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Dynamic and sequential patterning of the zebrafish posterior hindbrain by retinoic acid

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Abstract

A prominent region of the vertebrate hindbrain is subdivided along the anterior–posterior axis into a series of seven segments, or rhombomeres. The identity of each rhombomere is specified by the expression of conserved transcription factors, including Krox-20, vHnf1, Val (Kreisler, Mafb) and several Hox proteins. Previous work has shown that retinoic acid (RA) signaling plays a critical role in regulating the expression of these factors and that more posterior rhombomeres require higher levels of RA than more anterior rhombomeres. Models to account for RA concentration dependency have proposed either a static RA gradient or increasing time periods of RA exposure. Here, we provide evidence against both of these models. We show that early zebrafish rhombomere-specification genes, including *vhnf1* in r5–r6 and *hoxd4a* in r7, initiate expression sequentially in the hindbrain, each adjacent to the source of RA synthesis in paraxial mesoderm. By knocking down RA signaling, we show that progressively more posterior rhombomeres require increasingly higher levels of RA signaling, and *vhnf1* and *hoxd4a* expression are particularly RA-dependent. RA synthesis is required just at the time of initiation, but not for maintenance, of *vhnf1* and *hoxd4a* expression. Furthermore, a premature RA increase causes premature activation of *vhnf1* and *hoxd4a* expression. Our results support a new model of dynamic RA action in the hindbrain, in which a temporally increasing source of RA is required to sequentially initiate progressively more posterior rhombomere identities.

Keywords: Zebrafish; Hindbrain; Rhombomere; Segmentation; Retinoic acid; Retinaldehyde dehydrogenase; Hox; vHnf1; Valentino

Introduction

A major part of the vertebrate hindbrain is subdivided along the anterior–posterior axis into seven segments, or rhombomeres. A conserved set of transcription factors specifies the development and identity of each rhombomere (reviewed by Moens and Prince, 2002 and by Schneider-Maunoury et al., 1998). These factors include Krox-20, vHnf1, Val (Kreisler, Mafb) and several Hox proteins. For example, Val and Kreisler are required for the proper development of r5–r6 in zebrafish and mice, respectively, and Hoxb1 is important in r4 development. Because the proper expression of these factors is critical for the development of rhombomere-specific neurons and other cell types, understanding how these factors become properly initiated is an important problem.

Retinoic acid (RA) signaling plays a critical role in specifying rhombomere identity by acting upstream of these transcription factors, particularly in the posterior hindbrain (reviewed by Gavalas and Krumlauf, 2000 and by Maden, 2002). Studies in amniote embryos have shown that RA is required in both a concentration- and time-dependent manner for development of r5–r7, where more posterior rhombomeres require higher levels and longer time periods of RA signaling (Dupé et al., 1999; Gale et al., 1999; Niederreither et al., 2000; White et al., 2000; Dupé and Lumsden, 2001; Wendling et al., 2001). With the strongest and earliest loss of RA signaling, r5–r7 are lost, expression of *Krox-20* in r5 and *kreisler*, or *Mafb*, in r5–r6 are lost, and r3–r4 appear expanded. This phenotype is exemplified by

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mouse embryos mutant for *Raldh2*, which encodes an enzyme that synthesizes RA from retinal (Niederreither et al., 2000). Mild or late reduction of RA signaling causes loss of r7, accompanied by slight expansion of r5–r6 (reviewed by Begemann and Meyer, 2001 and Gavalas, 2002). The requirements for RA signaling in the zebrafish hindbrain are beginning to be elucidated (Begemann et al., 2001; Grandel et al., 2002; Linville et al., 2004; Begemann et al., 2004). The zebrafish *raldh2* mutant shows posterior hindbrain and spinal cord defects, including expansion of *val* expression and r5–r6, consistent with a mild defect in RA signaling in the hindbrain (Begemann et al., 2001; Grandel et al., 2002). It is not yet clear whether the role of RA in zebrafish r5–r7 development is both concentration- and time-dependent, as in other vertebrates.

An RA gradient across the hindbrain, with high posterior levels and lower anterior levels, would explain the concentration dependency (reviewed by Gavalas and Krumlauf, 2000; Maden, 2002). Although direct visualization of an RA gradient has been elusive, two findings support the presence of a hindbrain RA gradient. First, the RA source appears to be local. A high source of RA is likely generated by the expression of *raldh2* in somite tissue just posterior to the hindbrain (Niederreither et al., 1997; Berggren et al., 1999; Niederreither et al., 1999; Swindell et al., 1999; Chen et al., 2001; Begemann et al., 2001; Grandel et al., 2002). Hindbrain defects in zebrafish *raldh2* mutants can be rescued by wild-type cells placed in the somites (Begemann et al., 2001; Linville et al., 2004). Somite grafting experiments in chick and mouse embryos have further supported a role for mesodermal tissue just posterior to the hindbrain as a source for an RA signal (Itasaki et al., 1996; Gould et al., 1998; Grapin-Botton et al., 1998). Second, there is a putative sink for the gradient. Cyp26, an enzyme that metabolizes RA, is expressed in the anterior hindbrain and is proposed to function as a sink for RA (Swindell et al., 1999; Abu-Abed et al., 2001; Sakai et al., 2001; Kudoh et al., 2002; Dobbs-McAuliffe et al., 2004).

If an RA gradient exists, it does not appear to be static. For example, grafting experiments in chick embryos have shown that the ability of somite tissue, the putative source of an RA gradient, to induce ectopic *Hox* gene expression in the anterior hindbrain moves posteriorly during development (Itasaki et al., 1996). To account for the fact that more posterior rhombomeres require both higher levels of, and earlier exposure to, RA, duration of exposure to RA has been proposed as an alternative to a fixed anterior–posterior gradient of RA (Gavalas, 2002; Maden, 2002). In this model, cells of posterior rhombomeres, which develop later than more anterior rhombomeres, would be exposed to a constant source of RA for longer time periods before their specification, thus achieving a higher effective RA concentration. A recent study examining dynamic expression patterns of Cyp26 enzymes has provided further support that posterior rhombomeres experience longer exposure to RA than more anterior rhombomeres (Sirbu et al., 2005). However, it is still not clear how the temporal requirements for RA are integrated with its concentration dependence.

Here, we address the nature of RA action in the zebrafish posterior hindbrain. We show that the expression of early posterior rhombomere-specification genes, including *vhnf1* and *hoxd4a*, initiates sequentially in the hindbrain, near the source of RA synthesis in paraxial mesoderm. Using genetic and pharmacological approaches to knock down RA signaling, we show that progressively more posterior rhombomere markers require increasingly higher levels of RA signaling, and *vhnf1* and *hoxd4a* expression are particularly RA-dependent. RA synthesis is required for initiation, but not for maintenance, of *vhnf1* and *hoxd4a* expression, and RA signaling is not required until just at the time of initiation of their expression. We further show that a premature increase of RA can cause premature activation of *vhnf1* and *hoxd4a* expression. We also show that RA regulates *val* expression in r5–r6 through vHnf1. Our results from spatially and temporally manipulating RA levels and RA synthesis argue against a role for either a static gradient of RA signaling across the hindbrain RA or a requirement for temporal exposure to RA. Instead, our results support a role for a temporally increasing source of RA during hindbrain development. These results support a model of dynamic RA action in the hindbrain, in which a temporally increasing source of RA is required to sequentially initiate progressively more posterior rhombomere markers.

Materials and methods

Zebrafish stocks

Zebrafish (*Danio rerio*) were raised and staged as previously described (Westerfield, 1995; Kimmel et al., 1995). Time (hpf) refers to hours post-fertilization at 28.5°C. In some cases, embryos were raised for periods at room temperature, about 25°C. The wild-type stock used was AB. The *neckless*^{i26} line, a strong hypomorph for *raldh2* (*aldh1a2*—Zebrafish Information Network) has been described (Begemann et al., 2001). Homozygous *raldh2* mutant embryos were identified by PCR genotyping (Hernandez et al., 2004). Homozygous mutant embryos for *vhnf1*^{hi2169}, a likely null or strong hypomorphic allele, were identified by loss of *val* in r5/r6 (Sun and Hopkins, 2001).

RNA in situ hybridization

RNA in situ hybridizations were performed as previously described (Maves et al., 2002). The following cDNA probes were used: *raldh2* (Begemann et al., 2001); *hoxb1b* (Alexandre et al., 1996); *pax2a* (Krauss et al., 1991); *vhnf1* (Sun and Hopkins, 2001); *hoxb1a* (Prince et al., 1998a); *hoxd4a* (Prince et al., 1998b); *krox-20* (Oxtoby and Jowett, 1993); *valentino* (Moens et al., 1998); *ephrinb2a* (Cooke et al., 2001); *no tail* (Schulte-Merker et al., 1992). Embryos

were photographed using a Nikon SMZ1500 stereomicroscope and Nikon Coolpix 4500 digital camera or a Zeiss Axiophot 2 microscope. Images were assembled using Adobe Photoshop 7.

Pharmacological treatments

The following stock solutions were made and stored at −80°C: 100 mM 4-(Diethylamino)-benzaldehyde (DEAB; Aldrich) in DMSO; 10 mM or 1 mM all-*trans* retinoic acid (RA; Sigma) in DMSO. 10 mM BMS493 (Bristol Myers Squib; gift from Vicky Prince) in 50% ethanol was stored at −20°C. For embryo treatments, dilutions of these chemicals were made in Embryo Medium (EM) supplemented with penicillin (5000 U/L)/streptomycin (100 mg/L; Sigma). Embryos, still in chorions, were transferred into Petri dishes containing the treatment solution. For control treatments, sibling embryos were incubated in corresponding dilutions of DMSO or, for BMS493 treatments, 50% ethanol. All incubations were done in the dark.

To demonstrate the specificity of DEAB, we incubated embryos in DEAB with 1 nM RA and found that we could completely rescue the effects of DEAB (see Results). Concentrations of DEAB higher than 25 μM, or of BMS493 higher than 10 μM, showed non-specific effects and, in the case of DEAB, could not be rescued by RA. We tested the efficacy of DEAB inhibition of RA signaling by using a transgenic RA reporter line, RGYn2 (Perz-Edwards et al., 2001). With treatments initiating at 4 hpf (sphere stage), ≥5 μM DEAB concentrations cause loss of reporter expression, and lower DEAB concentrations cause reduction of expression (data not shown; see also Perz-Edwards et al., 2001). Furthermore, initiating 5 μM DEAB treatments at 10.3 hpf (1 somite stage (s)) causes loss of RA reporter expression (data not shown), showing that DEAB can function late. These controls all support the specificity and utility of these inhibitors in blocking RA signaling.

To quantify changes and variability in hindbrain RNA expression patterns after pharmacological manipulations, we sorted stained embryos into groups based on relative levels of expression and assigned each embryo within a group a score of 0, 1, 2, 3 or 4, where 0 is no hindbrain expression and 4 is wild-type level of hindbrain expression. In cases where we scored rhombomere length along the anterior–posterior axis, we sorted embryos into groups and assigned each embryo a score of 0, 1, 2 or 3, where 0 is no rhombomere present and 3 is wild type or longer rhombomere length. We found these approaches to give very reproducible results. Calculations and graphing were performed in Microsoft Excel.

Bead implantations

To make retinoic acid beads, AG1-X8 beads (200–400 mesh, Formate form; Bio-Rad) were quickly rinsed twice in EM with pen/strep and then were incubated in all-*trans* RA (Sigma), diluted to the appropriate concentration in DMSO, for 20 min at room temperature in the dark. Control beads were incubated in DMSO. Beads were then quickly rinsed twice in EM with pen/strep and stored on ice in the dark. All beads were prepared the same day they were used for implantation. Bead implants were carried out at 6 hpf (shield stage) as previously described (Maves et al., 2002). RA bead experiments were performed with little overhead lighting, and embryos with implanted RA beads were stored in the dark.

Results

The expression of rhombomere-specification transcription factors initiates sequentially near the source of RA synthesis

To address the roles of RA signaling in promoting early hindbrain development in zebrafish, we compared the initiation of expression of rhombomere-specification genes with respect to the source of RA synthesis. As a marker for the source of RA, we used *raldh2*, which is expressed in early mesendodermal cells and persists in paraxial mesoderm (Begemann et al., 2001; Grandel et al., 2002; Figs. 1A–D). The rhombomere-specification genes that we analyzed are *hoxb1b* (formerly called *hoxa1*; Alexandre et al., 1996) and *hoxb1a*, required for proper development of r4 (McClintock et al., 2002), *vhnf1*, required for the development of r5–r6 (Sun and Hopkins, 2001; Wiellette and Sive, 2003), and *hoxd4a*, which we find to be the earliest zebrafish *hox4* gene expressed in presumptive r7 (see below and data not shown). We find that the initial expression of each of these genes occurs sequentially in the presumptive hindbrain adjacent to *raldh2*-expressing cells (Fig. 1). *hoxb1b* expression initiates at about 6.5 hpf (McClintock et al., 2001; Fig. 1E). Over the next 3 h of development, *hoxb1b* continues to be expressed, with anterior-most expression at the r3/r4 boundary (McClintock et al., 2001; Figs. 1F–H). *vhnf1* expression initiates at about 8 hpf in cells posterior to the most anterior *hoxb1b*-expressing cells (compare Figs. 1I with F; see also Wiellette and Sive, 2003). *hoxb1a* initiates at about 9 hpf, with anterior-most expression at the r3/r4 boundary (Prince et al., 1998a; Figs. 1L–M). *hoxd4a* initiates at about 10.3 hpf (Fig. 1N), with anterior-most expression at the r6/r7 boundary (L. M., data not shown; Prince et al., 1998b). Thus, these genes with important functions in the development of r4–r7 turn on sequentially, and, with the exception of *hoxb1a*, increasingly more posteriorly in the presumptive hindbrain, near the source of RA synthesis in early mesendoderm and paraxial mesoderm.

Increasingly more posterior rhombomeres require increasingly higher levels of RA signaling

We next addressed the requirements for RA signaling in promoting expression of these rhombomere-specification

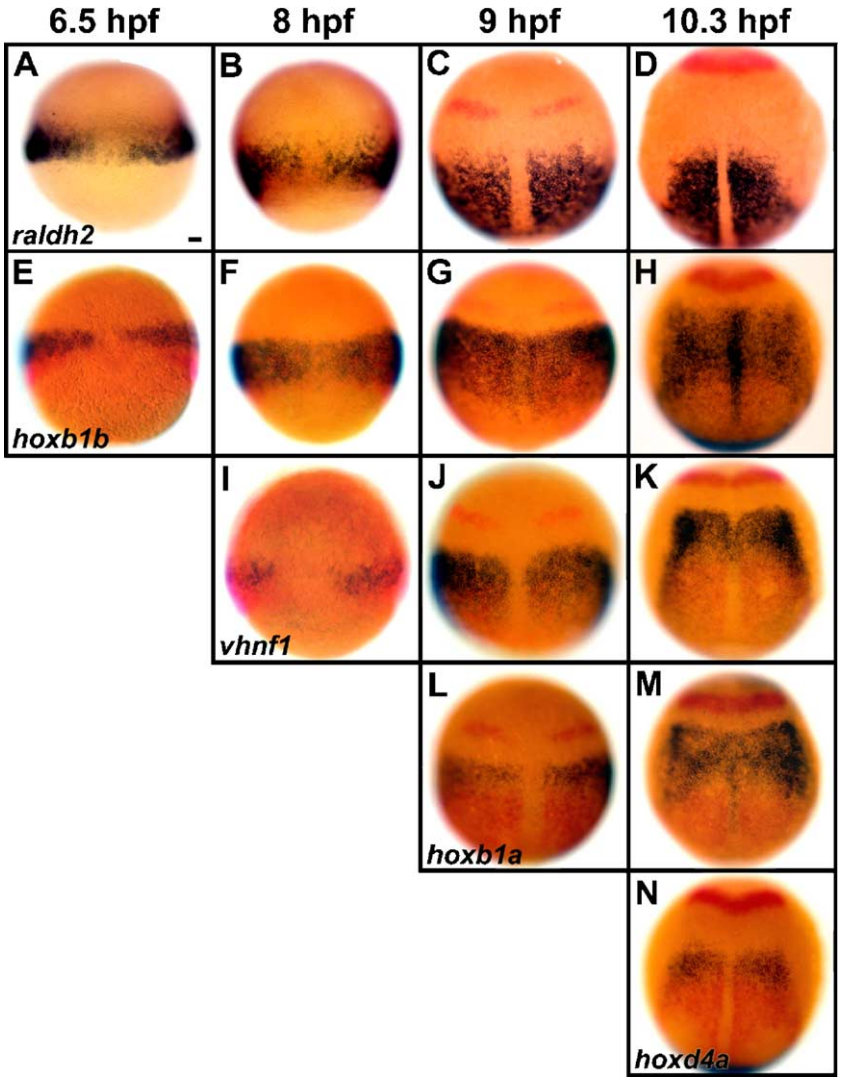

Fig. 1. Rhombomere-specification factors initiate expression sequentially near the source of RA synthesis. Wild-type embryos are stained in blue for *raldh2* (A–D), *hoxb1b* (E–H), *vhnf1* (I–K), *hoxb1a* (L–M) and *hoxd4a* (N). Other markers in red are *pax2a* to label the early midbrain–hindbrain boundary (MHB, Krauss et al., 1991; C–D, G–H, J–K, L–M, N) and *raldh2* (E–N). Dorsal views show anterior to the top. Stages are shown at the top of each column: 6.5 hpf (60% epiboly) (A, E), 8 hpf (80% epiboly) (B, F, I), 9 hpf (90% epiboly) (C, G, J, L), 10.3 hpf (1 somite (s)) (D, H, K, M, N). Double labels of *raldh2* (in red) and *hoxb1b* (E), *vhnf1* (I), *hoxb1a* (L) and *hoxd4a* (N) show that these four factors initiate near the source of RA synthesis. Scale bar: 50 μm.

factors in zebrafish. Previous studies show that zebrafish *raldh2* mutant embryos show posterior expansion of r3–r6, consistent with a reduced level of RA signaling (Begemann et al., 2001; Grandel et al., 2002). *hoxb1b* is slightly reduced in *raldh2* mutants (Grandel et al., 2002; Fig. 2B). *vhnf1* expression is slightly reduced in some *raldh2* mutants (Fig. 2F) but appears normal in others (not shown). We confirmed that r3–r6 are slightly posteriorly expanded in *raldh2* mutants, based on expression of *hoxb1a* in r4 (Fig. 2J), *krox-20* in r3 and r5 (Figs. 2J, N, R) and *val* in r5 and r6 (Fig. 2N; see also Table 1; Begemann et al., 2001; Grandel et al., 2002). *hoxd4a* is only subtly reduced in r7 and the anterior spinal cord in *raldh2* mutants (Fig. 2R; Table 1), in contrast to *hoxb4a*, which shows more severe reduction of expression in *raldh2* mutants (Begemann et al., 2001; Grandel et al., 2002). To further assess the effect on r7, we find that *efnb2a* expression in r7 appears reduced in *raldh2* mutants (Fig. 2V; although see Begemann et al., 2001). These results are consistent with expansion of r3–r6 and reduction of r7 in zebrafish *raldh2* mutants, similar to what has been observed upon mild reduction of RA signaling in other vertebrates (reviewed by Begemann and Meyer, 2001; Gavalas, 2002).

To further knock down RA signaling in zebrafish, we turned to the pharmacological inhibitors 4-(Diethylamino)-benzaldehyde (DEAB), a potent retinaldehyde dehydrogenase inhibitor, and BMS493, a pan-RA-receptor inhibitor (Russo et al., 1988; Wendling et al., 2000). We applied either of these inhibitors at 4 hpf, just before initiation of *raldh2* expression (Begemann et al., 2001; Grandel et al., 2002), and maintained embryos in the treatment bath. Treating embryos with low concentrations of either inhibitor

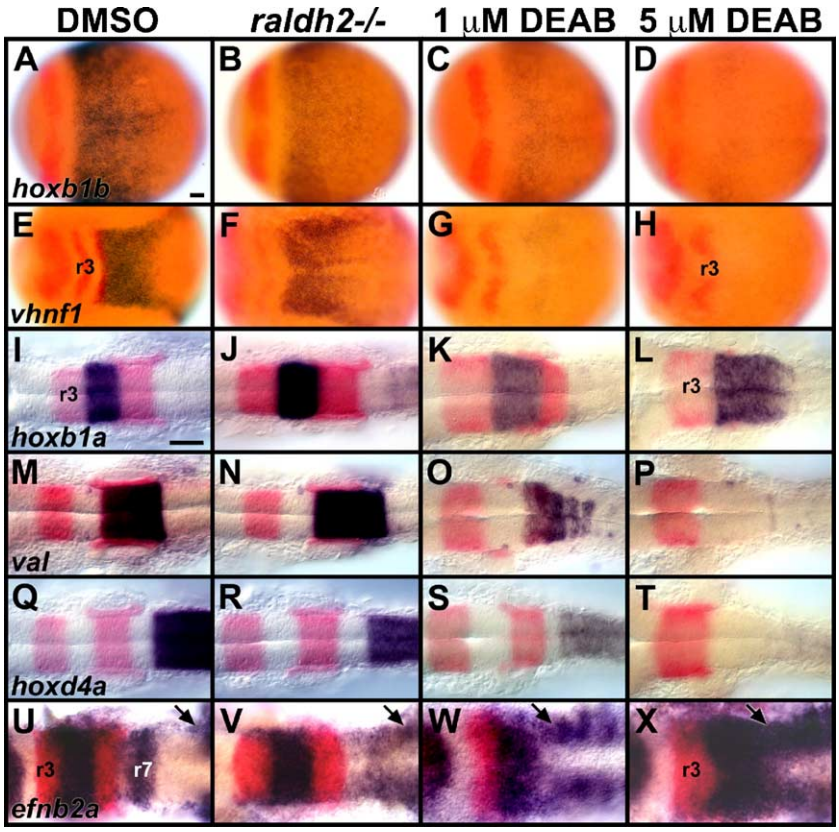

Fig. 2. Posterior rhombomere markers require progressively higher levels of RA signaling. Embryos are stained in blue for *hoxb1b* at 10 hpf (bud stage) (A–D), *vhnf1* at 10.3–10.6 hpf (1–2 s) (E–H), at 18–19 hpf (18–20 s), *hoxb1a* (I–L), *val* (M–P) and *hoxd4a* (Q–T) and *efnb2a* at 11.6 hpf (5 s) (U–X). Other markers in red are *pax2a*, to label the MHB (A–H) and inner ear (I–T), and *krox-20* (E–X), which labels r3 and r5 (Oxtoby and Jowett, 1993). Dorsal views show anterior to the left. Pharmacological treatment or genotype is shown at the top of each column. Control embryos treated with DMSO show wild-type expression patterns (A, E, I, M, Q, U). *raldh2* mutant embryos show subtle reduction in expression of *hoxb1b* (B, 7/7 embryos), *vhnf1* (F, 9/11 embryos), *hoxd4a* (R, 9/9 embryos) and *efnb2a* (V, 14/14 embryos show reduced expression in r7) and slight expansion of *hoxb1a* in r4 (J, 9/9 embryos), *krox-20* in r3 and r5 (J, N, R, 17/17 embryos) and *val* in r5/r6 (N, 8/8 embryos). 1 μM DEAB-treated embryos show more severe reduction in expression of *hoxb1b* (C, 18/18 embryos), *vhnf1* (G, 18/18 embryos), *krox-20* in r5 (K, O, S, 38/47 embryos), *val* in r5/r6 (O, 18/23) and *hoxd4a* (S, 24/24 embryos), greater expansion of domains of *krox-20* in r3 (K, O, S, 47/47 embryos) and *hoxb1a* in r4 (K, 21/21 embryos) and loss of *efnb2a* in r7 (W, 17/17 embryos). 1 μM DEAB-treated embryos can also show expansion of r5 and reduced r6 (not shown, 9/47 embryos). 5 μM DEAB-treated embryos show even more severe reduction of *hoxb1b* expression (D, 15/15 embryos), loss of expression of *vhnf1* (H, 15/15 embryos), *krox-20* in r5 (L, P, T, 31/41 embryos), *val* in r5–r6 (P, 20/28 embryos) and *efnb2a* in r7 (X, 17/17 embryos), and even greater expansion of domains of *krox-20* in r3 (L, P, T, 41/41 embryos) and *hoxb1a* in r4 (L, 12/12 embryos). The *val*-expressing cells in r4 are the Mauthner neurons (Moens et al., 1998). The arrows in U–X point to *efnb2a* expression in developing somites (Durbin et al., 1998), posterior to the r7 *efnb2a* domain in control (U) and *raldh2* mutant (V) embryos. r3, rhombomere 3 *krox-20* expression. Scale bars: 50 μm.

generates hindbrain phenotypes that resemble zebrafish *raldh2* mutants (Table 1; Grandel et al., 2002). Treating embryos with a series of higher concentrations of either inhibitor generates a series of more severe hindbrain phenotypes. With 1 μM DEAB, *hoxb1b* and *vhnf1* expression are severely reduced (Figs. 2C, G), and with 5 μM DEAB, *vhnf1* expression is lost (Fig. 2H). With intermediate inhibitor doses, we observe loss of r7, progressive reduction of r5 and r6 and posterior expansion of r3 and r4 (Table 1; Figs. 2K, O, S, W). For *efnb2a*, we interpret the strong hindbrain expression domain as expanded r4, with loss of the r7 domain (Fig. 2W). Finally, with the highest dose of either inhibitor that acts specifically (see below and Materials and methods), we observe loss of r5–r7 and greater posterior expansion of r3 and r4 (Table 1; Figs. 2L, P, T, X). This series of phenotypes very closely resembles those reported for intermediate and severe inhibition of RA signaling in other vertebrates (reviewed in Begemann and Meyer, 2001; Gavalas, 2002). These results show that, at the level of r5–r7, increasingly more posterior rhombomeres require increasingly higher levels of RA signaling.

Increasingly more posterior rhombomere markers require RA signaling at progressively later stages

In addition to concentration, timing of RA signaling has been proposed to be an important component of RA action in the posterior hindbrain (reviewed in Begemann and Meyer, 2001; Gavalas, 2002). Because *vhnf1* and *hoxd4a* have very strong requirements for RA signaling, we asked whether RA signaling is required for the initiation or maintenance of expression of *vhnf1* and *hoxd4a*. Following DEAB treatment beginning at 4 hpf, we find no initiation of hindbrain

Table 1
Rhombomere defects observed after RA inhibitor treatments at varying concentrations, compared to *raldh2* mutant phenotype

	Genotype	Number of embryos analyzed[a]	Normal hindbrain pattern	r3–r6 slightly enlarged, r7 reduced	r3–r5 enlarged, r6 reduced, r7 absent	r3–r4 enlarged, r5–r6 reduced, r7 absent	r3–r4 enlarged, r5–r7 absent
	Raldh2+/– or +/+	11	100%				
	Raldh2–/–	17		100%			
Inhibitor applied	Concentration						
DMSO	0.005%	34	**100%**				
DEAB	0.25 μM	40	40%	**60%**			
	0.5 μM	40	17%	30%	**38%**	15%	
	1 μM	47			19%	**72%**	9%
	5 μM	41				24%	**76%**
EtOH	0.05%	21	**100%**				
BMS493	1 μM	22	32%	**68%**			
	2.5 μM	25	4%	40%	**48%**	8%	
	5 μM	23		4%	13%	**74%**	9%
	10 μM	24			17%	**79%**	4%

[a] For all cases, rhombomeres were scored at 18 hpf (18 s) using expression of *val+krox-20* or *hoxd4a+krox-20*. Rhombomere size was judged as marker gene expression domain length along the anterior–posterior axis. Similar percentages were observed for either of the two probe combinations for a given treatment, and numbers were combined. r7 was scored by *hoxd4a* expression; the r7 marker *efnb2a* gave similar results (see Fig. 2).

expression of *vhnf1* or *hoxd4a* (Figs. 3A–D), showing that RA signaling is required for the initial expression of *vhnf1* and *hoxd4a*. To test whether RA signaling is required before or after the time of initiation of *vhnf1* and *hoxd4a* expression, we applied DEAB or BMS493 at a series of developmental stages. We find that inhibiting RA synthesis just before the initiation of *vhnf1* or *hoxd4a* expression is sufficient to block or severely reduce their expression (Fig. 3E). However, if embryos are treated with DEAB after *vhnf1* or *hoxd4a* expression is initiated, their expression appears normal (Fig. 3E). We also assessed when RA signaling is required for the specification of individual posterior rhombomeres, judging specification by the appearance of markers *krox-20* for r5, *val* (non-overlapping r5 *krox-20*) for r6 and *efnb2a* for r7. We find that specification of r5–r6 and r7 follows a similar temporal requirement for RA synthesis as *vhnf1* and *hoxd4a*, respectively (Fig. 3F). Inhibiting RA signaling with BMS493 at this series of stages gave similar results (data not shown). Therefore, RA synthesis and signaling are required for initiation, but not maintenance, of *vhnf1* and *hoxd4a* and for the progressive specification of r5–r7. Taken together, our results are consistent with those seen for time and concentration dependence of RA in chick and mouse embryos (Dupé and Lumsden, 2001; Wendling et al., 2001).

Because increasingly more posterior rhombomere markers initiate later in development and because specification of increasingly more posterior rhombomeres requires increasingly higher levels of RA signaling, we wondered whether increasingly more posterior rhombomere markers require an increasingly longer time period of RA signaling. To address this, we inhibited RA synthesis at 4 hpf and then added exogenous RA to the embryo medium at progressively later stages. The exogenous RA concentration used in these rescue experiments, 1 nM, causes only very subtle or no hindbrain defects in control embryos (Figs. 4A–B, E–F). While 5 μM DEAB treatments beginning at 4 hpf cause loss of *vhnf1* and *hoxd4a* expression (Figs. 4C, G), adding RA simultaneously with DEAB at 4 hpf can rescue the effects of DEAB (Figs. 4D, H), showing that DEAB is acting specifically to block RA synthesis. By adding RA at progressively later stages to DEAB-treated embryos, we find that RA is not needed for normal hindbrain expression of *vhnf1* or *hoxd4a* until the time when expression of either gene is normally initiated (Fig. 4I). Additionally, RA is not required for specification of r7 until 9–10 hpf (Fig. 4J). However, we also find that RA is not required for specification of r5–r6 until about 10 hpf, slightly later than r7 (Fig. 4J). This finding may appear to contradict the conclusion that more anterior rhombomeres require RA earlier than more posterior fates; however, this finding may reveal that r5–r6 fates, and *val* and r5 *krox-20* expression, may have additional inputs that support their specification in the absence of RA, whereas *vhnf1*, *hoxd4a* and r7 are more directly or solely dependent on RA. Taken together, these results contradict the hypothesis that increasingly more posterior rhombomeres require increasingly longer durations of RA signaling. Instead, our experiments demonstrate that a source of RA synthesis is required at distinct times for the specification of each posterior rhombomere. Because increasingly posterior rhombomeres require increasingly higher levels of RA, our results suggest that the source of RA increases during posterior hindbrain development.

An increased source of RA induces precocious expression of posterior rhombomere markers

If the source of RA increases during posterior hindbrain development and if this increasing source of RA promotes

the sequential induction of increasingly more posterior rhombomere markers, then generating a stronger endogenous RA source earlier should turn on posterior markers earlier. To locally increase the RA source, we placed RA beads into the mesendodermal margin, the site of *raldh2* expression (see Fig. 1), at 6 hpf, before *vhnf1* and *hoxd4a* normally initiate (see Fig. 1). The beads are placed unilaterally, near the presumptive hindbrain (Woo and Fraser, 1995). Control beads have no effect on the timing or position of *vhnf1* or *hoxd4a* expression (Figs. 5A, C, E, G). However, RA beads cause precocious expression of *vhnf1* unilaterally, near the RA bead, at 7 hpf (Fig. 5B). If these RA-bead-implanted embryos are allowed to develop further, they show anterior expansion of *vhnf1* on the RA-bead side (Fig. 5D). By 10 hpf, RA beads also cause precocious expression of *hoxd4a* unilaterally, near the RA bead (Fig. 5F). At later stages, these embryos show anterior expansion of *hoxd4a* on the RA-bead side (Fig. 5H). The anterior ectopic expansion of *vhnf1* and *hoxd4a* at later stages reveals that the increased RA causes *vhnf1* and *hoxd4a* to be induced and maintained in cells more anterior than would normally express these genes. These precocious inductions are concentration-dependent. *vhnf1* is induced strongly and with high frequency by 1 mM beads, as shown above, but 0.1 mM beads do not induce precocious *vhnf1* expression ($n = 8$). *hoxd4a* is induced strongly and with high frequency by 10 mM RA beads, as shown above, but, with 1 mM beads, *hoxd4a* is weakly induced at 10 hpf in only 4/11 embryos. These results reveal that *vhnf1* and *hoxd4a* are competent to respond to RA earlier than they normally initiate and that a stronger source of RA is sufficient to promote their early induction in more anterior cells. These results thus support our hypothesis that the RA source becomes stronger over time, providing a mechanism as to how RA progressively induces increasingly more posterior rhombomere fates during development (see Discussion).

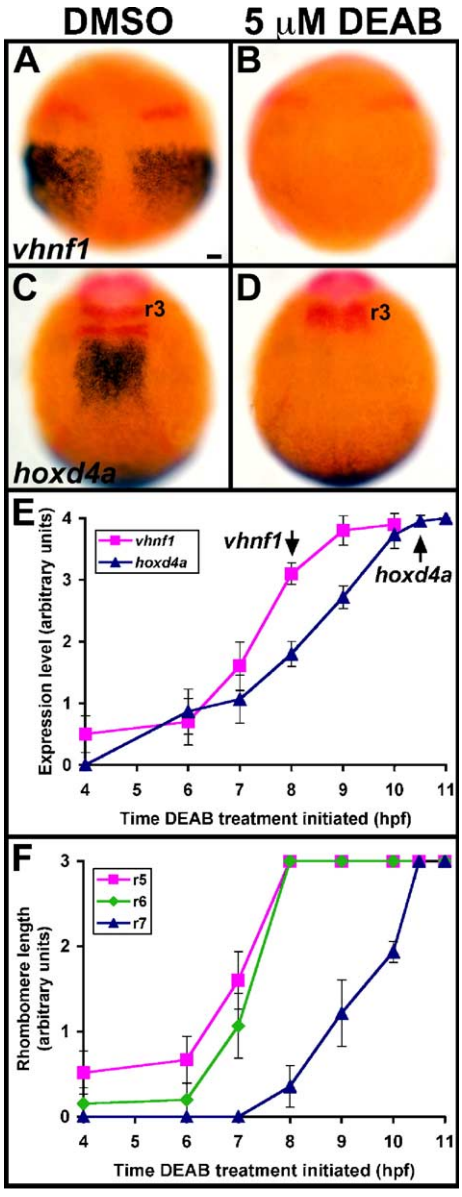

Fig. 3. RA synthesis is required for initiation of posterior hindbrain markers. (A–D) Control (A, C) and 5 μM DEAB-treated (B, D) embryos are stained in blue for *vhnf1* (A, B) and *hoxd4a* (C, D). Other markers in red are *pax2a* (A–D) and *krox-20* (C, D). Dorsal views show anterior to the top. Treatments were initiated at 4 hpf (sphere stage), and embryos were fixed at 9 hpf (90% epiboly) (A, B) or 10.6 hpf (2 s) (C, D). For *vhnf1*, 10/10 DEAB-treated embryos showed loss of expression. For *hoxd4a*, 10/10 DEAB-treated embryos showed loss of hindbrain expression as well as loss of r5 *krox-20* expression. r3, rhombomere 3 *krox-20* expression. Scale bar: 50 μm. (E) RA synthesis is required for initiation, but not maintenance, of *vhnf1* and *hoxd4a*. Embryos were treated with 5 μM DEAB beginning at stages between 4 hpf and 11 hpf (3 s). Arrows point to normal initiation times of *vhnf1* and *hoxd4a* expression. For *vhnf1* analysis, embryos were treated until 10.3 hpf (1 s) then were fixed and stained for *vhnf1* expression. For *hoxd4a* analysis, embryos were treated until 18 hpf (18 s) then were fixed and stained for *hoxd4a* and *krox-20* expression. Levels of hindbrain expression were scored on a 0–4 scale, with 0 representing no expression and 4 representing wild-type levels. $n = 10$ for each *vhnf1* data point. $n \geq 12$ for each *hoxd4a* data point. (F) Specification of r5–r6 and r7 has distinct temporal requirements for RA synthesis. The same series of treatments were used as for panel (E). For r5 analysis, *krox-20* expression was scored in 18 hpf embryos. For r6 analysis, *val* (non-overlapping r5 *krox-20*) expression was scored in 18 hpf embryos. For r7 analysis, *efnb2a* expression was scored in embryos fixed after treatment until 11.6 hpf (5 s). Rhombomere length was scored on a 0–3 scale, with 0 representing no expression and 3 representing wild type or longer lengths of expression domains. $n \geq 20$ for each r5 data point. $n \geq 10$ for each r6 data point. $n \geq 14$ for each r7 data point. Error bars represent 95% confidence interval.

RA acts through vHnf1 to regulate val expression

Our results above support a critical and likely direct role for RA for the expression of *vhnf1* and *hoxd4a* and for r7 specification. *vhnf1* acts upstream of *val* in r5–r6 specification (Sun and Hopkins, 2001). Because *vhnf1* and *val* expression are both dependent on RA (Fig. 2), we wanted to address the relationships among RA, *vhnf1* and *val*. Previous work has shown that, upon loss of RA signaling, *vhnf1* overexpression can activate *val* (Hernandez et al.,

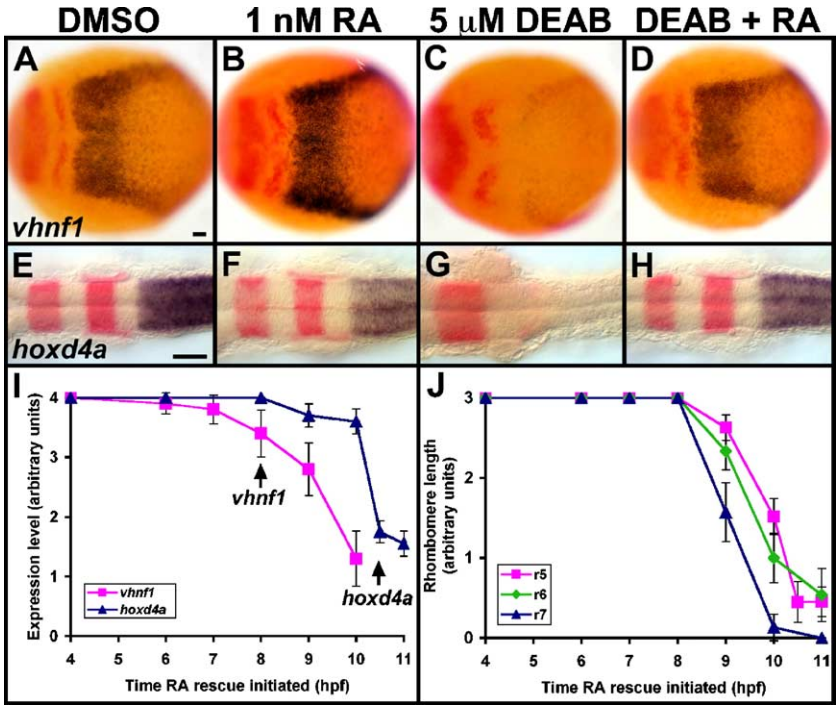

Fig. 4. RA rescues DEAB treatment and is not required until posterior marker initiation. (A–H) Control (A, E), 1 nM RA-treated (B, F), 5 μM DEAB-treated (C, G) and 5 μM DEAB + 1 nM RA-treated (D, H) embryos are stained in blue for *vhnf1* (A–D) and *hoxd4a* (E–H). Other markers in red are *pax2a* and *krox-20*. Dorsal views show anterior to the left. Treatments were initiated at 4 hpf (sphere stage), and embryos were fixed at 10.3 hpf (1 s) (A–D) or 18 hpf (18 s) (E–H). r3, rhombomere 3 *krox-20* expression. Scale bars: 50 μm. (I) RA can rescue DEAB treatment up to the time of either *vhnf1* or *hoxd4a* initiation. Embryos were treated with 5 μM DEAB beginning at 4 hpf then were additionally treated with 1 nM RA beginning at stages between 4 hpf and 11 hpf (3 s). Arrows point to normal initiation times of *vhnf1* and *hoxd4a* expression. For *vhnf1* analysis, embryos were treated until 10.3 hpf then were fixed and stained for *vhnf1* expression. For *hoxd4a* analysis, embryos were treated until 18 hpf then were fixed and stained for *hoxd4a* and *krox-20* expression. Levels of hindbrain expression were scored on a 0–4 scale, with 0 representing no expression and 4 representing wild-type levels. $n = 10$ for each *vhnf1* data point. $n \geq 18$ for each *hoxd4a* data point. 10/10 DEAB-treated/no RA controls showed little or no *vhnf1* expression (see Fig. 3E). 12/12 DEAB-treated/no RA controls showed no *hoxd4a* expression (see Fig. 3E). Other control embryos were treated with RA alone at each time point: *vhnf1* ($n = 10$ each time point) and *hoxd4a* ($n = 20$ each time point) expression appeared largely normal. (J) Rescue of r5–r6 and r7 has distinct temporal requirements for RA. The same series of treatments were used as for panel (I). For r5 analysis, *krox-20* expression was scored in 18 hpf embryos. For r6 analysis, *val* (non-overlapping r5 *krox-20*) expression was scored in 18 hpf (18 s) embryos. For r7 analysis, *efnb2a* expression was scored in embryos fixed after treatment until 11.6 hpf (5 s). Rhombomere length was scored on a 0–3 scale, with 0 representing no expression and 3 representing wild type or longer lengths of expression domains. $n \geq 20$ for each r5 data point. $n \geq 13$ for each r6 data point. $n \geq 13$ for each r7 data point. Error bars represent 95% confidence interval.

2004). To address whether RA acts through vHnf1 to activate *val*, we employed RA beads. In chick embryos, RA beads placed by r4–r6 can induce expression of the *val* ortholog *Mafb* anterior to r5–r6 in the hindbrain (Grapin-Botton et al., 1998). We find that RA beads can induce expression of both *vhnf1* and *val* in the anterior hindbrain (Figs. 6A–C). Compared to the *vhnf1* induction, this *val* induction appears more localized near its endogenous domain (Fig. 6B). When analyzed at later stages, these embryos continue to show ectopic *val* expression anterior to r5–r6 (Fig. 6C). *vhnf1* mutant embryos show essentially no *val* expression (Fig. 6E; Sun and Hopkins, 2001), and RA beads are not able to induce *val* expression in *vhnf1* mutant embryos (Fig. 6F). RA can still have effects in *vhnf1* mutant embryos as revealed by the altered r3 *krox-20* pattern (Fig. 6F). These results show that RA acts through vHnf1 to regulate *val* expression. Because *val* functions upstream of *Hox* gene expression in r5–r6 (Prince et al., 1998a), this pathway provides a mechanism for RA regulation of *Hox* gene expression in r5–r6.

Discussion

We have addressed the nature of RA activity during early patterning of the zebrafish hindbrain. Here, we discuss the roles of concentration-dependent RA signaling in activating gene expression and rhombomere identity. We also discuss how our results support a critical role for a temporally increasing source of RA during hindbrain development.

Conserved concentration-dependent requirements for RA signaling in posterior hindbrain patterning

We show that genes with important roles in early posterior rhombomere specification, *hoxb1b*, *vhnf1*, *hoxb1a* and *hoxd4a*, activate sequentially in the presumptive hindbrain, each adjacent to *raldh2*-expressing paraxial mesoderm, the source of RA synthesis (Fig. 1). We chose these genes because of their early expression in, and requirements for development of, r4 (*hoxb1b*, *hoxb1a*; McClintock et al., 2002), r5–r6 (*vhnf1*; Sun and Hopkins, 2001) and r7

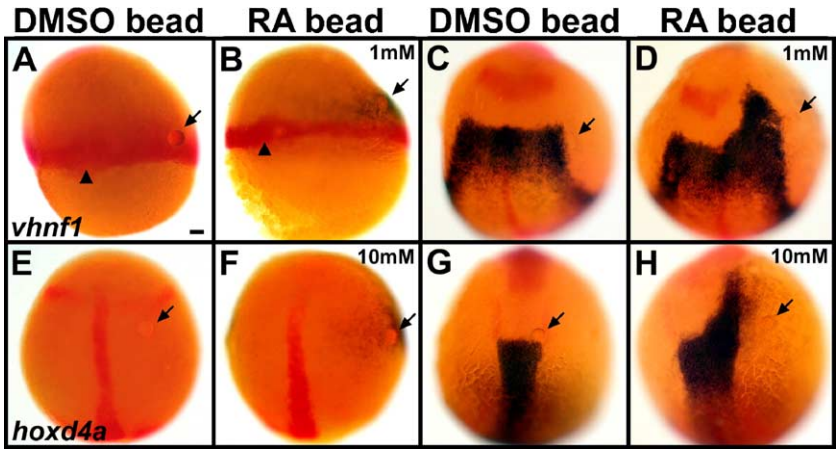

Fig. 5. RA beads can induce posterior hindbrain markers ectopically and prematurely. Embryos with control (A, C, E, G), 1 mM RA (B, D) or 10 mM RA (F, H) beads placed in the margin at 6 hpf (shield stage) and stained in blue for *vhnf1* at 7 hpf (65% epiboly) (A, B) or 10.3 hpf (1 s) (C, D) or for *hoxd4a* at 10 hpf (bud stage) (E, F) or 11.3 hpf (4 s) (G, H). Markers in red are *no tail* (*ntl*, Schulte-Merker et al., 1992), which labels the margin and the shield/dorsal midline (A–B) and the notochord (C–F) and also *pax2a* to label the MHB (C–H) and the early ear primordia (G–H). Dorsal views show anterior to the top. Arrows point to beads. Arrowheads point to the shield/dorsal midline (A–B). (A–D) *vhnf1* initiates prematurely on the RA-bead side (B) in 8/8 cases, compared with the control side (0/8) and with control bead embryos (A, 0/8). If bead-implanted embryos are allowed to develop further, to 10.3 hpf, 10/10 control embryos show normal *vhnf1* expression (C), and 9/9 embryos with 1 mM RA beads show anterior expansion of *vhnf1* on the RA-bead side (D). (E–H) *hoxd4a* initiates prematurely on the RA-bead side (F) in 7/7 cases, compared with the control side (0/7) and with control bead embryos (E, 0/10). If these embryos are allowed to develop further, to 11.3 hpf, 10/10 control embryos show normal *hoxd4a* expression (G), and 9/9 embryos with 10 mM RA beads show anterior expansion of *hoxd4a* on the RA-bead side (H). r3: rhombomere 3 *krox-20* expression. Scale bar: 50 μm.

(*hoxd4a*; Fig. 1), although the requirements for *hoxd4a* in zebrafish have not yet been determined. We addressed the requirements for RA signaling in activating expression of these genes. Previous studies have found that zebrafish

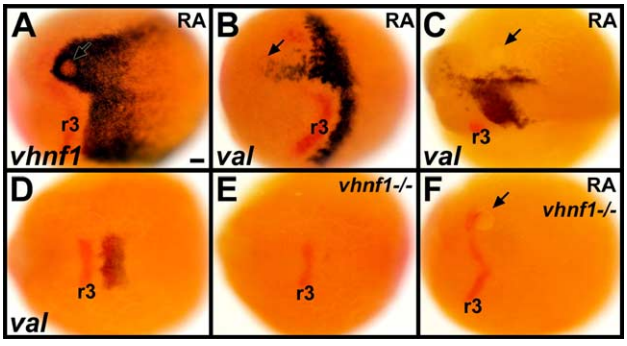

Fig. 6. RA induces *val* expression through vHnf1. (A–C) Embryos with 0.1 mM RA beads placed near the anterior presumptive hindbrain at 6 hpf (shield stage) are stained in red for *krox-20* and in blue for *vhnf1* at 10.3 hpf (1 s) (A), *val* at 10.3 hpf (B) or *val* at 15 hpf (12 s) (C). 13/13 0.1 mM RA beads induced *vhnf1* expression unilaterally in the anterior hindbrain, generally surrounding the bead (A). 10/10 0.1 mM RA beads induced *val* expression unilaterally in the anterior hindbrain (B). (D–F) Wild-type sibling (D), *vhnf1* mutant control (E) and *vhnf1* mutant embryo with a 0.1 mM RA bead placed near the anterior presumptive hindbrain at 6 hpf (F). Embryos are stained in blue for *val* and in red for *krox-20* at about 11.3 hpf (4 s). 3/8 control *vhnf1* mutant embryos showed no *val* expression; 5/8 showed some weak bilateral *val* expression (E). RA beads are unable to induce any *val* expression in *vhnf1* mutants (F). 6/9 *vhnf1* mutant embryos with RA beads showed no *val* expression (F); 3/9 showed some very weak bilateral *val* expression, similar to what is observed in control *vhnf1* mutant embryos. 14/14 wild-type sibling embryos with RA beads showed ectopic unilateral *val* expression. Dorsal views show anterior to the left. Arrows point to beads. r3: rhombomere 3 *krox-20* expression. Scale bar: 50 μm.

raldh2 mutants, or embryos exposed to moderate doses of the RA-receptor inhibitor BMS493, have mild defects in hindbrain patterning, including expansion of r3–r6 (Begemann et al., 2001; Grandel et al., 2002). More recent studies have shown that high doses of another RA-receptor inhibitor, or the Raldh inhibitor DEAB, cause severe posterior hindbrain defects in zebrafish, including loss of *val* expression (Linville et al., 2004; Begemann et al., 2004). We show that increasing doses of either BMS493 or DEAB generate a series of phenotypes that resemble the effects of increasing RA inhibition in other vertebrates, where r7 is reduced or lost first, then r6, then r5 (Table 1; Fig. 2). With the highest dose of either inhibitor that acts specifically, we observe loss of r5–r7 accompanied by posterior expansion of r3 and r4 (Table 1; Fig. 2), mimicking the most severe phenotypes of RA inhibition that have been reported in other vertebrates (reviewed by Begemann and Meyer, 2001; Gavalas, 2002). Thus, we demonstrate a series of RA concentration-dependent requirements for zebrafish posterior hindbrain development. The variety of approaches that have been used to inhibit RA signaling in several vertebrates, yielding very similar phenotypes, argues for the extremely conserved requirements for RA signaling in the vertebrate hindbrain, where progressively more posterior rhombomeres require increasingly higher levels of RA signaling for their development.

An increasing source of RA sequentially initiates the expression of posterior rhombomere markers

In addition to concentration, timing of RA signaling has been proposed to be an important component of RA action

in the posterior hindbrain (reviewed in Begemann and Meyer, 2001; Gavalas, 2002). Our finding that increasingly more posterior hindbrain genes initiate expression sequentially near the source of RA synthesis (Fig. 1) further suggests an important role for the timing of RA signaling in hindbrain patterning. We find that RA signaling is required in a time- as well as concentration-dependent manner for posterior rhombomere specification. We show that RA inhibition just before the time of initiation of *vhnf1* and *hoxd4a* is sufficient to block their expression (Fig. 3). We also show that the temporal requirements for RA signaling proceed in an anterior–posterior direction, with r5–r6 requiring RA earlier than r7 (Fig. 3), consistent with findings in other vertebrates (reviewed by Begemann and Meyer, 2001; Gavalas, 2002).

The role of timing as well as concentration of RA signaling has provided support against a static gradient of RA acting across the hindbrain. The prevailing hypothesis that integrates the time- and concentration-dependent requirements for RA is that more posterior rhombomeres need to be exposed to RA for longer time periods during development (Gavalas, 2002; Maden, 2002). We directly tested this hypothesis by rescuing DEAB treatments with RA at later and later time points. We find that RA is not needed for *vhnf1* or *hoxd4a* expression until just at the time when expression of either gene is normally initiated (Fig. 4). These experiments strongly argue against the idea that more posterior rhombomere markers require RA signaling for longer time periods during development in order to be activated. We then proposed an alternative hypothesis: that an increasing source of RA during development could account for both the time and concentration dependence of RA. We directly tested and provided evidence for this hypothesis by increasing the RA source with RA-labeled beads and then examining early initiation of posterior markers. Our RA bead experiments show that posterior markers *vhnf1* and *hoxd4a* not only respond to RA in a concentration-dependent manner but also are competent to respond to RA earlier than they normally initiate (Fig. 5). These experiments strongly support our hypothesis that an increasing source of RA sequentially initiates the expression of posterior rhombomere markers during hindbrain development. Despite the critical importance of understanding the temporal role of RA in *Hox* gene regulation, to our knowledge, only one other study has shown that RA can initiate *Hox* gene expression precociously during development (Roelen et al., 2002).

We propose that, as development proceeds, the source of RA increases locally to sequentially induce increasingly more posterior fates at higher and higher thresholds. Our hypothesis thus provides an alternative view to either a static gradient of RA acting across the hindbrain or more posterior rhombomeres requiring longer exposure to RA. The model in Fig. 7 illustrates our hypothesis. We assume that the wild-type source of RA increases linearly over time and induces increasingly more posterior fates at regular defined thresholds (Fig. 7A). Genes and rhombomeres that require only low levels of RA, such as *hox1* genes and r4, are thus initiated early, and those that require high levels of RA, such as *hoxd4a* and r7, can only be initiated at later stages when the source has grown. This scenario leads to the production of r3–r7 with equivalent lengths (Fig. 7B, wild type). Decreasing the RA source predicts rhombomere defects that correspond to those observed experimentally (Fig. 7). Interestingly, our assumption that the source of RA normally increases *linearly* over time does a remarkably good job of predicting the rhombomere phenotypes that are observed experimentally upon inhibiting RA signaling (Fig. 7; see also Table 1). An RA level of 87.5% of wild type would generate slightly expanded r3–r6 and reduced r7, corresponding to the zebrafish *raldh2* mutant phenotype. An RA level of 62.5% of wild-type would generate expanded r3–r5, reduced r6 and loss of r7, corresponding to a 0.5 μM DEAB treatment. An RA level of 37.5% of wild-type would lead to expanded r3 and r4 and loss of r5–

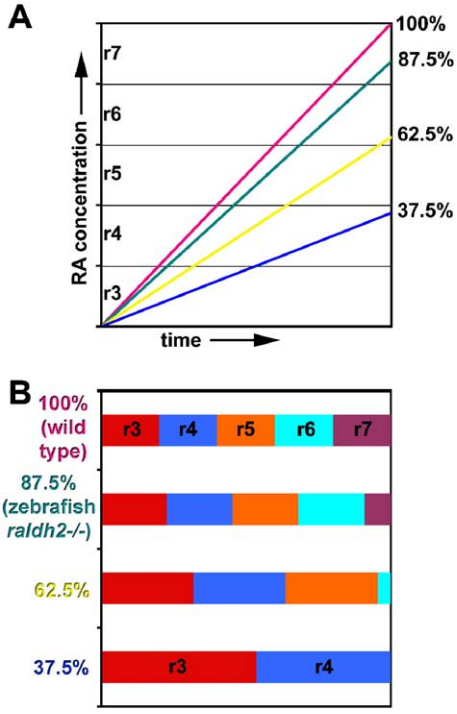

Fig. 7. Hypothetical model of RA effects on rhombomere identity. (A) We assume that the wild-type source of RA increases linearly over time (A, pink line) and induces more and more posterior fates at regular defined thresholds, noted on the *y* axis (A). We further assume that, as development proceeds (time, *x* axis in A), RA signaling will continue to induce a specific rhombomere fate until its concentration reaches the next higher threshold. We also assume that there is a time at which RA can no longer continue to induce hindbrain fates. This scenario leads to the production of r3–r7 with equivalent lengths (B, wild type). With decreasing RA sources (green, yellow, blue lines, A), the time it takes for the sources to achieve successive thresholds increases (A). To calculate predicted rhombomere lengths for each RA source in panel (A), we extrapolated the hypothetical RA-inhibition lines shown in panel (A) and assumed that the length of time between achieving each threshold would directly correspond to each rhombomere's length (B; data not shown).

r7, corresponding to a 5 μM DEAB treatment. Our model predicts that, at an 87.5% RA level, r3 increases by about 13%, the same value directly measured by Grandel et al. (2002) for the zebrafish *raldh2* mutant r3. However, our model also predicts that, at an 87.5% RA level, r5 and r6 each increase by about 13%, yet direct measurements of zebrafish *raldh2* mutants show that r5 increases only by about 7% and r6 increases by about 17% (Grandel et al., 2002).

Our model is supported by other experimental data. Increasing the RA source too early, achieving increasingly higher thresholds, would precociously induce increasingly more posterior rhombomere fates, similar to what we observe with increasing concentrations of RA beads (Fig. 5). Furthermore, our model predicts that decreasing the wild-type source at a late stage would cause the production of anterior rhombomere fates posterior to more posterior rhombomere fates; for example, a drop in RA levels to the r4 threshold at the time the r6 threshold has been reached should produce r4 fates posterior to r5. Late RA inhibitor treatments, which cause a patchy posterior spreading of r4–r6 markers, may support this prediction (Dupé and Lumsden, 2001; L.M., unpublished observations).

While sequential signaling of the RA source has been previously proposed (Maden, 2002), we suggest that a critical addition to this model is the importance of the temporally increasing RA source. A temporally increasing source of RA could be generated by an increasing number of cells expressing *raldh2*, as may occur during early zebrafish hindbrain patterning (see Fig. 1), or by successively more posterior mesoderm cells expressing increasing levels of *raldh2*, as may occur during early chick hindbrain patterning (Swindell et al., 1999). In support of our increasing RA source hypothesis, somite grafting experiments in chick embryos have shown that the ability of somite tissue to induce ectopic anterior *Hoxb4* expression moves posteriorly during development and seems to parallel changing levels of *raldh2* expression in the somites (Itasaki et al., 1996; Swindell et al., 1999). One advantage of our model is that it takes into account that morphogenetic movements during gastrulation could affect the ability of mesodermal tissues to signal to the hindbrain. As sequentially more posterior hindbrain genes are activated during zebrafish gastrulation, the mesodermal source of RA is shifting posteriorly relative to the presumptive hindbrain (Fig. 1). Hindbrain cells closest to the RA source at a given time will activate the appropriate rhombomere identity based on the level of RA. As these hindbrain neuroepithelial cells undergo cell divisions and cell movements, they shift anteriorly relative to the mesodermal source (Kimmel and Warga, 1987; Kimmel et al., 1994; Concha and Adams, 1998).

Many other experiments have argued against a static gradient of RA acting across the hindbrain (reviewed by Maden, 2002). In particular, maternal RA administration or RA bath treatments have been shown to at least partially rescue hindbrain patterning in vitamin-A-deficient rat and quail embryos, in *raldh2* mutant mice and in *raldh2* mutant fish (Gale et al., 1999; White et al., 2000; Niederreither et al., 2000; Mic et al., 2002; Begemann et al., 2001; Grandel et al., 2002). We show that even severe RA-synthesis inhibition can be completely rescued with an RA bath (Fig. 4). Mic et al. (2002) show that RA rescue of *Raldh2* mutant mice appears to activate endogenous local sources of RA, possibly helping to explain how a global RA treatment can promote a normal rescued pattern. Other factors that contribute to RA signaling or metabolism, such as the Cyp26 RA-degradation enzymes, play important roles in regulating RA activity in the hindbrain (reviewed by Maden, 2002). In zebrafish, the expression pattern of *cyp26a1* in the anterior hindbrain is complementary to that of early *hox* gene and *raldh2* expression (Kudoh et al., 2002; Dobbs-McAuliffe et al., 2004) and may support a function for Cyp26 as a sink for an RA gradient. The zebrafish *cyp26a1* mutant shows a reduced hindbrain and expanded anterior spinal cord phenotype that appears to be opposite to that of the *raldh2* mutant (Emoto et al., 2005). However, additional studies of the zebrafish *cyp26a1* mutant, as well as the examination of dynamic expression and functions of Cyp26 enzymes in mouse embryos, have suggested that these enzymes likely support dynamic activity of RA in the hindbrain (Sirbu et al., 2005; Emoto et al., 2005). In order to obtain further understanding of how RA regulates gene expression and rhombomere identity in the hindbrain, it will be necessary to identify the functions of additional *cyp26* genes. Furthermore, it is not yet known which RA receptors are functioning in the zebrafish hindbrain to mediate the signaling requirements that we have described here. In the future, it will be critical to determine which receptors are used in order to more completely dissect the direct targets and actions of RA.

Acknowledgments

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