which places *lacZ* (encoding β-galactosidase) under the transcriptional control of a retinoic acid response element (RARE) (Rossant et al., 1991). β-Galactosidase staining was performed for 1 hour with the substrate Salmon-gal (6-chloro-3-indolyl-β-D-galactopyranoside) (Labscientific) to produce a red reaction product; in some cases, staining was performed with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to produce a blue-green reaction product. Double staining to examine both RA activity (*RARE-lacZ*) and mRNA localization was performed by first staining 1 hour for β-galactosidase using Salmon-gal, followed by processing for whole-mount in situ hybridization as described (Tajbakhsh and Houzelstein, 1995).

Results

r3/r4/r5 gene expression boundaries in the early hindbrain

Disruption of *Cyp26a1* encoding an RA-degrading P450 enzyme has previously demonstrated that this gene functions in RA degradation for the anterior neural plate (Sakai et al., 2001; Abu-Abed et al., 2001). Transient expression of *Cyp26a1* has been previously reported in the anterior neural plate of mouse headfold stage embryos (Fujii et al., 1997; MacLean et al., 2001), but the location of its posterior boundary has not been defined. In order to define this boundary, we compared expression of *Cyp26a1* with that of *Krox20* (Egr2 – Mouse Genome Informatics), which is a marker for presumptive r3 at the headfold stage (Wilkinson, 1993), with *Hoxb1* which is expressed continuously throughout the posterior hindbrain up to the presumptive r3/r4 boundary at the headfold stage (Wilkinson et al., 1989), and with *Epha2* which is an early hindbrain marker limited to presumptive r4 and the adjacent mesoderm (Becker et al., 1994). Owing to the early stages examined here, the rhombomeric (r) territories of gene expression described throughout the results will refer to presumptive rhombomeres. Embryos double-stained for *Cyp26a1* and *Hoxb1* expression exhibited a gap between the two domains, indicating that *Cyp26a1* is not expressed in r3, whereas embryos double-stained for *Cyp26a1* and *Krox20* exhibited no gap between these two domains, suggesting that *Cyp26a1* is expressed posteriorly to r2 (Fig. 1A-C). Double-staining for *Cyp26a1* and *Epha2* revealed a gap between their expression domains where presumptive r3 lies (Fig. 1D-E). These results demonstrate that *Cyp26a1* is expressed in the anterior hindbrain down to the r2/r3 boundary.

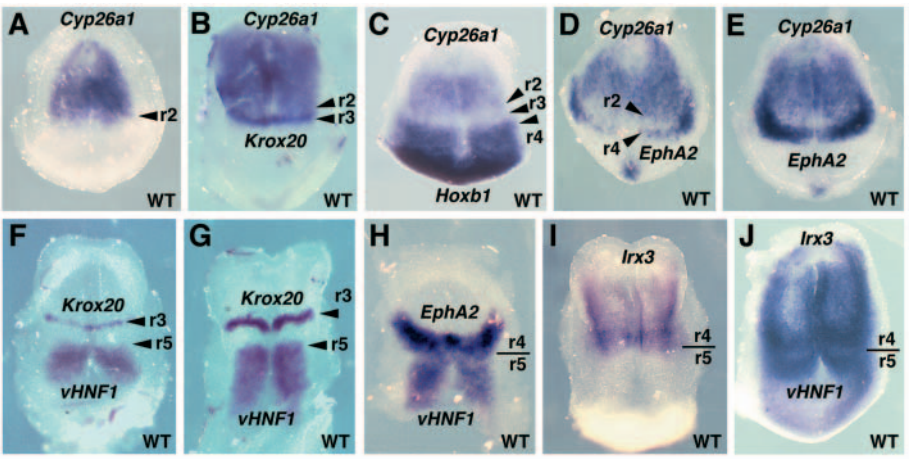

Fig. 1. Boundaries of *Cyp26a1*, *vHnf1* and *Irx3* expression in the early mouse hindbrain. Anterior is oriented towards the top in all panels, and all embryos are wild type (WT). (A-E) *Cyp26a1* mRNA at E7.75-E8.0 is localized anteriorly with its posterior extent to presumptive rhombomere 2 (r2) of the hindbrain, as determined by whole-mount in situ hybridization. Shown is an E7.75 embryo stained for expression of *Cyp26a1* (A), and an E8.0 embryo double-stained for expression of *Cyp26a1* and *Krox20*, a known marker for r3 (B). Also shown is an E7.75 embryo double-stained for expression of *Cyp26a1* and *Hoxb1*, a known marker for r4 (C); neither gene is expressed in r3. Double-staining for *Cyp26a1* and *Epha2* (a marker for presumptive r4 and the adjacent mesoderm) at E7.75 (D) and E7.9 (E) demonstrates a space between the two expression domains where presumptive r3 lies; expression of *Epha2* in the mesoderm adjacent to r4 overlaps the lateral regions of presumptive r3 from this viewpoint. (F,G) Expression of *vHnf1* is localized posteriorly with its anterior border at the r4/r5 boundary Shown are embryos double-stained for expression of *vHnf1* and *Krox20* at E8.0 when *Krox20* is expressed only in r3 (F) and at E8.25 when *Krox20* is expressed strongly in r3 and weakly in r5 (G). There is a gap between *Krox20* and *vHnf1* expression at both stages, indicating that *vHnf1* expression extends to r5 but not into r4. (H) Double staining for *Epha2* and *vHnf1* shows that *vHnf1* is expressed in r5 up to the r4/r5 boundary. (I) Expression of *Irx3* at E8.25 is localized anteriorly with a posterior border at the r4/r5 boundary when compared with the expression pattern of *vHnf1* (G). (J) Double staining for *Irx3* and *vHnf1* expression at E8.0 provides evidence that these two expression domains meet at r4/r5.

In zebrafish embryos, *vHnf1* has been found to be expressed in the posterior hindbrain up to the r4/r5 boundary (Wiellette and Sive, 2003; Hernandez et al., 2004). In mouse embryos, *vHnf1* expression has been reported in the posterior hindbrain at E8.0-E8.5 (Coffinier et al., 1999; Barbacci et al., 1999), but its anterior boundary has not been defined. E8.0 mouse embryos double stained for *vHnf1* and *Krox20* (which is limited to r3 at E8.0) exhibited a gap between the two domains, indicating that *vHnf1* is not expressed in r4 (Fig. 1F). A similar gap was observed at E8.25 when *Krox20* is expressed strongly in r3 and weakly in r5, indicating that *vHnf1* expression overlaps the r5 domain of *Krox20* (Fig. 1G; also see Fig. 2M for *Krox20* r5 expression). Double-staining for *Epha2* (which is limited to r4) and *vHnf1* revealed that their expression domains meet without a gap (Fig. 1H). These results indicate that *vHnf1* is expressed in the posterior hindbrain up to the r4/r5 boundary.

The zebrafish *iro7* gene is expressed in the anterior hindbrain down to the r4/r5 boundary (Lecaudey et al., 2004). The mouse homolog, *Irx3*, is also expressed in the anterior neural plate (Bosse et al., 1997), but its anterior boundary is not defined. At E8.25, *Irx3* was expressed in the anterior hindbrain down to approximately the r4/r5 boundary (Fig. 1I) when compared with the previously established r5 expression border of *vHnf1* at E8.25 (Fig. 1G). Double-staining for *Irx3* and *vHnf1* expression at E8.0 provided further evidence that the posterior border of *Irx3* expression is in r4 as it lies directly adjacent to the *vHnf1* r5 domain (Fig. 1J).

Shifting boundaries of RA activity along the hindbrain

RA synthesis during early mouse hindbrain development is controlled by *Raldh2* expressed in the paraxial mesoderm (Niederreither et al., 2000), and RA degradation is controlled at least in part by *Cyp26a1* expressed in the anterior neural plate (Sakai et al., 2001; Abu-Abed et al., 2001). A similar situation exists in *Xenopus*, chick and zebrafish embryos (Hollemann et al., 1998; Swindell et al., 1999; Kudoh et al., 2002). This has led to the hypothesis that RA synthesized in the paraxial mesoderm may diffuse anteriorly across the hindbrain until it meets the *Cyp26a1* expression domain, perhaps establishing a posterior-high gradient of RA activity across the hindbrain. Such RA is presumably needed for induction of various genes including *Hoxb1* whose expression in r4 requires two retinoic acid response elements (RAREs) located 3′ and 5′ of the promoter (Marshall et al., 1994; Studer et al., 1994). Among the various vertebrate embryo models of hindbrain development, the mouse is unique in that an RA-reporter transgene (*RARE-lacZ*) has been constructed that allows RA activity to be detected during the early stages of hindbrain development (Rossant et al., 1991). *RARE-lacZ* expression is completely eliminated in the hindbrain of *Raldh2*$^{-/-}$ embryos and can be induced in all embryonic cells of wild-type embryos following treatment with excess RA, thus demonstrating that this transgene is indeed a faithful reporter for endogenous RA (Niederreither et al., 1999; Mic et al., 2002). Previous studies using *RARE-lacZ* embryos have not defined the anterior boundary of RA activity during the headfold stages, although it is clear that RA is present in the hindbrain up to the r5 boundary at E8.25-E9.25 (Sakai et al., 2001; Mic et al., 2002). Here, we have examined wild-type headfold stage embryos to determine if RA exists anterior to r5.

As our studies above have now defined r2/r3 as the posterior boundary of *Cyp26a1* expression, we examined embryos double-stained for *Cyp26a1* and *RARE-lacZ* to determine the anterior extent of RA activity in the mouse hindbrain. At E7.4, expression of *Cyp26a1* expression was observed, but not *RARE-lacZ* (Fig. 2A). At E7.5, weak *RARE-lacZ* expression was observed with an ill-defined anterior boundary near the node, but by E7.6 *RARE-lacZ* expression now extended further anterior up to the *Cyp26a1* domain with a well-defined boundary at r2/r3 (Fig. 2B,C). The initiation of *RARE-lacZ*

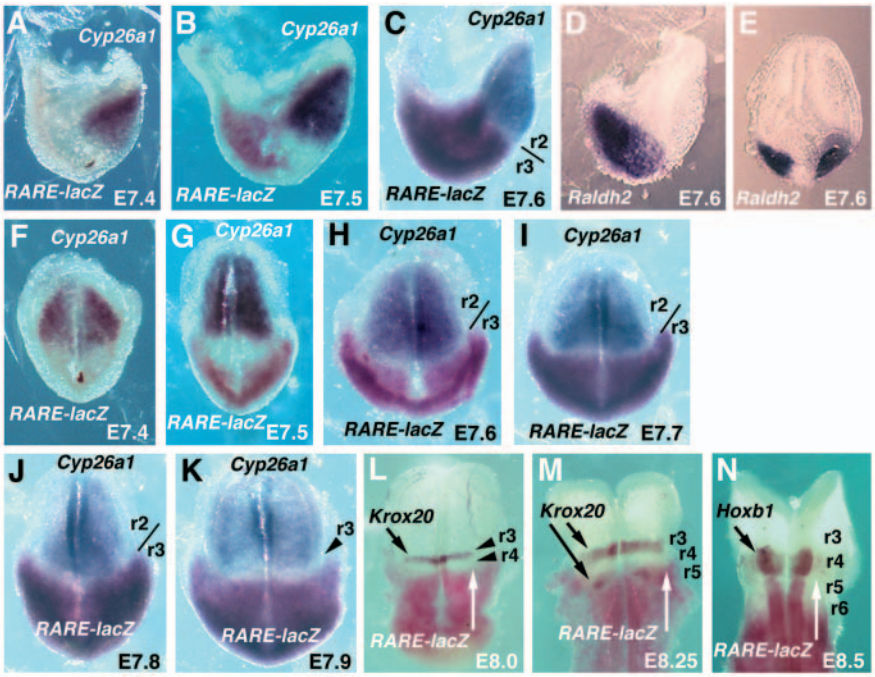

Fig. 2. Boundaries of hindbrain RA activity along the anteroposterior axis. All embryos are homozygous for the *RARE-lacZ* RA-indicator transgene; stages are indicated on each panel. Anterior is oriented towards the right in A-D (lateral views) and towards the top in E-O (dorsal views). (A-C) Embryos were double-stained for *RARE-lacZ* expression (β-galactosidase activity), which is observed posteriorly, followed by *Cyp26a1* expression (in situ hybridization), which is observed anteriorly with its posterior border at r2. *RARE-lacZ* expression is first seen at E7.5 (B) and the r2/r3 division marked in C indicates the anterior extent of *RARE-lacZ*, which reaches r3 at E7.6. (D,E) *Raldh2* expression detected posteriorly in the paraxial mesoderm at E7.6. (F-K) Dorsal view of embryos double-stained for expression of *RARE-lacZ* and *Cyp26a1* from E7.4-E7.9. *RARE-lacZ* expression has reached r3 by E7.6 (H), but begins to clear from r3 by E7.9 (K). (L-N) Double-stained embryos showing *RARE-lacZ* expression (white arrows indicate its anterior extent at the r4/r5 boundary) and expression of either *Krox20* in r3 at E8.0 (L), *Krox20* in both r3 and r5 at E8.25 (M) and *Hoxb1* in r4 at E8.5 (N).

expression correlates with the initiation of *Raldh2* expression as we observed no *Raldh2* expression at E7.4, weak expression at E7.5 and strong expression at E7.6 in the posterior paraxial mesoderm (Fig. 2D-E; data not shown). Dorsal views of a series of double-stained embryos from E7.4-E7.8 demonstrates the initiation of *RARE-lacZ* expression at E7.5, and the formation of an r2/r3 boundary of RA activity from E7.6-E7.8 (Fig. 2F-J). The border formed between *Cyp26a1* and *RARE-lacZ* expression is very dynamic, but embryos at E7.8 provided evidence that the two domains do temporarily abut without an intervening gap (Fig. 2J). At E7.9, we observed a clearing of *RARE-lacZ* expression in r3, indicating the occurrence of a posterior shift in the RA boundary to ~r3/r4 (Fig. 2K). E8.0 and E8.25 embryos double stained for *Krox20* and *RARE-lacZ* revealed that RA activity had retreated to an r4/r5 boundary by E8.0 as the anterior boundary of *RARE-lacZ* expression overlapped that of *Krox20* in r5 (Fig. 2L-M). Thus, RA activity clears from r4 by E8.0-E8.25. E8.5 embryos double-stained for *Hoxb1* (now limited to r4) and *RARE-lacZ* demonstrated that RA activity still remained in r5 and further posterior, thus indicating that a stable RA activity boundary at r4/r5 was being maintained (Fig. 2N). These findings indicate that RA does not form a stable gradient across the early hindbrain, but instead displays shifting boundaries of RA activity first at r2/r3 then at r4/r5. Our results also demonstrate that RA exists transiently in r3 and r4 during the time when *Hoxb1* expression initiates in the hindbrain.

RA induction of *Cyp26c1* in r4

Although the expression domain of *Cyp26a1* is positioned properly to account for the formation of an r2/r3 boundary of RA activity, it cannot account for the subsequent shift to r4/r5. Previous studies have shown that a related enzyme encoded by *Cyp26c1* is expressed in r2 and r4 as well as the adjacent head mesoderm in E8.0-E8.5 mouse embryos (Tahayato et al., 2003). A third related mouse enzyme encoded by *Cyp26b1* is expressed in r3 and r5 at approximately E8.25, but this is clearly after *Cyp26c1* expression is already apparent in r4 (MacLean et al., 2001) (I.O.S. and G.D., unpublished).

Wild-type E7.75 headfold embryos double-stained for *Cyp26c1* and *RARE-lacZ* revealed a domain of *Cyp26c1* expression limited to the head mesoderm anterior to the r2/r3 boundary of RA activity, and thus not in a position to regulate RA levels in the hindbrain at this stage (Fig. 3A,B). E7.75 *Raldh2*$^{-/-}$ embryos double stained for *Cyp26c1* and *RARE-lacZ* demonstrated that *RARE-lacZ* expression was completely lost when *Raldh2* function was lost, whereas *Cyp26c1* expression was maintained in the head mesoderm (Fig. 3A,B). Wild-type embryos stained for *Cyp26c1* expression from E7.75-E8.0 revealed that the early domain of head mesoderm expression intensifies up to E8.0 and that expression in r4 begins at about E7.9 then intensifies at E8.0 (Fig. 3C-E). Comparison of E8.25-E8.5 wild-type and *Raldh2*$^{-/-}$ embryos double-stained for *Cyp26c1* and *Raldh2* demonstrated that a loss of RA synthesis results in a loss of *Cyp26c1* expression in r4 but expression remains in r2 and head mesoderm (n=5; Fig. 3F-I). *Cyp26c1* expression in the head mesoderm of *Raldh2*$^{-/-}$ embryos is intensified and expanded posteriorly, and the r2 expression domain is wider along the anteroposterior axis (Fig. 3H-I). Thus, *Cyp26c1* expression initiates in r4 at approximately the same time that we observe a shift in RA activity from r2/r3 to r4/r5, and RA is required for r4 induction of *Cyp26c1*.

RA is required for early expansion of *Hoxb1* expression to r3/r4 border

RA generated by *Raldh2* has previously been shown to be required for r4 expression of *Hoxb1* in E8.25-E8.5 mouse embryos (Niederreither et al., 2000). We examined the effect of a loss of RA synthesis on the early anterior expansion of *Hoxb1* expression. The normal expansion of *Hoxb1* expression from the node to the r3/r4 boundary is shown in wild-type embryos examined from E7.5-E8.0 (Fig. 4A-C). By E8.0, *Hoxb1* is continuously expressed from the node to r3/r4 and has not yet been restricted to r4 (Fig. 4C). *Raldh2*$^{-/-}$ embryos at E8.0 completely lacked the anterior expansion of *Hoxb1* to r3/r4, thus resembling wild-type E7.5 embryos in which *Hoxb1* expression was limited anteriorly to a region near the node

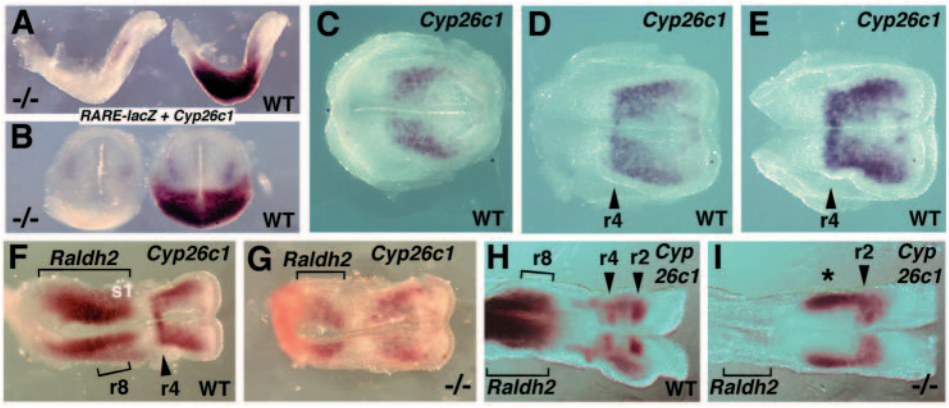

Fig. 3. RA generated by *Raldh2* is required for *Cyp26c1* expression in r4. Anterior is oriented towards the right in all panels except B, where anterior is towards the top. (A,B) Lateral (A) and dorsal (B) views of E7.75 *Raldh2*$^{-/-}$ (–/–) and wild-type (WT) embryos double stained for expression of *RARE-lacZ* (β-galactosidase activity) observed posteriorly followed by staining for *Cyp26c1* mRNA (whole-mount in situ hybridization) observed anteriorly. The *Raldh2*$^{-/-}$ embryo lacks *RARE-lacZ* expression, but *Cyp26c1* expression at this stage (head mesoderm) is not affected. (C-E) *Cyp26c1* expression in dorsal view of wild-type embryos at E7.75 (C), E7.9 (D) and E8.0 (E) showing its initial expression in r4, with the remaining expression occurring in head mesoderm. (F-I) Dorsal view of double staining for expression of *Raldh2* (posterior) and *Cyp26c1* (anterior) in *Raldh2*$^{-/-}$ and wild-type embryos at E8.25 (F-G) and E8.5 (H-I). *Raldh2*$^{-/-}$ embryos (marked by a large reduction in *Raldh2* mRNA) lack the r4 domain of *Cyp26c1* expression while retaining the r2 expression domain normally observed by E8.5. *Raldh2*$^{-/-}$ embryos also exhibit an abnormal posterior extension of *Cyp26c1* expression in head mesoderm at E8.5 marked by an asterisk (I). The anterior extent of *Raldh2* mRNA in wild-type embryonic mesoderm is somite 1 (s1), which is adjacent to rhombomere 8 (r8) at E8.25 (F).

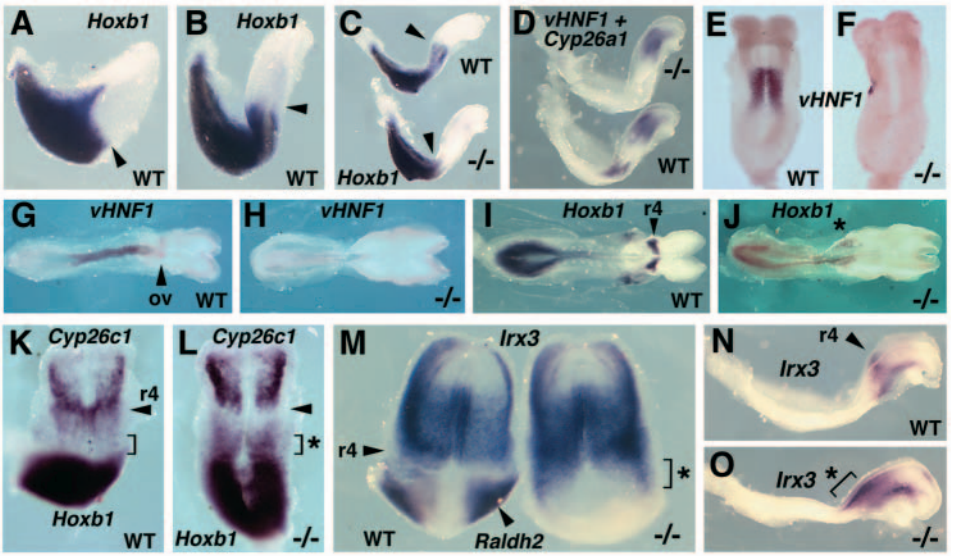

Fig. 4. Requirement of RA for induction of *Hoxb1* and *vHnf1*, and for repression of *Irx3*. Anterior is towards the right (A-D,G-J,N-O) or towards the top (E-F,K-M). (A-C) *Hoxb1* expression normally exhibits an anterior extension into the hindbrain, but this extension is eliminated in *Raldh2*$^{-/-}$ embryos. Arrowheads indicate the anterior extent of *Hoxb1* expression, which is at the level of the node in a wild-type embryo at E7.5 (A), and anterior to the node in the hindbrain at E7.75 (B) and E8.0 (C). The anterior expansion of *Hoxb1* expression into the hindbrain is eliminated in an *Raldh2*$^{-/-}$ embryo (C). (D) Double in situ hybridization at E8.0, showing a lateral view of *vHnf1* expression posteriorly in the hindbrain and *Cyp26a1* expression anteriorly. The *Raldh2*$^{-/-}$ embryo lacks *vHnf1* expression, but *Cyp26a1* is not affected by a loss of RA. (E,F) A dorsal view of *vHnf1* expression at E8.25, showing a complete loss of *vHnf1* mRNA in an *Raldh2*$^{-/-}$ embryo. (G,H) *vHnf1* expression at E8.5 is normally present in the anterior spinal cord, as well as the posterior hindbrain up to the otic vesicle (ov), but an *Raldh2*$^{-/-}$ embryo lacks *vHnf1* mRNA in these neural tissues. (I,J) Neural expression of *Hoxb1* at E8.5 is normally limited to r4 in the hindbrain and to the posterior spinal cord, but an *Raldh2*$^{-/-}$ embryo lacks the r4 expression domain and instead exhibits a weak expression domain shifted to the most posterior region of the hindbrain (asterisk). (K,L) Double-staining for expression of *Cyp26c1* (anterior) and *Hoxb1* (posterior) at E8.25 demonstrates that both overlap in r4 of wild-type embryo, but that the *Raldh2*$^{-/-}$ embryo lacks expression of both in the region where r4 should have developed (arrowhead). The brackets indicate a region where the *Raldh2*$^{-/-}$ embryo retains expression of *Hoxb1* in the most posterior region of the hindbrain (asterisk), whereas this domain of *Hoxb1* expression is normally lost by E8.25. (M) Double staining for expression of *Irx3* and *Raldh2* in E7.9 wild-type and *Raldh2*$^{-/-}$ embryos demonstrates the normal posterior border of *Irx3* expression at r4 separated from the *Raldh2* domain, whereas the mutant exhibits a loss of *Raldh2* expression and an expansion of *Irx3* expression into the posterior hindbrain (bracket and asterisk). (N,O) *Irx3* expression at E8.25 is normally present in the anterior hindbrain with a posterior limit at r4 (arrowhead), but an *Raldh2*$^{-/-}$ embryo exhibits a posterior expansion of *Irx3* expression to the hindbrain/spinal cord junction (bracket and asterisk).

(*n*=3; Fig. 4A,C). These findings demonstrate that RA generated by *Raldh2* is required for the initial expansion of *Hoxb1* expression throughout the hindbrain up to r3/r4.

RA regulates *vHnf1* and *Irx3* during r4/r5 boundary formation

Recent studies in zebrafish suggest that two homeodomain genes, *vHnf1* and *iro7* (*Irx3*), play crucial roles in establishment of the r4/r5 boundary and r4 restriction of *Hoxb1* expression (Wiellette and Sive, 2003; Lecaudey et al., 2004; Hernandez et al., 2004). Those studies demonstrated that *vHnf1* functions as a repressor for *Hoxb1*, that *vHnf1* and *iro7* function as mutual repressors on opposite sides of the r4/r5 boundary, and that RA is required for *vHnf1* expression. We examined these genes in mouse embryos that lack RA synthesis. E8.0 wild-type and *Raldh2*$^{-/-}$ embryos double stained for *Cyp26a1* and *vHnf1* demonstrated that a loss of RA results in a complete loss of *vHnf1* expression in the hindbrain, but no effect on *Cyp26a1* expression in the anterior neural plate (*n*=3; Fig. 4D). *Raldh2*$^{-/-}$ embryos examined at E8.25-E8.5 lacked *vHnf1* expression in the hindbrain as well as the spinal cord (*n*=3; Fig. 4E-H).

Raldh2$^{-/-}$ embryos at E8.25-E8.5 lacked the r4 domain of *Hoxb1* expression, but exhibited residual *Hoxb1* expression at the posterior hindbrain/spinal cord junction that would normally be eliminated by this stage (*n*=3; Fig. 4I-L). *Raldh2*$^{-/-}$ embryos double stained for *Cyp26c1* and *Hoxb1* demonstrated a complete loss of expression of both genes in r4, but retention of *Hoxb1* expression at the posterior hindbrain/spinal cord junction lying just posterior to the remaining head mesodermal domain of *Cyp26c1* (Fig. 4K-L). This residual posterior domain of *Hoxb1* expression may be the remnants of that observed near the node in E8.0 *Raldh2*$^{-/-}$ embryos (Fig. 4C). These findings suggest that mouse *vHnf1* may function in repression of *Hoxb1*, as the loss of *vHnf1* observed in *Raldh2*$^{-/-}$ embryos may result in a failure to repress this RA-independent posterior domain of *Hoxb1* expression.

Raldh2$^{-/-}$ embryos examined at E7.9-E8.25 exhibited a posterior expansion of *Irx3* expression in the hindbrain (*n*=6; Fig. 4M-O). Whereas *Irx3* expression in the anterior hindbrain normally displayed an r4 posterior boundary, a loss of RA synthesis resulted in an expansion posteriorly throughout the hindbrain to the spinal cord junction. This abnormal posterior domain of *Irx3* expression lies at approximately the same location where *Hoxb1* expression is abnormally retained in the mutant posterior hindbrain (compare Fig. 4J with Fig. 4O). These results suggest that the effect of RA on mouse *Irx3* expression may be due to the requirement of RA for expression of *vHnf1*, which may function (as it does in zebrafish) as a repressor of both *Irx3* and *Hoxb1* in the posterior hindbrain up to the r4/r5 boundary.

Requirement of *vHnf1* for r4/r5 gene expression boundary

We examined the effect of a loss of *vHnf1* function on expression of *Hoxb1* in the mouse hindbrain. Disruption of

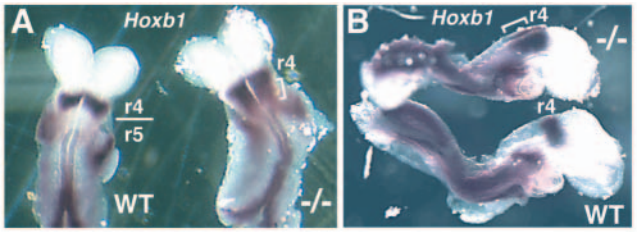

Fig. 5. *vHnf1* is required to define r4/r5 gene expression boundary in mouse embryos. (A,B) Dorsal and lateral views of *Hoxb1* expression in wild-type and conditional *vHnf1*$^{-/-}$ embryos. The normal r4/r5 boundary of *Hoxb1* expression is indicated in wild-type embryos. *vHnf1*$^{-/-}$ embryos exhibit expansion of *Hoxb1* expression posterior to the r4/r5 boundary in the hindbrain (brackets).

mouse *vHnf1* has previously been shown to result in an extra-embryonic defect of visceral endoderm formation, leading to embryonic lethality prior to hindbrain formation (Coffinier et al., 1999; Barbacci et al., 1999). A conditional *vHnf1* mutant mouse has allowed the function of this gene to be examined at later stages (Coffinier et al., 2002). Here, conditional *vHnf1*$^{-/-}$ embryos were generated from matings with mice containing the *Mox2-Cre* (MORE) transgene, which stimulates Cre/lox-mediated deletion throughout the epiblast following implantation, but does not affect extra-embryonic tissues (Tallquist and Soriano, 2000). *Hoxb1* expression in E8.5 conditional *vHnf1*$^{-/-}$ embryos was observed posterior to the normal r4/r5 boundary, indicating a failure to restrict *Hoxb1* expression to r4 (n=3; Fig. 5A-B). These findings indicate that the function of *vHnf1* as a *Hoxb1* repressor is conserved in mouse and zebrafish.

Physiological dose of RA rescues r3/r4 and r4/r5 gene expression boundaries

Previous studies have demonstrated that *Raldh2*$^{-/-}$ embryos can be rescued by low-dose maternal dietary RA supplementation which was quantitated to provide embryonic RA in the normal physiological range (Mic et al., 2003). Following such RA supplementation and analysis at E8.0, we found that *RARE-lacZ* expression was recovered in *Raldh2*$^{-/-}$ embryos, and that the pattern of *RARE-lacZ* expression observed in RA-treated wild-type embryos was indistinguishable from that of untreated wild-type embryos (n=3; Fig. 6A; compare to Fig. 2L). The anterior boundary of *RARE-lacZ* expression in such RA-rescued *Raldh2*$^{-/-}$ embryos was similar to that of wild type, suggesting that RA degradation anteriorly by Cyp26 enzymes may be preventing this low dose of RA from acting anteriorly. Following RA supplementation and analysis at E8.75, *Raldh2*$^{-/-}$ embryos exhibited a recovery of *Hoxb1* expression restricted to r4 (n=3; Fig. 6B,C) and a recovery of *Cyp26c1* expression in r4 (n=3; Fig. 6D,E). RA supplementation of *Raldh2*$^{-/-}$ embryos also resulted in a recovery of *vHnf1* expression (n=3; Fig. 6F-G) and the *Irx3* expression domain was now restricted posteriorly to the r4/r5 boundary (n=4; Fig. 6H).

These observations indicate that a low dose of exogenous RA can mimic the local endogenous RA synthesis function of *Raldh2* in paraxial mesoderm. The uneven distribution of RA in treated *Raldh2*$^{-/-}$ embryos follows the normal pattern probably because of *Cyp26a1* and *Cyp26c1*, which are expressed relatively normally (Fig. 4D; Fig. 6E). It is clear that anterior cells of the embryo are responsive to RA, as previous studies have shown that treatment of embryos with a high dose of RA induces *RARE-lacZ* in all cells of the embryo (Rossant et al., 1991; Mic et al., 2002). Thus, it is clear that RA is normally functioning locally in a paracrine fashion and that a low dose of RA can mimic this paracrine function, whereas a high dose of RA results in abnormal systemic distribution.

Excess RA induces anterior shifts in *Cyp26c1* domain and *Hoxb1/vHnf1* boundary

Our results suggest that *Cyp26c1* may provide an RA degradation function in r4 to limit the anterior border of RA activity, and that this border may be crucial for establishing a border between *Hoxb1* and *vHnf1* at the r4/r5 boundary.

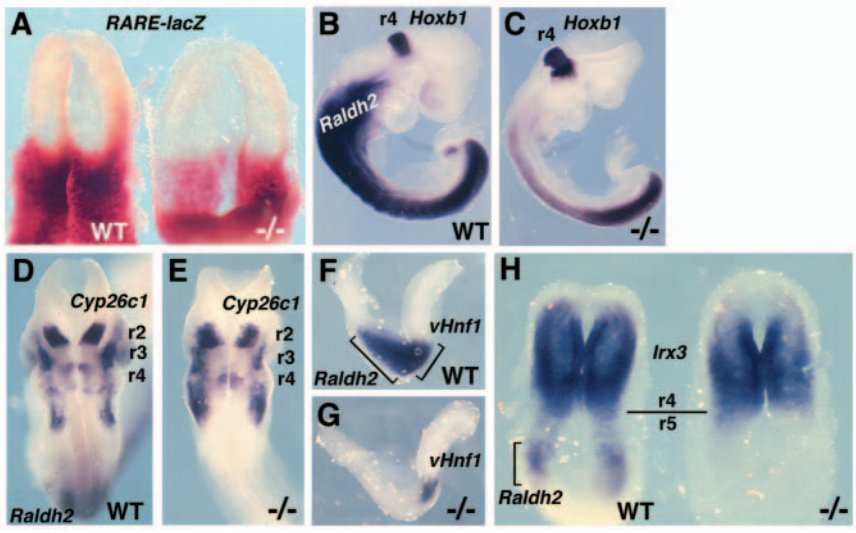

Fig. 6. Maternal dietary RA supplementation rescues r3/r4 and r4/r5 gene expression boundaries in *Raldh2*$^{-/-}$ embryos. Embryos were subjected to low-dose maternal dietary RA supplementation from E6.75-E8.0 (A,F-H) or from E6.75-E8.25 (B-E). (A) *RARE-lacZ* expression in RA-treated wild-type and *Raldh2*$^{-/-}$ embryos at E8.0. The *Raldh2*$^{-/-}$ embryo exhibits an anterior border of *RARE-lacZ* expression similar to that observed in a wild-type littermate, although the intensity of staining was less than that observed in the wild-type embryo. (B-E) RA treatment of wild-type and *Raldh2*$^{-/-}$ embryos followed by analysis at E8.75 demonstrates that RA administration rescues r4 expression of *Hoxb1* (B,C) and *Cyp26c1* (D,E) in *Raldh2*$^{-/-}$ embryos. RA-treated wild-type embryos exhibited relatively normal expression of *Hoxb1* and *Cyp26c1*. All embryos were also simultaneously stained for *Raldh2* expression, which appeared only in wild-type embryos as indicated. (F-H) RA treatment of *Raldh2*$^{-/-}$ embryos followed by analysis at E8.0 results in posterior hindbrain expression of *vHnf1* (F-G) and relatively normal r4/r5 restriction of *Irx3* (H). Embryos were double stained for *Raldh2* expression, which was observed only in wild-type embryos (brackets).

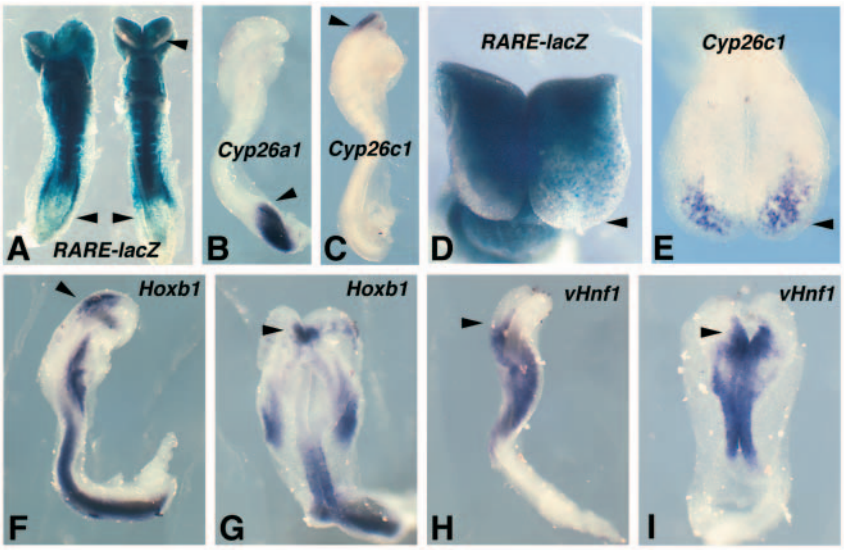

Fig. 7. Teratogenic dose of RA induces anterior shifts in the *Cyp26c1* expression domain and the *Hoxb1/vHnf1* expression boundary. All embryos were subjected to a 20 mg/kg dose of RA at E7.5, then analyzed 18 hours later at E8.25 (see Figs 2-4 for untreated controls). (B,C,F,H) Lateral view; (G,I) dorsal view; (A) ventral view; (D,E) anterior view. (A) *RARE-lacZ* expression is observed to the anterior tip of some embryos (left embryo), but in other embryos *RARE-lacZ* expression is reduced at the anterior tip (upper arrowhead in right embryo). *RARE-lacZ* expression in all embryos is greatly reduced posteriorly in the tailbud (lower arrowheads) when compared with the trunk. (B-C) *Cyp26a1* expression is observed in the tailbud (arrowhead) similar to untreated embryos (MacLean et al., 2001), whereas *Cyp26c1* expression has been shifted to the anterior-most region of the embryo (arrowhead). A lateral view shows that most *Cyp26c1* expression is neural, with mesodermal expression being greatly reduced. (D,E) Comparison of anterior expression patterns of *RARE-lacZ* and *Cyp26c1* demonstrates that the abnormal domain of *Cyp26c1* expression lies in the region where *RARE-lacZ* expression first clears anteriorly. (F,G) The anterior domain of *Hoxb1* expression (arrowhead) has been shifted further anteriorly from where it should normally reside at r4. (H-I) The anterior border of *vHnf1* expression (arrowhead) has been shifted further anteriorly. This abnormal anterior border of *vHnf1* expression lies posterior to the abnormal expression domains of *Cyp26c1* and *Hoxb1*.

Genetic studies of the related genes *Cyp26a1* and *Cyp26b1* have revealed that both have a function in RA degradation (Sakai et al., 2001; Abu-Abed et al., 2001; Yashiro et al., 2004), but genetic studies for *Cyp26c1* have not been reported. In order to test this potential function for *Cyp26c1*, we treated *RARE-lacZ* embryos with a teratogenic dose of RA to disrupt the normal boundary of RA activity. Previous studies have shown that a 20 mg/kg dose of RA administered at E7.5 leads to induction of *RARE-lacZ* expression throughout the anterior region of the embryo when examined 6 hours after treatment (Rossant et al., 1991), thus eliminating the normal RA boundary in the hindbrain. When we administered a 20 mg/kg dose of RA at E7.5, we found that some embryos examined at E8.25 (18 hours after treatment) still exhibited a shift in *RARE-lacZ* expression completely to the anterior tip (n=9/15), but that some embryos exhibited clearing of *RARE-lacZ* expression at the anterior-most region of the embryo (n=6/15) and that all embryos exhibited reduced *RARE-lacZ* expression posteriorly in the tailbud (Fig. 7A). *Cyp26a1* expression following this treatment was observed in the tailbud where *RARE-lacZ* expression was reduced, consistent with its known function in RA degradation (n=3; Fig. 7B); expression was similar to that in untreated embryos, which normally downregulate *Cyp26a1* anteriorly and upregulate expression in the tailbud by E8.25 (MacLean et al., 2001). However, this RA treatment resulted in a shift in *Cyp26c1* expression to the anterior-most region of the embryo (n=7; Fig. 7C; compare with untreated control in Fig. 3F). A closer comparison demonstrates that this new *Cyp26c1* expression domain lies in approximately where *RARE-lacZ* expression first begins to clear anteriorly (Fig. 7D-E). This provides evidence that *Cyp26c1* is functioning to degrade RA in this anteriorly shifted domain.

Previous studies have shown that a 20 mg/kg RA dose administered at E7.5 results in an anterior shift in hindbrain *Hoxb1* expression observed 18 hours later at E8.25 (Conlon and Rossant, 1992). We also found that *Hoxb1* expression in the brain was shifted anteriorly with this RA treatment (n=5; Fig. 7F,G), plus we observed that *vHnf1* expression was also shifted anteriorly under these conditions (n=6; Fig. 7H,I). RA treatment resulted in a coordinate shift in the *Hoxb1* and *vHnf1* expression domains such that the new posterior border of *Hoxb1* expression in the brain lies at approximately the same location as the new anterior border of *vHnf1* expression (Fig. 7G,I). These findings provide evidence that *Cyp26c1* functions to restrict the anterior boundary of RA, and that this RA boundary helps determine the location of the *Hoxb1/vHnf1* boundary.

Discussion

Mechanism of RA action during establishment of hindbrain *Hoxb1* expression

The studies reported here provide new information on how RA-synthesizing and RA-degrading enzymes cooperate in regulation of hindbrain gene expression, resulting in restricted expression domains for *Hoxb1* and its repressor *vHnf1*, as well as formation of an *Irx3/vHnf1* boundary at r4/r5. Previous genetic studies have demonstrated that *Raldh2* functions in RA synthesis (Niederreither et al., 1999; Mic et al., 2002) and that P450 enzymes encoded by the Cyp26 family function as RA-degrading enzymes (Sakai et al., 2001; Abu-Abed et al., 2001; Yashiro et al., 2004), but the effect of these enzymes on the spatiotemporal localization of RA activity in the hindbrain has been unclear. By carefully examining mouse head-fold stage embryos carrying the *RARE-lacZ* RA-reporter transgene, we have shown that RA activity coincides with the onset of *Raldh2* expression in the trunk paraxial mesoderm and that RA is secreted from these cells and travels anteriorly. Furthermore, double-labeling experiments have revealed that distinct boundaries of RA activity are created by expression of

Cyp26a1, which provides a short-lived r2/r3 boundary, and later of *Cyp26c1*, which provides a long-lived r4/r5 boundary. In addition, through analysis of *Raldh2*$^{-/-}$ mouse embryos, we discovered that hindbrain expression of *vHnf1* is completely eliminated and that *Irx3* expression expands posteriorly, thus indicating that repression of *Hoxb1* and *Irx3* posterior to r5 is indirectly controlled by RA via induction of *vHnf1*. This conclusion was supported by our observation that *vHnf1*$^{-/-}$ embryos exhibit expansion of *Hoxb1* expression posterior to r4.

Previous studies on chick embryos treated with RA receptor antagonists provided evidence that hindbrain patterning requires graded responses to RA, with a higher RA concentration being required posteriorly (Dupé and Lumsden, 2001). Our findings with mouse embryos demonstrate that the length of RA exposure is also important for regulating hindbrain gene expression as RA activity was observed for only 6-8 hours in r3-r4 (E7.6-E7.9), but for at least 20 hours in r5-r8 (E7.6-E8.5). The studies we present also make it clear that a stable RA gradient is not established across the hindbrain, but that the initial gradient of RA entering the posterior hindbrain is converted by RA-degrading enzymes into RA boundaries that shift over time such that anterior tissues receive a short pulse of RA and posterior tissues receive a long pulse of RA.

Our studies also reveal that the initial RA boundary at r2/r3 is independent of RA activity, as *Cyp26a1* expression does not require RA, but that the shift to an r4/r5 boundary is dependent upon RA to activate *Cyp26c1* expression in r4. Our findings provide evidence that RA is present in presumptive r3, r4 and r5 to directly regulate the activity of RA receptors binding the 3′ and 5′ RAREs of *Hoxb1*, which have previously been shown to be required for induction up to the r3/r4 border and repression in r3/r5, respectively (Marshall et al., 1994; Studer et al., 1994). Interestingly, the RA present in r5 can repress *Hoxb1* not only directly through the previously established 5′ RARE, but also indirectly by inducing *vHnf1* in the posterior hindbrain up to the r4/r5 border. A model of hindbrain gene expression incorporating the role of shifting boundaries of RA activity is presented in Fig. 8.

It is unclear how RA-liganded RA receptors bound to the 5′ RARE of *Hoxb1* result in r3/r5 repression whereas RA-liganded RA receptors bound to the 3′ RARE of *Hoxb1* stimulate widespread induction in the posterior hindbrain up to r4. It is possible that additional transcriptional regulatory proteins expressed in r3 and/or r5 bind near the 5′ RARE and interact with an RA-liganded RA receptor in such a fashion that results in *Hoxb1* repression in r3 and r5 rather than induction. In addition, as a loss of *Cyp26a1* function in mouse embryos results in ectopic expression of *Hoxb1* in r3 (but not r5) and presumably higher RA activity in r3 (Sakai et al., 2001; Abu-Abed et al., 2001), this suggests that the 5′ RARE is insufficient to repress *Hoxb1* in r3. Perhaps the relative activities of the 3′ and 5′ RAREs are determined by levels of RA such that a higher than normal level of RA in r3 disrupts 5′ RARE repression and/or allows 3′ RARE induction of *Hoxb1*.

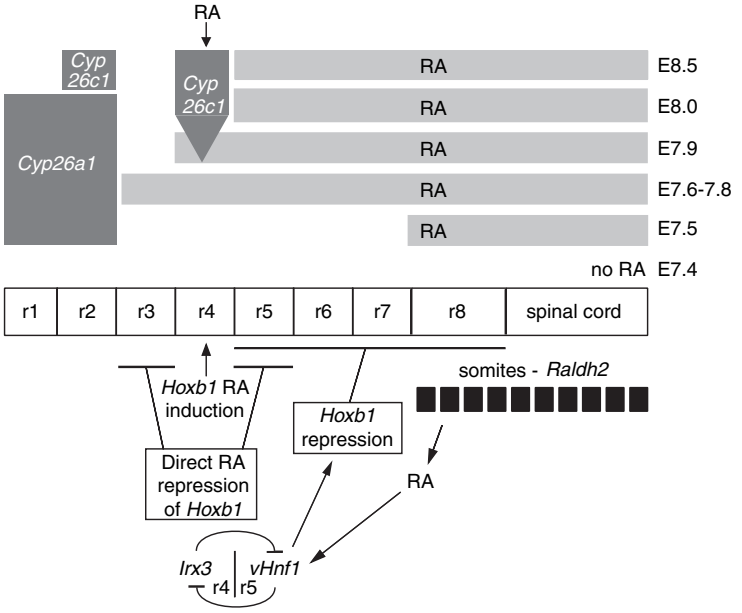

Fig. 8. Role of shifting RA boundaries during posterior hindbrain segmentation. A model for RA action during mouse hindbrain development is shown. Our findings demonstrate that RA generated by *Raldh2* in the paraxial mesoderm travels anteriorly to presumptive r3 and r4 during establishment of *Hoxb1* expression, but that this is very transient. Initially, RA forms an early anterior boundary at r2/r3 (next to the r2 border of *Cyp26a1* expression) followed soon after by a late anterior boundary at r4/r5 (next to the r4 border of *Cyp26c1* expression). RA is therefore present in r3/r4/r5 to directly regulate *Hoxb1* induction and repression through previously described 3′ and 5′ RAREs (Marshall et al., 1994; Studer et al., 1994). We also demonstrate that *vHnf1* requires RA for posterior hindbrain expression, and that *vHnf1* is needed to limit the posterior extent of *Hoxb1* expression to help establish the r4/r5 expression boundary for *Hoxb1*. Thus, RA acts directly to induce *Hoxb1* expression and then RA acts both directly and indirectly (through induction of *vHnf1*) to restrict *Hoxb1* expression to r4. Also shown is a mutual repression between *Irx3* and *vHnf1* at the r4/r5 boundary, which has been demonstrated in zebrafish (Lecaudey et al., 2004) and is supported by our findings in mouse.

RA signaling is required for neural expression of *vHnf1*

Our findings shed more light on the mechanism whereby *vHnf1* is induced in the posterior hindbrain. We find that RA activity is required for induction of mouse *vHnf1* as previously reported for zebrafish *vHnf1* (Hernandez et al., 2004). However, other previous studies have suggested that *Hoxb1* may be sufficient to induce *vHnf1* in zebrafish (Choe and Sagerström, 2004). Our studies suggest that *Hoxb1* is not sufficient for *vHnf1* induction as we find persistent expression of *Hoxb1* in the posterior hindbrain and anterior spinal cord of *Raldh2*$^{-/-}$ embryos, but a complete lack of *vHnf1* expression in this domain of *Hoxb1* expression. Thus, we suggest that RA signaling is needed to activate *vHnf1*. A direct effect of RA is plausible as a DR1 retinoid response element has been identified in the promoter region of mouse *vHnf1* (Power and Cereghini, 1996). As the *vHnf1* DR1 response element was found to bind retinoid receptors less efficiently than a DR5 retinoid response element (such as those present near the RARβ and *Hoxb1* genes), it was suggested that *vHnf1* may be less responsive to RA than genes with DR5 retinoid response elements

(Power and Cereghini, 1996). We suggest that this lower responsiveness of *vHnf1* to RA may be crucial to the mechanism whereby its expression domain is limited to a more posterior boundary than that of *Hoxb1*. The r5 anterior limit of *vHnf1* expression is in fact closer to the paraxial mesodermal source of RA than the r4 anterior limit of *Hoxb1*, and we have demonstrated that RA activity is transient in r4 but more long lived in r5.

Hoxb1 expression in the absence of RA activity

Studies on quail embryos reported that vitamin A deficiency (VAD) results in a complete absence of r4-r8, with the hindbrain consisting of an enlarged r1-r3 (devoid of *Hoxb1* expression) joined to the anterior spinal cord, which retained expression of *Hoxb1* (Maden et al., 1996). A similar phenotype was also observed in mouse *Raldh2*$^{-/-}$ embryos at E8.25-E8.5, which lacked the characteristic r4 stripe of *Hoxb1* expression, but retained a diffuse domain of *Hoxb1* expression in the posterior-most region of the hindbrain next to the spinal cord junction (Niederreither et al., 2000). However, results from chick embryos treated with retinoid receptor antagonists suggested that RA deficiency leads to elimination of r5-r8 but with an enlarged r4 remaining because of residual expression of *Hoxb1* and other r4 markers in the posterior-most region of the hindbrain adjacent to the spinal cord (Dupé and Lumsden, 2001). Results from VAD rat embryos also suggested that r4 is not totally eliminated (Baybutt et al., 2000). The residual *Hoxb1* expression observed in the posterior hindbrain of mouse *Raldh2*$^{-/-}$ embryos was originally not interpreted as an indication of r4 character, possibly owing to the observation of an extension of the *Krox20* r3 expression domain all the way to the spinal cord junction (Niederreither et al., 2000). We note that the r4 expression domain of *Cyp26c1* is completely missing in the posterior hindbrain of *Raldh2*$^{-/-}$ embryos (but an expanded r2 expression domain was observed), providing further evidence that at least some aspects of r4 character have been lost in the absence of RA activity. In addition, residual expression of *Hoxb1* in the posterior hindbrain of *Raldh2*$^{-/-}$ embryos may be due to the loss of *vHnf1* expression, which normally functions to repress *Hoxb1* in that location. Rather than being an indicator of r4 character, this residual *Hoxb1* expression may be the remnants of that which normally occurs independent of RA in posterior neuroectoderm up to the level of the node prior to anterior expansion of *Hoxb1* expression into the hindbrain (Forlani et al., 2003).

Conserved function for RA boundaries

The model of mouse hindbrain RA activity proposed here is likely to be conserved in other vertebrate embryos. *Raldh2* expression occurs in the trunk paraxial mesoderm of all vertebrate embryos analyzed and *Cyp26* homologs expressed in the hindbrain exist as well (see Introduction). Studies on zebrafish embryos have demonstrated that *vHnf1* functions as a repressor of *Hoxb1* in r5 (Wiellette and Sive, 2003) and it was recently reported that RA is required for *vHnf1* expression in the zebrafish hindbrain (Hernandez et al., 2004). In addition, zebrafish *Irx3* (*iro7*) and *vHnf1* function as mutual repressors needed to establish an r4/r5 expression boundary (Lecaudey et al., 2004). Thus, shifting boundaries of RA activity that regulate the spatiotemporal expression patterns of *Hoxb1*, *vHnf1* and *Irx3* may be a general feature of vertebrate hindbrain development. However, there have been no reports of methods to localize RA activity in the hindbrain of other vertebrates to directly test this. Thus, our studies highlight the importance of using mouse embryos carrying the *RARE-lacZ* transgene as a model system to decipher RA function, as this is the only system in which the location of RA activity can be determined during development.

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References

Abu-Abed, S., Dollé, P., Metzger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* **15**, 226-240.

Ang, H. L., Deltour, L., Hayamizu, T. F., Zgombic-Knight, M. and Duester, G. (1996). Retinoic acid synthesis in mouse embryos during gastrulation and craniofacial development linked to class IV alcohol dehydrogenase gene expression. *J. Biol. Chem.* **271**, 9526-9534.

Barbacci, E., Reber, M., Ott, M.-O., Breillat, C., Huetz, F. and Cereghini, S. (1999). Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. *Development* **126**, 4795-4805.

Baybutt, R. C., Hu, L. and Molteni, A. (2000). Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type II pneumocytes. *J. Nutr.* **130**, 1159-1165.

Becker, N., Seitanidou, T., Murphy, P., Mattei, M. G., Topilko, P., Nieto, M. A., Wilkinson, D. G., Charnay, P. and Gilardi-Hebenstreit, P. (1994). Several receptor tyrosine kinase genes of the Eph family are segmentally expressed in the developing hindbrain. *Mech. Dev.* **47**, 3-17.

Begemann, G., Schilling, T. F., Rauch, G. J., Geisler, R. and Ingham, P. W. (2001). The zebrafish *neckless* mutation reveals a requirement for *raldh2* in mesodermal signals that pattern the hindbrain. *Development* **128**, 3081-3094.

Blumberg, B., Bolado, J., Jr., Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Bosse, A., Zulch, A., Becker, M. B., Torres, M., Gomez-Skarmeta, J. L., Modolell, J. and Gruss, P. (1997). Identification of the vertebrate Iroquois homeobox gene family with overlapping expression during early development of the nervous system. *Mech. Dev.* **69**, 169-181.

Chen, Y. L., Pollet, N., Niehrs, C. and Pieler, T. (2001). Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of *Xenopus* embryos. *Mech. Dev.* **101**, 91-103.

Choe, S.-K. and Sagerström, C. G. (2004). Paralog group 1 hox genes regulate rhombomere 5/6 expression of *vhnf1*, a repressor of rostral hindbrain fates, in a *meis*-dependent manner. *Dev. Biol.* **271**, 350-361.

Clagett-Dame, M. and DeLuca, H. F. (2002). The role of vitamin A in mammalian reproduction and embryonic development. *Annu. Rev. Nutr.* **22**, 347-381.

Coffinier, C., Thépot, B., Babinet, C., Yaniv, M. and Barra, J. (1999). Essential role for the homeoprotein vHNF1/HNF1β in visceral endoderm differentiation. *Development* **126**, 4785-4794.

Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M. and Barra, J. (2002). Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129**, 1829-1838.

Cohen, D. R., Cheng, C. W., Cheng, S. H. and Hui, C. C. (2000). Expression of two novel mouse *Iroquois* homeobox genes during neurogenesis. *Mech. Dev.* **91**, 317-321.

Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly

induces anterior ectopic expression of murine *Hox-2* genes in vivo. *Development* **116**, 357-368.

Dickman, E. D., Thaller, C. and Smith, S. M. (1997). Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* **124**, 3111-3121.

Downs, K. M. and Davies, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255-1266.

Duester, G. (2000). Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur. J. Biochem.* **267**, 4315-4324.

Dupé, V. and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.

Forlani, S., Lawson, K. A. and Deschamps, J. (2003). Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. *Development* **130**, 3807-3819.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163-4173.

Gavalas, A. (2002). ArRAnging the hindbrain. *Trends Neurosci.* **25**, 61-64.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

Goddard, J. M., Rossel, M., Manley, N. R. and Capecchi, M. R. (1996). Mice with targeted disruption of *Hoxb-1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.

Grandel, H., Lun, K., Rauch, G. J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Küchler, A. M., Schulte-Merker, S., Geisler, R. et al. (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* **129**, 2851-2865.

Haselbeck, R. J., Hoffmann, I. and Duester, G. (1999). Distinct functions for *Aldh1* and *Raldh2* in the control of ligand production for embryonic retinoid signaling pathways. *Dev. Genet.* **25**, 353-364.

Hernandez, R. E., Rikhof, H. A., Bachmann, R. and Moens, C. B. (2004). *vhnf1* integrates global RA patterning and local FGF signals to direct posterior hindbrain development in zebrafish. *Development* **131**, 4511-4520.

Holland, L. Z. and Holland, N. D. (1996). Expression of *AmphiHox-1* and *AmphiPax-1* in amphioxus embryos treated with retinoic acid: Insights into evolution and patterning of the chordate nerve cord and pharynx. *Development* **122**, 1829-1838.

Hollemann, T., Chen, Y. L., Grunz, H. and Pieler, T. (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361-7372.

Hunt, P., Wilkinson, D. and Krumlauf, R. (1991). Patterning the vertebrate head: Murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. *Development* **112**, 43-50.

Kastner, P., Mark, M. and Chambon, P. (1995). Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell* **83**, 859-869.

Kolm, P. J., Apekin, V. and Sive, H. (1997). *Xenopus* hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

Krumlauf, R. (1993). *Hox* genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet.* **9**, 106-112.

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.

Lecaudey, V., Anselme, I., Rosa, F. and Schneider-Maunoury, S. (2004). The zebrafish Iroquois gene *iro7* positions the r4/r5 boundary and controls neurogenesis in the rostral hindbrain. *Development* **131**, 3121-3131.

Lufkin, T. (1996). Transcriptional control of *Hox* genes in the vertebrate nervous system. *Curr. Opin. Genet. Dev.* **6**, 575-580.

MacLean, G., Abu-Abed, S., Dollé, P., Tahayato, A., Chambon, P. and Petkovich, M. (2001). Cloning of a novel retinoic-acid metabolizing cytochrome P450, *Cyp26B1*, and comparative expression analysis with *Cyp26A1* during early murine development. *Mech. Dev.* **107**, 195-201.

Maden, M. (2002). Retinoid signalling in the development of the central nervous system. *Nat. Rev. Neurosci.* **3**, 843-853.

Maden, M., Gale, E., Kostetskii, I. and Zile, M. H. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.

Mangelsdorf, D. J., Umesono, K. and Evans, R. M. (1994). The retinoid receptors. In *The Retinoids: Biology, Chemistry, and Medicine,* 2nd edn (ed. M B. Sporn, A. B. Roberts and D. S. Goodman), pp. 319-349. New York: Raven Press.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370**, 567-571.

Mic, F. A., Haselbeck, R. J., Cuenca, A. E. and Duester, G. (2002). Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of *Raldh2* null mutant mice. *Development* **129**, 2271-2282.

Mic, F. A., Molotkov, A., Benbrook, D. M. and Duester, G. (2003). Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc. Natl. Acad. Sci. USA* **100**, 7135-7140.

Niederreither, K., Subbarayan, V., Dollé, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.* **21**, 444-448.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.

Pöpperl, H., Bienz, M., Studer, M., Chan, S.-K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R. (1995). Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell* **81**, 1031-1042.

Power, S. C. and Cereghini, S. (1996). Positive regulation of the vHNF1 promoter by the orphan receptors COUP-TF1/Ear3 and COUP-TFII/Arp1. *Mol. Cell. Biol.* **16**, 778-791.

Reijntjes, S., Gale, E. and Maden, M. (2003). Expression of the retinoic acid catabolising enzyme CYP26B1 in the chick embryo and its regulation by retinoic acid. *Gene Exp. Patt.* **3**, 621-627.

Reijntjes, S., Gale, E. and Maden, M. (2004). Generating gradients of retinoic acid in the chick embryo: *Cyp26C1* expression and a comparative analysis of the Cyp26 enzymes. *Dev. Dyn.* **230**, 509-517.

Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguère, V. (1991). Expression of a retinoic acid response element-*hsplacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.

Sakai, Y., Meno, C., Fujii, H., Nishino, J., Shiratori, H., Saijoh, Y., Rossant, J. and Hamada, H. (2001). The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213-225.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P. W., Boncinelli, E. and Mavilio, F. (1990). Sequential activation of *HOX2* homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346**, 763-766.

Studer, M., Pöpperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265**, 1728-1732.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. *Nature* **384**, 630-634.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282-296.

Tahayato, A., Dollé, P. and Petkovich, M. (2003). *Cyp26c1* encodes a novel retinoic acid-metabolizing enzyme expressed in the hindbrain, inner ear, first branchial arch and tooth buds during murine development. *Gene Exp. Patt.* **3**, 449-454.

Tajbakhsh, S. and Houzelstein, D. (1995). *In situ* hybridization and β-galactosidase: a powerful combination for analysing transgenic mice. *Trends Genet.* **11**, 42.

Tallquist, M. D. and Soriano, P. (2000). Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extraembryonic gene function. *Genesis* **26**, 113-115.

Van der Wees, J., Schilthuis, J. G., Koster, G. H., Diesveld-Schipper, H., Folkers, G. E., van der Saag, P. T., Dawson, M. I., Shudo, K., van der Burg, B. and Durston, A. J. (1998). Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. *Development* **125**, 545-556.

White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, P. F. and Clagett-Dame, M. (1998). Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the

rat are prevented by feeding pharmacological levels of all-*trans*-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95**, 13459-13464.

Wiellette, E. L. and Sive, H. (2003). *vhnf1* and Fgf signals synergize to specify rhombomere identity in the zebrafish hindbrain. *Development* **130**, 3821-3829.

Wilkinson, D. G. (1992). Whole mount *in situ* hybridization of vertebrate embryos. In *In Situ Hybridization: A Practical Approach* (ed. D. G. Wilkinson), pp. 75-83. Oxford: IRL Press.

Wilkinson, D. G. (1993). Molecular mechanisms of segmental patterning in the vertebrate hindbrain and neural crest. *BioEssays* **15**, 499-505.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.

Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y. and Hamada, H. (2004). Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing limb. *Dev. Cell* **6**, 411-422.