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Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development

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Retinoic acid (RA) is essential for normal vertebrate development, including the patterning of the central nervous system. During early embryogenesis, RA is produced in the trunk mesoderm through the metabolism of vitamin A derived from the maternal diet and behaves as a morphogen in the developing hindbrain where it specifies nested domains of Hox gene expression. The loss of endogenous sources of RA can be rescued by treatment with a uniform concentration of exogenous RA, indicating that domains of RA responsiveness can be shaped by mechanisms other than the simple diffusion of RA from a localized posterior source. Here, we show that the cytochrome p450 enzymes of the Cyp26 class, which metabolize RA into polar derivatives, function redundantly to shape RA-dependent gene-expression domains during hindbrain development. In zebrafish embryos depleted of the orthologs of the three mammalian *CYP26* genes *CYP26A1*, *CYP26B1* and *CYP26C1*, the entire hindbrain expresses RA-responsive genes that are normally restricted to nested domains in the posterior hindbrain. Furthermore, we show that Cyp26 enzymes are essential for exogenous RA to rescue hindbrain patterning in RA-depleted embryos. We present a 'gradient-free' model for hindbrain patterning in which differential RA responsiveness along the hindbrain anterior-posterior axis is shaped primarily by the dynamic expression of RA-degrading enzymes.

KEY WORDS: Retinoic acid, Hindbrain, Cyp26, Hox, Morphogen, Zebrafish

INTRODUCTION

Retinoic acid (RA) is a known teratogen with crucial roles in the patterning of the vertebrate nervous system. In the hindbrain, RA is essential for the establishment of the anterior-posterior pattern, as demonstrated by embryos in which RA is depleted either dietarily, pharmacologically or genetically (Begemann et al., 2004; Dupe and Lumsden, 2001; Maden et al., 1996; Niederreither et al., 1999). RA is produced in the anterior paraxial mesoderm by the activity of aldehyde dehydrogenase 1 family, member A2 (Aldh1a2), which oxidizes retinal to RA (Begemann et al., 2001; Gavalas, 2002; Niederreither et al., 1999). RA either diffuses or is transported from the paraxial mesoderm into the adjacent central nervous system. RA directly regulates gene expression through its nuclear hormone receptor (RAR) and co-receptor (RXR), which bind RA response elements (RAREs) in the enhancers of target genes (Bastien and Rochette-Egly, 2004). In the hindbrain, RA regulates the expression of 3′-Hox genes through direct (in the case of Hox-1 and Hox-4 genes) or indirect (in the case of Hox-3 genes) mechanisms (Gould et al., 1998; Hernandez et al., 2004; Marshall et al., 1994; Nolte et al., 2003; Studer et al., 1994; Zhang et al., 2000). Other anterior RA-responsive genes (Hox-1 family genes) are expressed earlier and at lower RA concentrations than the more-posterior RA-responsive genes (Hox-4 family genes) (Dupe and Lumsden, 2001; Maves and Kimmel, 2005; Simeone et al., 1990). Based on the effects of switching the RAREs of the Hox-1 and Hox-4 genes, Gould et al. proposed that Hox-1 genes are expressed at more anterior levels than Hox-4 genes because their RAREs are more sensitive to RA (Gould et al., 1998).

These data have lead to a model in which a continuous spatio-temporal gradient of RA through the hindbrain generates nested domains of RA-responsive gene expression. These domains are then resolved by secondary mechanisms into non-overlapping domains that correspond with the morphological segments of the hindbrain – the rhombomeres (r). However, a number of observations suggest that an RA gradient is neither detectable nor required for normal hindbrain development. First and foremost, embryos depleted of endogenous RA can be fully rescued by a uniform concentration of exogenous RA (Begemann et al., 2004; Begemann et al., 2001; Gale et al., 1999; Grandel et al., 2002; Mic et al., 2002; Niederreither et al., 2000). Second, this rescue can be accomplished by a range of RA concentrations and over a range of developmental stages (Dupe and Lumsden, 2001; Maves and Kimmel, 2005). Third, when RA responsiveness is measured by the expression of a RARE-LacZ reporter, no gradient of expression is detected in the hindbrain. Instead, distinct boundaries of reporter expression that shift over time are detected (Rossant et al., 1991; Sirbu et al., 2005). Finally, in contrast to earlier findings (Gould et al., 1998), recent evidence has suggested that, in the context of their intact enhancers, a Hox-1 RARE is equally responsive to RA as a Hox-4 RARE (Nolte et al., 2003).

These data suggest that cells in the presumptive hindbrain neuroepithelium can be patterned by RA in a manner that is independent both of concentration and of duration of exposure, necessitating a new model for RA-dependent hindbrain patterning. Here, we propose such a model based on the hindbrain patterning defects caused by the prevention of RA metabolism by the cytochrome P450 enzymes of the Cyp26 class. The Cyp26 enzymes (Cyp26a1, Cyp26b1 and Cyp26c1) have been proposed to function in the regulation of RA-dependent gene expression through their ability to metabolize RA into hydroxylated polar derivatives (Fujii et al., 1997; White et al., 1996). In the mouse tailbud and limbs, loss

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of Cyp26 function leads to increased RA-dependent gene expression, spina bifida and caudal agenesis similar to the teratogenic effects of high concentrations of exogenous RA (Abu-Abed et al., 2001; Sakai et al., 2001; Yashiro et al., 2004).

With regard to hindbrain patterning, *cyp26a1* is expressed during gastrulation in the anterior neurectoderm (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002). Based on this expression domain, it was hypothesized that localized regions of RA synthesis in the anterior trunk mesoderm and degradation in the anterior neural plate provide a classical 'source-and-sink' mechanism for the spatial regulation of RA in the central nervous system (Kudoh et al., 2002; Swindell et al., 1999). However, *cyp26a1* mutants in fish and mouse exhibit relatively subtle hindbrain-patterning defects inconsistent with a global role for *cyp26a1* in hindbrain patterning (Abu-Abed et al., 2001; Emoto et al., 2005; Kudoh et al., 2002; Sakai et al., 2001). The recent identification of other cyp26 genes has suggested that these may participate in shaping RA responsiveness in the hindbrain (Abu-Abed et al., 2002; Gu et al., 2005; MacLean et al., 2001; Reijntjes et al., 2005; Reijntjes et al., 2004; Sirbu et al., 2005; Tahayato et al., 2003; Taimi et al., 2004; Zhao et al., 2005). Here, we demonstrate that the zebrafish orthologs of mammalian CYP26B1 and CYP26C1 function redundantly with *cyp26a1* to pattern the hindbrain, because embryos depleted of all three proteins exhibit a profound posterior transformation of the hindbrain. Furthermore, we demonstrate that cyp26 genes are responsible for the ability of exogenous RA to rescue embryos depleted of endogenous sources of RA. In embryos depleted of Cyp26 activity, the low RA concentrations that normally rescue the RA-depleted hindbrain are highly teratogenic. Based on our results, we present a 'gradient-free' model for RA-dependent hindbrain patterning in which the spatially regulated inactivation of RA by Cyp26 enzymes is responsible for the establishment of RA-responsive gene-expression domains in the hindbrain.

MATERIALS AND METHODS

Cloning

Cyp26b1 was initially identified as an EST (Nelson, 1999), and we cloned the 5′ end of the coding sequence with the SMART RACE kit (Clontech). *cyp26c1* was identified in a Blast search of the zebrafish genome sequence using the human CYP26C1 protein sequence and was then amplified from 12 hour post fertilization (hpf) whole-zebrafish-embryo cDNA.

Morpholinos, RNA in situ hybridizations and genotyping

Table 1 summarizes the sequences of the morpholinos (MOs) used in this study, the combinatorial depletion experiments performed and their outcomes. To determine the efficacy of *cyp26b1* MO1, we made a *cyp26b1*-GFP fusion construct by cloning a fragment of the 5′ UTR and 5′ coding sequence including the MO target sequence into pCS2-GFP_{LT}XLT. The resulting plasmid was linearized with *Not*I and mRNA was prepared for injection with the SP6 mMessage mMachine kit (Ambion). Embryos were injected with 470 pg mRNA plus 5 ng MO. Embryo lysates were prepared as described (Waskiewicz et al., 2001), separated on 4-12% NuPAGE Bis-Tris gels and transferred to Invitrolon PVDF membranes (Invitrogen). For immunoblotting, we used anti-GFP (Torrey Pines, 1:2000) and anti-Actin (SantaCruz, sc1616, 1:200), and proteins were detected with SuperSignal West Dura substrate (Pierce) according to the manufacturer's directions. Quantitation of the GFP bands showed that *cyp26b1* MO blocked 95-98% of the translation of the injected *cyp26b1*-GFP mRNA.

We used RT-PCR to determine the efficacy of *cyp26c1* MO1, which is targeted to the exon-3–intron-3 splice junction of the pre-mRNA. RT-PCR analysis with multiple primer pairs revealed no detectable wild-type transcript. Three alternately spliced transcripts were detected and sequenced: two resulted in the introduction of a premature stop codon either by the inclusion of intron 3 or the exclusion of exon 3. A third transcript resulted in the deletion of the last 18 bases of exon 3, which leaves the transcript in frame but deletes six amino acids, several of which are conserved across Cyp26 family members.

All of the experiments described in this manuscript used *cyp26b1* MO1 and *cyp26c1* MO1; however, *cyp26b1* MO2 and *cyp26c1* MO2 gave the same phenotypes. Unlike *cyp26c1* MO1, *cyp26c1* MO2 was toxic at higher concentrations. Our control MO was targeted to the *dead-end* mRNA and eliminates primordial germ cells but does not affect other aspects of development (Weidinger et al., 2003). To assay for redundancy between the cyp26 genes, MOs were injected alone or in combination into embryos from a *cyp26a1*$^{+/-}$ intercross (Emoto et al., 2005). In order to control for non-specific effects due to MO injections, all embryos were injected with a total of 5 ng MO as determined by measuring the diameter of the injected bolus in mineral oil (see Table 1).

Two-color RNA in situ hybridizations were performed, essentially as described (Prince et al., 1998), except that Iodo-Nitrotetrazolium Violet (Sigma) was used as the red Alkaline Phosphatase substrate. Embryos were de-yolked and flat-mounted for photomicroscopy using a Zeiss Axioplan II microscope. After photographing, individual embryos were un-mounted and genotyped for the *cyp26a1* mutation as described (Emoto et al., 2005).

Drug treatments

Dechorionated embryos from wild-type or *cyp26a1*$^{+/-}$ parents were incubated in the dark in pharmacological agonists and antagonists of the RA-metabolism pathway as follows:

4-(diethylamino)benzaldehyde [DEAB, an inhibitor of retinaldehyde dehydrogenases (Russo et al., 1988); Aldrich]: 10 μM, beginning at 50% epiboly [5.25 hpf (Kimmel et al., 1995)];

R115866, a specific inhibitor of Cyp26 enzymes (Janssen Pharmaceutica): 10 μM, beginning at dome stage (4.33 hpf);

all-trans RA (Sigma): 0.1-100 nM, beginning at 50% epiboly.

Table 1. Summary of combinatorial *cyp26* knock-down experiments and their outcomes

Treatment	Summary of phenotype	
	Cyp26a1+	Cyp26a1–/–
5 ng control MO	None	Very mild (expanded r4)
2.5 ng cyp25b1 MO1 + 2.5 ng control MO	None	Very mild (expanded r4)
2.5 ng cyp25c1 MO1 + 2.5 ng control MO	None	Medium (up to cerebellum)
2.5 ng cyp25b1 MO1 + 2.5 ng cyp26c1 MO	None	Severe (up to cerebellum)
5 nM RA	None	Severe (throughout brain)
10 μM DEAB + 5 nM RA	None	Severe (throughout brain)

Morpholino sequences:

Cyp26b1 MO1 (ATG)	5′-CTCGAAGAGCATGGCTGTGAACGTC-3′
Cyp26b1 MO2 (exon-2–intron-2 splice)	5′-ATTGACCTTACCTTCCTCCTTTTGC-3′
Cyp26c1 MO1 (exon-3–intron-3 splice)	5′-AAACTCGGTTATCCTCACCTTGCGC-3′
Cyp26c1 MO2 (intron-1–exon-2 splice)	5′ GGAACCCTGTCACAACATAACAGAG-3′
Control (dead-end MO)	5′-GCTGGGCATCCATGTCTCCGACCAT-3′

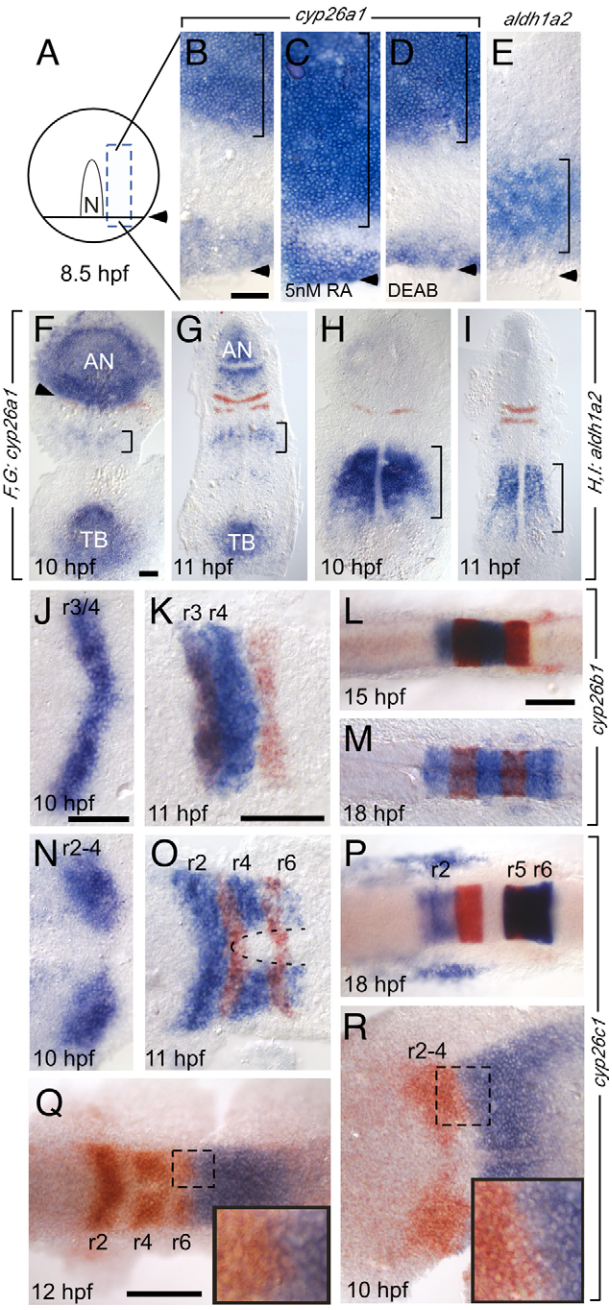

Fig. 1. Expression of the cyp26 genes in the developing hindbrain. (**A-R**) Whole-mount in situ hybridizations during the hindbrain patterning period. All embryos are shown as dorsal views. Anterior is to the top in A-I and to the left in J-R. In situ probes are noted in brackets beside the panels, embryonic age is noted in hours post fertilization (hpf). (A) Schematic of an 80% epiboly (8.5 hpf) embryo. The dotted box is the region shown in the flat-mounted embryos in B-E; the arrowhead indicates the advancing margin of the epiblast. During gastrulation, *cyp26a1* (B-D,F,G) is expressed in the ectoderm (bracket in B-D) anterior to the domain of RA synthesis indicated by *aldh1a2* expression (bracket in E). (C,D) Ectodermal *cyp26a1* expression expands in the presence of sub-teratogenic concentrations of RA (C), but is established independent of RA (D). (F,G) *cyp26a1* expression recedes anteriorly at the onset of somitogenesis. *krox20* (red) is shown in r3 and r5. Arrowhead indicates the posterior limit of *cyp26a1* expression. Bracket marks weak *cyp26a1*expression in the anterior trunk mesoderm. (H,I) *aldh1a2* expression during early somitogenesis. Bracket shows expression in trunk mesoderm. (J-R) Dynamic *cyp26b1* (J-M) and *cyp26c1* (N-R) expression during somitogenesis. *krox20* expression in r3 and r5 is in red in J-P; *cyp26b1* expression is shown in blue in J-M; and cyp26c1 expression is shown in blue in N-R. In Q,R, *cyp26c1* is in red whereas *hoxd4* (Q) and *vhnf1* (R) are in blue. Insets in Q,R correspond to the dotted boxes. Dotted curve in O indicates the *cyp26c1*-free domain in ventral r3-r6. Scale bars: 100 μM. Scale bar in B is for B-E; scale bar in F is for F-I; scale bar in J is for J,N,R; scale bar in K is for K,O; scale bar in L is for L,M,P. AN; anterior neurectodermal expression; TB, tailbud expression.

In each case, the compound was diluted to 1000 times its final concentration in DMSO and then diluted 1000-fold in embryo medium. Controls were treated with carrier alone (0.1% DMSO). For retinal treatments, embryos were injected at the one-cell stage with 1 nl of 20 pmol/nl all-trans retinal in DMSO (Sigma), and controls were injected with 1 nl DMSO alone.

RESULTS

cyp26b1 and *cyp26c1* are expressed dynamically during hindbrain development

Mammalian genomes contain three CYP26 genes: *CYP26A1*, *CYP26B1* and *CYP26C1*. Cloning of the zebrafish *cyp26a1* ortholog has been described (White et al., 1996). We cloned zebrafish homologs of *cyp26b1* and *cyp26c1*, and examined their expression during development. *Cyp26c1* has been previously described as *cyp26d1* (Gu et al., 2005) and *cyp26b1-like* (Kawakami et al., 2005) (ZDB gene 050714-2). Based on two lines of evidence, we argue that this gene is in fact the ortholog of mammalian *CYP26C1*. First, phylogenetic analysis of zebrafish cyp26 genes places it in the same clade as mouse and human *CYP26C1* genes with moderate bootstrap support (see Fig. S1A in the supplementary material). Second, *CYP26A1* and *CYP26C1* are adjacent to one another on human chromosome 10q23-q24, and current zebrafish genomic sequence data and radiation-hybrid data places zebrafish *cyp26a1* and *cyp26c1*, *cyp26d1- and cyp26b1-like* in regions of zebrafish linkage groups (LGs) 12 and 17, respectively, which show synteny to human chromosome 10 (see Fig. S1B in the supplementary material) (Woods et al., 2005). Because draft genomes for other tetrapod vertebrates also show *CYP26A1* and *CYP26C1* as adjacent genes, it is likely that they were adjacent genes in the ancestral vertebrate genome and that, following the proposed genome duplication early in teleost evolution, the duplicate of *cyp26c1* was lost from LG 12 and the duplicate of *cyp26a1* was lost from LG 17. Henceforth, we refer to the gene previously named *cyp26d1* or *cyp26b1-like* as *cyp26c1*.

The expression patterns of zebrafish *cyp26a1*, *cyp26b1* and *cyp26c1* have been described (Dobbs-McAuliffe et al., 2004; Gu et al., 2005; Kudoh et al., 2002; Zhao et al., 2005). We focus here on their expression during hindbrain development. During gastrulation, *cyp26a1* is expressed in the anterior neurectoderm (bracket in Fig. 1B) and in a narrow domain at the margin at 8.5 hpf (arrowhead in Fig. 1B). The posterior limit of *cyp26a1* expression at 8.5 hpf abuts the anterior limit of *hoxb1b* expression at the r3-r4 boundary (Kudoh et al., 2002), but rapidly recedes anteriorly to lie at the r2-r3 boundary at 10 hpf and lies further anterior still at 11 hpf (Fig. 1F,G). As described previously, *cyp26a1* is directly RA-inducible, even at sub-teratogenic concentrations of RA (5 nM), which cause the ectodermal domain of expression to expand towards the margin (Fig.

1C) (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002; Loudig et al., 2000; White et al., 1996). In spite of its strong RA-inducibility, the early anterior neurectodermal expression of *cyp26a1* is established independently of RA, evident because it is unaffected in embryos treated with 4-(diethylamino)benzaldehyde (DEAB), a specific inhibitor of retinaldehyde dehydrogenase (Fig. 1D) (Dobbs-McAuliffe et al., 2004; Sirbu et al., 2005). Throughout the hindbrain-patterning period, neurectodermal *cyp26a1* expression lies significantly anterior to that of *aldh1a2*, which is restricted to the anterior mesoderm (Fig. 1E,H,I) (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002; Sirbu et al., 2005; Swindell et al., 1999). *cyp26a1* is also expressed in the tailbud and in a crescent in the anterior trunk mesoderm immediately anterior to the *aldh1a2*-expressing domain (brackets in Fig. 1F,G).

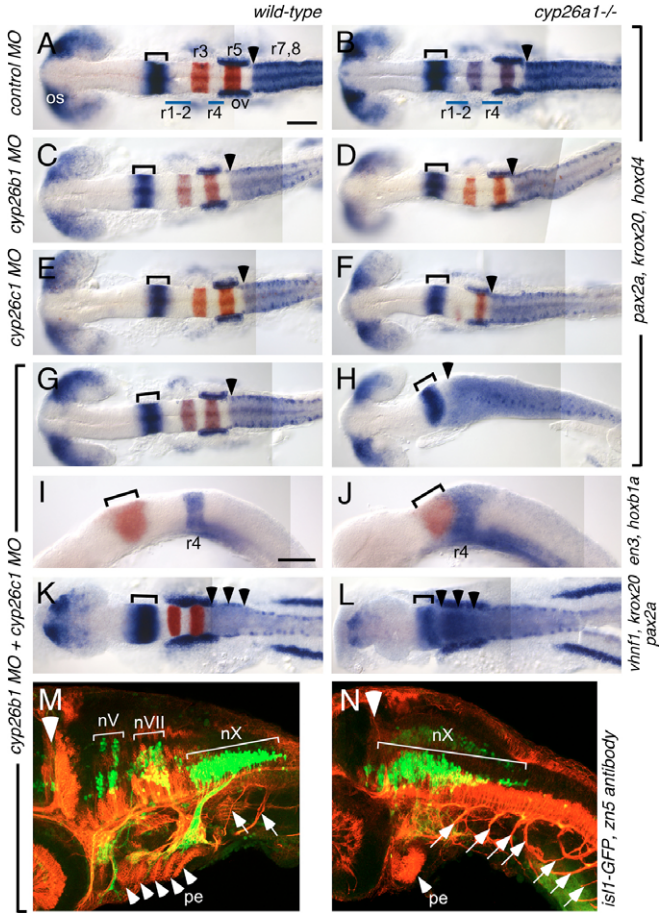

Fig. 2. *cyp26b1* and *cyp26c1* function redundantly with *cyp26a1* to pattern the hindbrain. Whole-mount RNA in situ hybridizations at 18 hpf (**A-J**) and 13 hpf (**K**,**L**) and immunostaining at 48 hpf (**M**,**N**) in wild-type (left column) and *cyp26a1*$^{-/-}$ (right column) embryos injected with MOs as shown on the left. (A-H) *pax2a* (blue) marks the optic stalk (os), posterior midbrain and cerebellum (bracket), and the otic vesicles (ov); whereas *hoxd4* (also blue) marks the r7-r8 territory and *krox20* (red) marks r3 and r5. MO depletion of Cyp26b1 and/or Cyp26c1 does not affect this pattern in wild-type embryos (C,E,G), but progressively posteriorizes the hindbrain in *cyp26a1*$^{-/-}$ embryos (D,F,H). Arrowhead marks the r6-r7 boundary, which is shifted to the anterior hindbrain in Cyp26-depleted embryos. (I,J) *en3* (red) marks the posterior midbrain and cerebellum (bracket) and *hoxb1a* (blue) marks r4, which is shifted anteriorly in Cyp26-depleted embryos. (K,L) *pax2a* (blue) and *krox20* (red) are expressed as described above. *vhnf1* (also blue) is expressed in the posterior hindbrain up to the r5-r6 boundary (arrowheads) and is also shifted anteriorly in Cyp26-depleted embryos. (M,N) The *isl1*-GFP transgene (green) marks cranial motor neurons (nV: trigeminal motor neurons in r2 and r3; nVII: facial motor neurons in r4-6; nX: vagal motor neurons in r8) whereas the zn5 antibody (red) marks spinal motor neurons (arrows), pharyngeal arch endoderm (pe, arrowheads mark individual pharyngeal arches) and other structures. The large white arrowhead indicates the mid-hindbrain boundary. In Cyp26-depleted embryos, the motor neurons of the vagus nerve (nX) are expanded anteriorly, as are the spinal motor neurons. Scale bars: 100 μm. Scale bar in A is for A-H,K,L; scale bar in I is for I,J.

cyp26b1 and *cyp26c1* are expressed in the developing hindbrain in a dynamic, rhombomere-restricted fashion. *cyp26b1* expression is initiated in r3 and r4 beginning at tailbud stage (10 hpf; Fig. 1J). This expression slowly expands to include r2 by the 12-somite stage (15 hpf; Fig. 1L), and r5 and r6 by the 20-somite stage (19 hpf; Fig. 1M). *cyp26c1* expression overlaps with, but precedes, *cyp26b1* expression at each stage. *cyp26c1* expression is initiated earlier, before the end of gastrulation (9 hpf), in presumptive r2 through r4 in a domain that abuts the anterior limit of *vhnf1* (also known as *tcf2* – Zebrafish Information Network; ZDB gene 020104-1) expression at the presumptive r4-r5 boundary (Fig. 1N,R). Expression rapidly expands posteriorly to include r6 by the 6-somite stage (12 hpf), at which time the posterior limit of *cyp26c1* expression abuts the anterior limit of *hoxd4* (also known as *hoxd4a* – Zebrafish Information Network) expression (Fig. 1O,Q). At the same time, *cyp26c1* (but not *cyp26b1*) expression is downregulated in r3. *cyp26c1* (but not *cyp26b1*) expression is excluded from the ventral-most hindbrain above the anterior tip of the notochord during the early somite stages (dotted line in Fig. 1). Although the significance of this ventral exclusion of *cyp26c1* expression for RA distribution is not known, we find that the ventral hindbrain is more sensitive to exogenous RA than are more dorsal hindbrain regions (see Fig. S2 in the supplementary material). By 14 hpf, *cyp26c1* expression is downregulated in r2 to r4 and is strongly up-regulated in r5 and r6 (Fig. 1P).

Unlike *cyp26a1*, neither *cyp26b1* nor *cyp26c1* are globally upregulated by exogenous RA (data not shown, and see Fig. S3B,E in the supplementary material). RA is also not required for the normal onset of their expression, evident because both genes are expressed in DEAB-treated embryos (see Fig. S3C,F in the supplementary material). However, we did observe effects on the expression of both *cyp26b1* and *cyp26c1* at the 3-somite stage that suggest that both genes are affected indirectly by RA-dependent patterning events in the hindbrain (see Fig. S3 in the supplementary material). Briefly, in 100 nM RA, r4 is expanded anteriorly, and with it the r4 expression of *cyp26b1* and *cyp26c1*, whereas in 10 μM DEAB r2 and r3 are expanded posteriorly, and with them the r2 and r3 expression of *cyp26b1* and *cyp26c1*. Sirbu et al. (Sirbu et al., 2005) showed that *cyp26c1* expression in r4 is dependent upon RA. We do not see clear evidence of this in the zebrafish, although the r4 domain of *cyp26c1* expression is consistently reduced at the 3-somite stage in DEAB-treated embryos (see Fig. S3B in the supplementary material).

cyp26b1 and *cyp26c1* contribute to normal hindbrain patterning

We tested the function of Cyp26b1 and Cyp26c1 by knocking-down their function using antisense MOs. We performed all of our experiments in embryos generated by intercrossing *cyp26a1* heterozygotes so that we could examine Cyp26b1 and Cyp26c1 function both in the presence and in the absence of Cyp26a1 function (Table 1). The hindbrain phenotype of *cyp26a1* mutants is

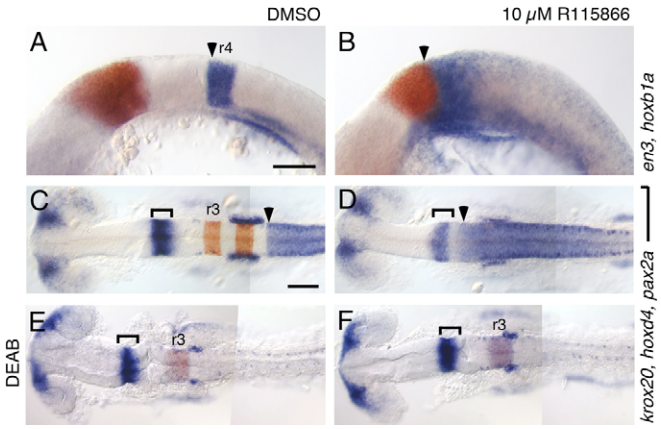

Fig. 3. A selective antagonist of Cyp26 enzymes recapitulates the *cyp26a1*; *cyp26b1*; *cyp26c1* phenotype. RNA in situ hybridizations with the markers described in Fig. 2. (**A-F**) Compared with DMSO-treated controls (A,C,E), treatment with 10 μM R115866 (B,D,F) causes an anterior shift of *hoxb1a* (arrowhead in A,B) and *hoxd4* (arrowhead in C,D) towards the presumptive cerebellum, marked by *en3* (red in A,B) and by *pax2a* (brackets in C-F). This effect of R115866 is reversed by co-treatment with 10 μM DEAB (E,F). Scale bars: 100 μm. Scale bar in A is for A,B; Scale bar in C is for C-F.

subtle: r4 [marked by *hoxb1a* and bounded by the r3 and r5 stripes of *krox20* (also known as *egr2b* – Zebrafish Information Network)] is slightly expanded in length and the anterior hindbrain (r1-r3) is slightly reduced (Fig. 2A,B, Fig. 4A,B and see Fig. S4A,B in the supplementary material) (Emoto et al., 2005). Furthermore, the posterior-most hindbrain – marked by high levels of *hoxd4* expression, and comprising r7 and the long unsegmented 'vagal' rhombomere, r8, which lies between the segmented hindbrain and the first somite (Lumsden, 1990) – is expanded in length as described previously (Emoto et al., 2005) (Fig. 2A,B and Fig. 4A,B).

Depleting embryos of either Cyp26b1 or Cyp26c1, or of both enzymes, caused no brain-patterning phenotype in wild-type embryos, aside from a subtle shortening of the hindbrain (Fig. 2 and see Fig. S4 in the supplementary material, left columns). However, depleting both enzymes strongly enhanced the *cyp26a1*$^{-/-}$ hindbrain phenotype (Fig. 2 and see Fig. S4 in the supplementary material, right columns). In *cyp26b1* MO-injected *cyp26a1*$^{-/-}$ embryos, r4 is further expanded (Fig. 2D and see Fig. S4C,D in the supplementary material) and the r6-r7 boundary is shifted slightly towards r5 (arrowhead in Fig. 2C,D). Knocking-down *cyp26c1* caused a stronger enhancement of the *cyp26a1*$^{-/-}$ phenotype, consistent with its earlier onset of expression in the presumptive hindbrain. In these embryos, r3 is strongly reduced or absent, whereas r4 is expanded anteriorly so that its anterior limit lies adjacent the posterior limit of *en3* (also known as *eng2b* – Zebrafish Information Network) expression in the presumptive cerebellum (Fig. 2F and see Fig. 4F in the supplementary material). The r6-r7 boundary is again shifted anteriorly, but remains posterior to a narrow r5 (arrowhead in Fig. 2F).

Embryos depleted of all three Cyp26 proteins have a strongly posteriorized hindbrain (Fig. 2G-L and see Fig. S4G,H in the supplementary material). Both r3 and r5 are eliminated (Fig. 2G,H) and r4 abuts the cerebellum (Fig. 2I,J and see Fig. S4G,H in the supplementary material). The anterior limit of *vhnf1* expression, which, by the 8-somite stage (13 hpf), marks the r5-r6 boundary, is also shifted to abut the cerebellum (arrowheads in Fig. 2K,L). The r6-r7 boundary of *hoxd4* expression is similarly shifted, coming to lie within a few cell diameters of cerebellum (arrowhead in Fig. 2H). Thus, three RA-responsive genes (*hoxb1a*, *vhnf1* and *hoxd4*) that normally form nested expression domains in the hindbrain are all expanded into the anterior-most hindbrain in embryos depleted of all three Cyp26 proteins. In spite of this strong transformation of the hindbrain, the patterning of the mid- and fore-brain, marked by *pax2a*, *otx2*, *dlx2a* and *eomes*, appears unaffected, except for a decrease in length detectable in *cyp26a1* single mutants (data not shown) (Emoto et al., 2005).

We examined the neuronal organization of Cyp26-depleted embryos. In *cyp26a1*$^{+}$ embryos injected with control MOs or with *cyp26b1* and/or *cyp26c1* MOs, we observed normal patterns of cranial and spinal motor-nerve differentiation (Fig. 2M). However, in *cyp26a1*$^{-/-}$ embryos injected with both *cyp26b1* MO and *cyp26c1* MO, the vagal neurons (nX in Fig. 2M,N) characteristic of r8 are expanded to the mid-hindbrain boundary (large arrowhead in Fig. 2M,N) and spinal motor roots (arrows in Fig. 2M,N) extend from hindbrain levels into a disorganized branchial region (small arrowheads in Fig. 2M,N). This occurs in spite of the fact that there are no somites to innervate at this level (data not shown). These neuronal phenotypes are consistent with our analysis of marker gene expression, in which the RA-inducible hox gene characteristic of r7-8 (*hoxd4*) is expanded anteriorly throughout the hindbrain region.

A pharmacological inhibitor of Cyp26 activity phenocopies Cyp26 depletion

Pharmacological antagonists that inhibit RA metabolism have been developed as tools for the treatment of dermatological diseases and cancer (Njar, 2002; Njar et al., 2006). The compound R115866 is a highly selective antagonist of Cyp26a1 activity in vitro, and exerts retinoidal effects in adult rats (Stoppie et al., 2000). Its effects on Cyp26b1 and Cyp26c1 have not been examined. We observed that treating zebrafish embryos with 10 μM R115866 caused a phenotype identical to that of embryos depleted of all three Cyp26 enzymes (compare Fig. 2J,H with Fig. 3B,D). This suggests that R115866 inhibits Cyp26b1 and Cyp26c1 as effectively as does knocking-down their expression with MOs, and confirms our above observation that Cyp26 activity is essential for normal hindbrain patterning. The effects of R115866 treatment are completely reversed by the addition of DEAB, so that embryos treated with both drugs resemble embryos treated with DEAB alone (Fig. 3E,F). This demonstrates that, as for other phenotypes observed in *cyp26a1*-mutant fish and mice (Emoto et al., 2005; Niederreither et al., 2002), the posteriorized hindbrain phenotype caused by blocking all Cyp26 activity is due to the accumulation of excess RA and not to the absence of bioactive Cyp26-generated RA derivatives. Although such derivatives have been observed to have significant retinoidal effects in cells and in embryos, and have been postulated to have functions in vivo (Idres et al., 2002; Pijnappel et al., 1993), we see no evidence for their having a role in hindbrain patterning.

Cyp26a1 protects against RA teratogenicity.

The long-standing observation that depletion of endogenous RA can be rescued by treatment with a low concentration of exogenous RA demonstrates that a RA gradient is not strictly necessary for hindbrain patterning (Begemann et al., 2004; Begemann et al., 2001; Gale et al., 1999; Niederreither et al., 2000). However, the basis of this rescue phenomenon has not been determined, and has significant implications for the mechanism of hindbrain patterning. We hypothesized that Cyp26 enzymes enable this rescue by inactivating exogenous RA in a patterned manner. We tested the roles of the cyp26 genes by performing the RA-rescue experiment in Cyp26-depleted embryos.

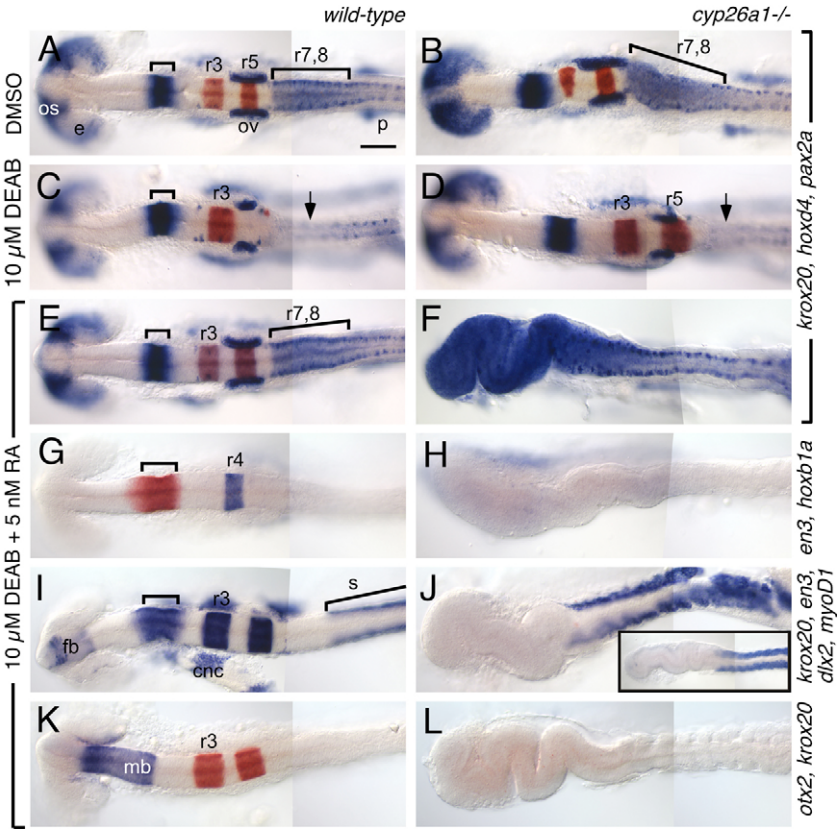

Fig. 4. *cyp26a1* protects the hindbrain from exogenous RA. Wild-type (left column) and *cyp26a1*$^{-/-}$ (right column) embryos treated with DMSO (**A**,**B**), 10 μM DEAB (**C**,**D**) or 10 μM DEAB + 5 nM RA (**E-L**). RNA in situ hybridizations use the markers described in Fig. 2, except for I,J, which is a mix of *en3* (bracket), *krox20* (r3, r5), *dlx2* [cranial neural crest (cnc) and forebrain (fb)] and *myoD* (somites; s). Large bracket in A indicates the r7-r8 region, which is elongated in *cyp26a1* mutants (B). (C) In DEAB-treated embryos, posterior rhombomeres (r5-r8) are absent (arrow indicates the absence of high *hoxd4* expression characteristic of r7-r8). (D) This phenotype is partially rescued in *cyp26a1* mutants, as seen by rescue of r5 but not of r7-r8. (E-L) The DEAB phenotype is fully rescued in wild-type embryos by treatment with 5 nM RA (E,G,I,K) whereas, in *cyp26a1* mutants, this low dose of RA causes strong posteriorization of the brain (F,H,J,L). This phenotype resembles that of wild-type embryos treated with 200 nM RA (inset in J). Scale bar: 100 μm. os: optic stalk; e: eye; p: pronephros.

The effects of blocking RA synthesis with DEAB in zebrafish have been described previously (Begemann et al., 2004; Maves and Kimmel, 2005). They include the loss of posterior hindbrain identities (r5-r8, Fig. 4C), expansion of anterior hindbrain identities (r2-r4), and a dramatic anterior shift of paraxial- and lateral-plate mesoderm-derived tissues (pronephros and somites; Fig. 4C). In wild-type (*cyp26a1*$^{+/+}$ and *cyp26a1*$^{+/-}$) DEAB-treated embryos, this phenotype is rescued by treatment with between 0.5 and 10 nM RA: concentrations that are non-teratogenic or weakly teratogenic in wild-type embryos (Fig. 4E,G,I,K and data not shown). In the experiments described below, we used 5 nM RA as our 'rescuing' concentration. Whereas in wild-type embryos 5 nM RA is non-teratogenic, it strongly posteriorizes *cyp26a1*$^{-/-}$ embryos, either in the presence or in the absence of DEAB, causing anterior expansion of r7-r8 identity (Fig. 4E,F and data not shown) and the loss of all brain regions anterior to r7: r3 and r5 (marked by *krox20*; Fig. 4E,F,I-L); r4 (marked by *hoxb1a*; Fig. 4G,H); the cerebellum and posterior tectum (marked by *en3*; Fig. 4G-J); the diencephalon and midbrain (marked by *otx2*; Fig. 4K,L); and the telencephalon and eyes [marked by *dlx2* (Fig. 4I,J) and by *eomes* (data not shown)]. Embryos posteriorized in this manner typically exhibited an accordion-like folding of the anterior neural tube. Exactly the same effects are observed in *cyp26a1*$^{-/-}$ embryos treated with 5 nM RA in the absence of DEAB (data not shown). This phenotype strongly resembles the effects of 40-fold-higher levels of RA on wild-type embryos (inset in Fig. 4J), demonstrating that it is the ability of *cyp26a1* to inactivate RA that enables RA-deficient embryos to be rescued by exogenous RA. Furthermore, these results demonstrate that *cyp26a1* is able to protect embryos from the potentially teratogenic effects of low concentrations of RA. We did not see a similar sensitivity to exogenous RA in *cyp26b1* and/or *cyp26c1* MO-injected embryos.

We asked why, under normal circumstances, *cyp26b1* and *cyp26c1* can compensate for a lack of *cyp26a1* (Fig. 2), whereas, in the presence of 5 nM RA, they cannot (Fig. 4). A total of 5 nM RA is sufficient to induce expression of *cyp26a1* far posterior to its normal limit in the hindbrain (Fig. 1C). In spite of this, the expressions of *cyp26b1* and *cyp26c1* are initiated at the correct anterior-posterior level, and subsequent hindbrain patterning is unaffected (Fig. 5C,G and data not shown). By stark contrast, in *cyp26a1*$^{-/-}$ embryos treated with 5 nM RA, *cyp26b1* and *cyp26c1* are not expressed, and the entire brain is strongly posteriorized (Fig. 4 and Fig. 5D,H).

cyp26a1 protects against potentially teratogenic RA precursors

Our findings demonstrate that *cyp26a1* protects against the potentially teratogenic effects of RA. Maternally-derived RA is present at very low levels in zebrafish eggs and early embryos prior to the onset of embryonic RA synthesis, and is therefore unlikely to be a teratogenic risk (Costaridis et al., 1996). However, the levels of maternally loaded retinal – the immediate precursor of RA – are higher (9 pmol/egg) (Costaridis et al., 1996; Lampert et al., 2003). We asked whether Cyp26a1 protects against teratogenicity of RA precursors. We increased retinal levels in wild-type and *cyp26a1*$^{-/-}$ eggs by injecting retinal directly into the yolk of one-cell stage embryos. Although wild-type embryos tolerate over ten-times the normal amount of retinal in the yolk (data not shown), *cyp26a1*$^{-/-}$ embryos are strongly posteriorized by only a three-times the normal amount of retinal (27 pmol; Fig. 6). The teratogenic effects of a small increase in RA precursor in the absence of Cyp26a1 demonstrates that Cyp26a1 may normally play an important role in protecting the embryo against the potentially teratogenic effects of maternally derived RA precursors. They also suggest that the RA

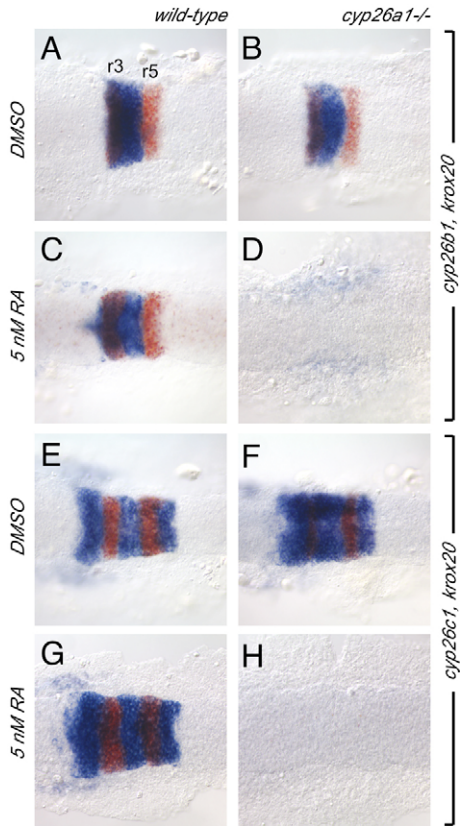

Fig. 5. Exogenous RA disrupts *cyp26b1* and *cyp26c1* expression in *cyp26a1*$^{-/-}$ embryos but not in wild type. *cyp26b1* (**A-D**) and *cyp26c1* (**E-H**) expression (blue) is established normally in wild-type (A,E) and *cyp26a1*$^{-/-}$ (B,F) embryos at the 6-somite stage (12 hpf). *cyp26b1* and *cyp26c1* expression is also established normally in wild-type embryos treated with a sub-teratogenic concentration of RA (5 nM; C,G), but not in *cyp26a1*$^{-/-}$ embryos treated with 5 nM RA (D,H). *krox20* expression is shown in red.

biosynthetic enzyme Aldh1a2 is unable to buffer changes in the levels of its substrate. Because retinal is itself derived directly from dietary vitamin A, it may be expected to fluctuate depending on maternal diet. These observations emphasize the crucial importance of a tightly regulated RA-degradative pathway in nervous system patterning.

DISCUSSION

A gradient-free model for hindbrain patterning by retinoic acid

A robust model for the mechanism of hindbrain patterning must explain the following observations: (1) RA is essential for normal hindbrain development; however, neither the concentration of RA nor the localization of its synthesis are crucial for this pattern; and (2) RA-dependent gene expression occurs in a spatio-temporal sequence, with anterior RA-responsive genes being expressed earlier than posterior ones; however, the duration of RA exposure is not crucial for this temporal sequence (Maves and Kimmel, 2005).

We have identified a crucial role for Cyp26 RA-metabolizing enzymes in establishing hindbrain pattern, because depleting them alone and in combination leads to a progressive posteriorization of the hindbrain. In fully Cyp26-depleted embryos, three RA-dependent genes that normally form nested expression domains with distinct anterior limits (*hoxb1a*, r3-r4; *vhnf1*, r4-r5; and *hoxd4*, r6-r7) are all expanded up to the anterior-most hindbrain (Fig. 7A). Based on the dynamic expression of the cyp26 genes in the hindbrain and on the effects of depleting embryos of Cyp26 activity, we propose a gradient-free model for RA-dependent events in hindbrain patterning, in which RA degradation by Cyp26 enzymes determines progressively more-posterior limits of RA-dependent gene expression in a step-wise manner (Fig. 7B). We note that cyp26 genes are expressed similarly, although not identically, in tetrapods, predicting a similar combinatorial role for Cyp26 enzymes in mammalian hindbrain development.

In step 1, complete by 9 hpf, the anterior limit of *hoxb1b* and *hoxb1a* – the functional homologs of mammalian *HOXA1* and *HOXB1* – are established by the posterior limit of *cyp26a1* expression. This event establishes the r3-r4 boundary (Kudoh et al., 2002), the first morphological boundary in the hindbrain (Moens et al., 1998). This function can be compensated for by *cyp26c1*, because the anterior limit of r4 is strongly affected only in the absence of both *cyp26a1* and *cyp26c1*. In step 2, complete by 11 hpf, the anterior limit of the next RA-responsive gene, *vhnf1*, is determined by the posterior limit of *cyp26c1* expression at the r4-r5 boundary. This function can be partially compensated for by *cyp26b1*. In step 3, complete by 12 hpf, the anterior limit of the last RA-responsive gene, *hoxd4*, is determined by the posterior limit of *cyp26c1* at the r6-r7 boundary, a function that can also be compensated for by the overlapping expression of *cyp26b1*. Some of the mechanistic underpinnings of this model and its broader implications are discussed further below.

A similar model was previously proposed based on the correspondence between *cyp26a1* and *cyp26c1* expression domains and boundaries of RA-dependent reporter-gene expression in the mouse (Sirbu et al., 2005). These authors predicted that *cyp26a1* establishes the r2-r3 boundary and that *cyp26c1* subsequently establishes the r4-r5 boundary. Our combinatorial functional analysis of *cyp26* genes confirms this model in the general sense that Cyp26 activity determines sequential boundaries of RA-responsive gene expression in the hindbrain. However, our observations demonstrate a different and broader role for cyp26 genes in hindbrain patterning, involving all three cyp26 genes functioning to establish three sequential RA-responsiveness boundaries: r3-r4, r4-r5 and r6-r7. We do not observe a function for Cyp26 enzymes at the r2-r3 boundary: r1-r3 are entirely lost while r4 identity shifts anteriorly to abut the forming cerebellum.

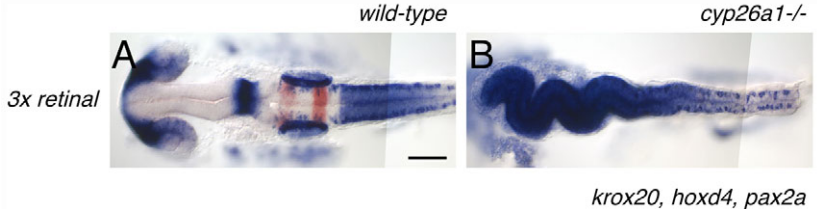

Fig. 6. *cyp26a1* protects against teratogenic effects of the RA precursor retinal. Wild-type (**A**) and *cyp26a1*$^{-/-}$ (**B**) embryos injected with 20 pmol retinal at the one-cell stage. Wild-type embryos are only mildly affected by approximately triple the normal levels of retinal, whereas *cyp26* mutants are strongly posteriorized, with *hoxd4* expression extending throughout the brain. Scale bar: 100 μm.

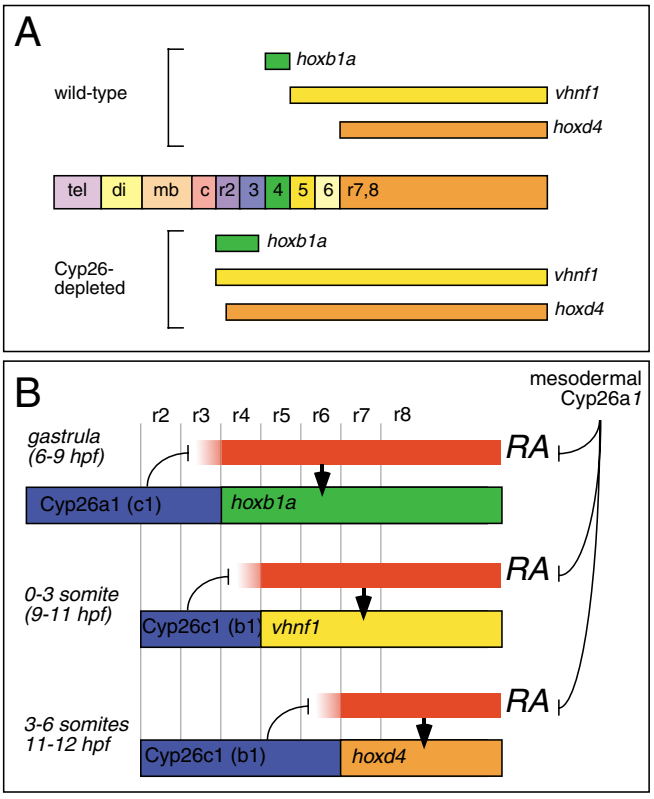

Fig. 7. A model for hindbrain patterning through regulated RA inactivation by the Cyp26 enzymes. (**A**) RA-responsive gene expression in Cyp26-depleted embryos. Embryos depleted of all three cyp26 genes experience unpatterned RA signaling; as a result, the three RA-responsive genes examined in this study – *hoxb1a* (green), *vhnf1* (yellow) and *hoxd4* (orange) – are expressed throughout the transformed hindbrain. (**B**) A 'gradient free' model for hindbrain patterning through regulated RA inactivation. Dynamic patterns of Cyp26 expression in the hindbrain (blue bars) antagonize RA-dependent gene expression by eliminating RA (red bars) first in the anterior hindbrain (6-9 hpf), then in r2-r4 (9-11 hpf), and then in r2-r6 (11-12 hpf). At each point, sequential RA-responsive genes (colored bars) are limited to progressively more posterior rhombomeres. At the same time, Cyp26a1-dependent RA degradation in the trunk mesoderm suppresses global RA levels (black hammers on right side). tel: telencephalon; di; diencephalon; mb: midbrain; c: cerebellum.

We find that *cyp26a1* and *cyp26c1* are both required to establish the anterior limit of *hoxb1a* expression at the r3-r4 boundary, and that all three genes are required to establish the r4-r5 and r6-r7 boundaries. This degree of redundancy is unexpected given the lack of overlap between the expression domains of the cyp26 genes in the hindbrain. The posterior limit of *cyp26a1* expression lies in the anterior hindbrain (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002; Sirbu et al., 2005), whereas both *cyp26b1* and *cyp26c1* mark, sequentially, the r4-r5 and r6-r7 boundaries. However, it is important to notice that *cyp26a1* is also expressed in the anterior trunk mesoderm near the RA source, where it probably functions to reduce global RA levels (Emoto et al., 2005; Niederreither et al., 2002). We propose that the severe posteriorization of Cyp26-depleted embryos results from the combined effects of depleting segment-restricted Cyp26 activity within the hindbrain and increasing global RA levels due to the loss of Cyp26a1 activity in the anterior trunk mesoderm (Fig. 7B).

Because hindbrain patterning is unaffected in *cyp26b1; cyp26c1*-depleted embryos when *cyp26a1* is wild type, we hypothesize that redundant mechanisms can control boundaries of RA-dependent gene expression in the hindbrain, but that these mechanisms are overridden in *cyp26a1* mutants in which global RA levels are elevated. One trivial possibility is that our MOs have not fully depleted Cyp26b1 and Cyp26c1 activity; however, our validation experiments with these MOs indicate that they deplete over 95% of the wild-type gene products. A second possibility is that non-homogeneous expression of RARs or RXRs in the hindbrain may modulate RA responsiveness. During the stages when RA is patterning the zebrafish hindbrain, two receptors – RARαa and RARαb – are expressed throughout the hindbrain, but RARαb mRNA levels are higher in the presumptive r5 and r6 whereas RARαa mRNA levels are higher posterior to the presumptive r6-r7 boundary (Hale et al., 2006). Furthermore, RXRγ, a RA co-receptor, is exclusively expressed posterior to the r6-r7 boundary (Tallafuss et al., 2006). By increasing the RA response, these non-homogeneously distributed RARs and RXRs may help to establish the r4-r5 and r6-r7 boundaries. A third possibility is that spatially restricted transcription factors repress RA-responsive gene expression even when ligand and receptor are present. Iro7 (also known as Irx7 – Zebrafish Information Network) is a TALE homeodomain protein expressed in the anterior hindbrain that represses *vhnf1* expression anterior to the r4-r5 boundary (Lecaudey et al., 2004). Other TALE homeodomain proteins have been shown to repress transcription from retinoid-responsive elements by binding to RXR retinoid receptors and recruiting general co-repressors to the complex (Bartholin et al., 2006). Thus, Iro7 may compensate for Cyp26b1 and Cyp26c1 by directly suppressing RA-responsive gene expression anterior to the r4-r5 boundary. Finally, a diffusion gradient of RA from its source in the anterior trunk mesoderm may compensate for the absence of hindbrain Cyp26 expression. RA can act as a classical morphogen, specifying distinct rhombomere identities at different threshold concentrations (Dupe and Lumsden, 2001; Maves and Kimmel, 2005), and an RA gradient may initiate nested domains of RA-responsive gene expression in the hindbrain when *cyp26b1* and *cyp26c1* are depleted. We note that any or all of the mechanisms we have proposed above (a diffusion gradient, receptor expression or other transcription factors that modulate RAR activity) may contribute to hindbrain patterning under normal circumstances. However, none of these mechanisms are sufficient to limit RA responsiveness in the hindbrain when all three Cyp26 enzymes are depleted.

cyp26a1 is required to establish hindbrain pattern in the absence of a localized source of RA

We have shown that, in the zebrafish, *cyp26a1* is essential for the ability of exogenous RA to rescue embryos in which endogenous RA synthesis is inhibited. Although 5 nM RA can fully rescue the hindbrain and anterior trunk patterning defects of wild-type embryos in which RA synthesis is inhibited with DEAB, in *cyp26a1*$^{-/-}$ embryos it causes a strong posteriorization similar to that normally caused by 40 times more RA. From this, we conclude that Cyp26a1 is responsible for the normal pattern generated in the presence of otherwise teratogenic amounts of RA.

How does Cyp26a1 protect the embryos from exogenous RA? We observe that, in embryos treated with 5 nM RA, *cyp26a1* expression expands throughout the epiblast. This expanded expression presumably eliminates the excess RA and allows the normal onset of expression of *cyp26b1*, *cyp26c1* and other redundant factors described above that can modulate RA-responsive gene expression in the

hindbrain, and the hindbrain develops normally under these conditions. In *cyp26a1* mutants treated with 5 nM RA, the excess RA is not eliminated, *cyp26b1* and *cyp26c1* expression is not initiated, and the entire brain is transformed to posterior hindbrain and/or anterior spinal cord identity. Unlike in untreated *cyp26a1* mutants, *cyp26b1* and *cyp26c1* cannot compensate for the lack of *cyp26a1* because they are not expressed.

The phenotype of *cyp26a1*$^{-/-}$ embryos treated with 5 nM RA is significantly more severe than that of embryos depleted of all three Cyp26 enzymes in the absence of exogenous RA. In the former, the entire brain is transformed to an r7 and/or r8 identity whereas, in the latter, only the hindbrain territory is transformed. This difference may be because, in untreated embryos, RA simply does not diffuse as far as the midbrain, so Cyp26 enzymes are not required to inactivate it there. However, we have noted a surprisingly sharp anterior limit of RA-responsiveness in Cyp26-depleted embryos that corresponds with the posterior limit of the presumptive cerebellum. It is possible that other mechanisms prevent RA signaling anterior to this boundary. The development of the mid- and fore-brain has been shown to require active repression of gene expression by unliganded RARs (Koide et al., 2001), a mechanism that is expected to be easily destabilized by the presence of RA. It seems likely that multiple mechanisms exist that protect the mid- and fore-brain from the teratogenic effects of RA.

Regulation of cyp26 expression

A major outstanding question is how is cyp26 expression normally regulated in the hindbrain? Kudoh et al. (Kudoh et al., 2002) showed that the normal posterior limit of *cyp26a1* in the hindbrain is established by signals (FGFs and Wnts) from the margin because, in embryos treated with antagonists of these pathways, the *cyp26a1* boundary is shifted posteriorly. Although *cyp26a1* is directly inducible by RA, its anterior neurectodermal expression arises independently of RA (Dobbs-McAuliffe et al., 2004; Sirbu et al., 2005) (our work). The factors that positively regulate *cyp26a1* in the anterior neurectoderm remain to be identified.

We also do not know how *cyp26b1* and *cyp26c1* expression is initiated in r2-r4, or what regulates their subsequent expansion into r5 and r6. In general, the mechanisms controlling gene expression in the anterior rhombomeres are poorly understood in any vertebrate (Moens and Prince, 2002). Similar to other anterior hindbrain genes, the initiation of *cyp26b1* and *cyp26c1* expression is independent of RA, because both genes are expressed in DEAB-treated embryos. *cyp26b1* and *cyp26c1* expression is also independent of the prior establishment of hindbrain boundaries by Cyp26a1, because both genes are expressed normally in *cyp26a1* mutants and in embryos in which *cyp26a1* expression is globally up-regulated by sub-teratogenic concentrations of RA. The early expression domain of *cyp26b1* and *cyp26c1* in r3 and r4 is similar to that of *iro7*, suggesting that they may be downstream of, or co-regulated with, *iro7* (Lecaudey et al., 2004). Modern genetic and genomic resources available for the zebrafish will allow the important mystery of cyp26 regulation to be addressed in the future.

Implications for the regulation of retinoic acid during hindbrain patterning

The model we propose for hindbrain patterning through localized RA inactivation by Cyp26 enzymes (Fig. 7B) accounts for a number of previously unexplained aspects of hindbrain patterning. First among these is the observation that embryos depleted of endogenous RA can be rescued by exogenous RA. This rescue can be achieved over a 20-fold range of RA concentrations, indicating that RA-dependent gene expression is also not strictly concentration-dependent. By generating a stepwise pattern of RA degradation during hindbrain development, Cyp26 enzymes eliminate the need for a continuous RA gradient. Secondly, a major tenet of the RA morphogen model has been that more-posterior RA-responsive genes, such as the hox-4 genes, are less sensitive to RA than more-anterior ones, such as the hox-1 genes (Gould et al., 1998); however, this has recently been challenged by the observation that, in the context of the intact enhancer, the RARE of *hoxd4* is no less sensitive to RA than the RARE of *hoxa1* (Nolte et al., 2003). Furthermore, posterior RA-responsive genes do not require a longer exposure to RA than anterior ones, as has been proposed (Dupe and Lumsden, 2001; Sirbu et al., 2005), because identical concentrations of RA applied shortly before the normal initiation of expression are sufficient to rescue this expression in RA-depleted embryos, irrespective of the anterior limit of the RA-responsive gene in question (Maves and Kimmel, 2005). According to our model, the anterior limit of *hoxb1a*, *vhfn1* and *hoxd4* are determined not by different RA concentrations or length of exposure of cells to RA, but simply by the posterior limit of Cyp26 activity at the time of their expression onset. In its most extreme version, each 'step' in the model is essentially a binary decision in which cells posterior to the Cyp26 domain experience RA and initiate RA-responsive gene expression appropriate for that developmental time, while cells within the Cyp26 domain do not. What determines which RA-responsive genes are available to be expressed at a given time is the subject of ongoing studies. Recent work has shown that, within a Hox cluster, the timing of hindbrain expression may be regulated by the progressive opening of chromatin rather than the local accumulation of active transacting factors (Chambeyron et al., 2005).

A RA-dependent patterning mechanism that does not require the formation of a stable gradient in either space or time is expected to be robust to environmental fluctuations. RA is a potent teratogen that is derived from dietary sources of vitamin A, so a robust mechanism for controlling its activity is particularly important. This control does not appear to be exerted at the level of RA biosynthesis, because we have observed that, in the absence of Cyp26a1, even low amounts of precursor are highly teratogenic. Our step-wise model for hindbrain patterning by RA is robust in that it tolerates a broad range of environmental conditions.

Although our model provides robustness to the hindbrain-patterning process and explains how patterning can be established in the presence of uniform RA, our data does not rule out the possibility that other mechanisms act redundantly with RA degradation to pattern the hindbrain under normal circumstances. Indeed, our observation that hindbrain Cyp26b1 and Cyp26c1 activity is dispensable when global RA levels are kept in check by Cyp26a1 suggests that such mechanisms are at work. It is possible that a RA-responsive pre-pattern is established by a transient RA-diffusion gradient, but that Cyp26 enzymes are required to 'lock in' this pattern. As with other developmental processes, it is likely that RA-dependent nervous system patterning events are controlled by overlapping, redundant mechanisms that modulate RA signaling at multiple levels. Our work demonstrates that Cyp26-dependent RA degradation is a crucial component of this complex regulation.

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Note added in proof

Recently, Uehara et al. (Uehara et al., 2006) reported an anterior expansion of retinoic acid responsiveness in *Cyp26a1*$^{-/-}$*; Cyp26c1*$^{-/-}$ double mutant mice that is similar to the phenotype we have observed in *cyp26a1*$^{-/-}$*; cyp26c1* MO-injected embryos, consistent with an evolutionarily conserved role for Cyp26 enzymes in shaping retinoic acid responsiveness in the hindbrain.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/1/02706/DC1

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