Regionalized metabolic activity establishes boundaries of retinoic acid signalling

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The competence of a cell to respond to the signalling molecule retinoic acid (RA) is thought to depend largely on its repertoire of cognate zinc finger nuclear receptors. XCYP26 is an RA hydroxylase that is expressed differentially during early *Xenopus* development. In *Xenopus* embryos, XCYP26 can rescue developmental defects induced by application of exogenous RA, suggesting that the enzymatic modifications introduced inhibit RA signalling activities *in vivo*. Alterations in the expression pattern of a number of different molecular markers for neural development induced upon ectopic expression of XCYP26 reflect a primary function of RA signalling in hindbrain development. Progressive inactivation of RA signalling results in a stepwise anteriorization of the molecular identity of individual rhombomeres. The expression pattern of XCYP26 during gastrulation appears to define areas within the prospective neural plate that develop in response to different concentrations of RA. Taken together, these observations appear to reflect an important regulatory function of XCYP26 for RA signalling; XCYP26-mediated modification of RA modulates its signalling activity and helps to establish boundaries of differentially responsive and non-responsive territories.

Keywords: CYP26/hindbrain segmentation/retinoic acid/*Xenopus laevis*

Introduction

Retinoic acid (RA) and RA metabolites, the retinoids, are required for differentiation and tissue maintenance. Deficiencies in the metabolism of retinoids are associated with severe defects of vertebrate embryonic development (reviewed in Maden *et al.*, 1998; Zile, 1998), and administration of excess RA during vertebrate embryogenesis alters pattern formation in the limb buds and in the developing nervous system (reviewed in Eichele, 1989; Durston *et al.*, 1998). Retinoids directly regulate transcription via interaction with specific Cx-type zinc finger nuclear receptors; two structurally and functionally distinct classes of such receptors have been identified: the retinoic acid receptor (RAR) and the retinoid receptor (RXR).

RAR activates transcription only as a heterodimer with RXR (Kliwer *et al.*, 1992; Leid *et al.*, 1992), whereas RXR also functions in the form of a homodimer (Zhang *et al.*, 1992a). RXR homodimer and RAR–RXR heterodimer differ in the binding activity of different RA metabolites. All-*trans*-RA (ATRA) can only activate RAR–RXR heterodimers, whereas 9-*cis*-RA can activate transcription both via the RAR–RXR heterodimer and via the RXR homodimer (Heyman *et al.*, 1992; Zhang *et al.*, 1992b). In vertebrates, both classes of receptors exist in the form of three different isotypes and numerous isoforms, which are expressed differentially in complex patterns during embryogenesis and whose activity is modulated further by the activity of co-activators and co-repressors (reviewed in Beato *et al.*, 1995; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995).

The amount of RA that will interact with its cognate receptors is influenced further by two different intracellular activities. One is provided by high affinity cellular RA-binding proteins (CRABPs) which are expressed in different isoforms with temporally and spatially restricted expression characteristics during vertebrate embryogenesis. The exact function of these RA-binding proteins is not fully understood, but overexpression of xCRABP in *Xenopus* embryos appears to result in developmental defects that resemble those induced by RA itself (Dekker *et al.*, 1994), indicating a positive regulatory function of xCRABP on RA signalling. The other activity that influences RA signalling is defined by an RA-metabolizing enzyme that has been identified only recently and is termed P450RAI or CYP26. CYP26 belongs to the cytochrome P450 (CYP) superfamily of haem-binding monooxygenases and metabolizes RA into various derivatives; CYP26 gene transcription is RA inducible and defines the posterior neural plate and neural crest cells as major expression domains in the developing mouse embryo (White *et al.*, 1996, 1997; Fujii *et al.*, 1997).

Neural development has been found to be particularly affected by RA signalling. Application of excess RA to gastrulating *Xenopus* embryos results in severe truncations of anterior neural and mesodermal structures (Durston *et al.*, 1989; Sive *et al.*, 1990). Excess RA further has been demonstrated to cause abnormal development of the hindbrain, in that Krox20, which is normally expressed in the rhombomeres 3 and 5, is condensed into a single band at the anterior end of the hindbrain (Papalopulu *et al.*, 1991). In mouse embryos, RA similarly alters segmental expression of Krox20 and of different Hox genes; the effects observed are indicative of a homeotic transformation of rhombomeres 2/3 to a more posterior rhombomere 4/5 identity (Marshall *et al.*, 1992). RA signalling can also be increased by ectopic expression of constitutively active versions of RA receptors, resulting in the suppression of anterior

neural structures (Blumberg *et al.*, 1997). Embryonic development under conditions of reduced RA signalling has been investigated in different ways. Most significantly, RA-deficient quail embryos have been described to completely lack hindbrain structures posterior to rhombomere 3 (Maden *et al.*, 1996). A disturbed segmental organization of the hindbrain was also observed upon ectopic expression of dominant-negative variants of RA receptors in *Xenopus* embryos (Blumberg *et al.*, 1997; Kolm *et al.*, 1997; van der Wees *et al.*, 1998). Effects obtained include loss of Krox20 expression in rhombomere 5. Taken together, these studies support the idea that RA provides an essential signalling activity for the development of the posterior hindbrain and that increased levels of RA result in the transformation of individual segments within the anterior hindbrain to a more posterior character.

RA signalling has also been implicated in the process of neuronal differentiation. Ectopic expression of a combination of RAR α and RXR β was described to result in the formation of ectopic primary neurons (Sharpe and Goldstone, 1997), whereas a dominant-negative version of RAR α interferes with primary neurogenesis in *Xenopus* embryos (Blumberg *et al.*, 1997; Sharpe and Goldstone, 1997). Similarly, vitamin A-deficient quail embryos fail to extend neurites into the periphery of the neural tube (Maden *et al.*, 1998).

A final approach to study the role of RA in development has been provided with the generation of RAR and RXR null alleles in mice (reviewed in Kastner *et al.*, 1995). In marked contrast to single RAR null mutants, a series of RAR compound null mutants was found to recapitulate the complete spectrum of developmental defects associated with vitamin A deficiency (Lohnes *et al.*, 1994). In contrast, RXR α appears to constitute the main RXR implicated in developmental functions (Kastner *et al.*, 1997). Thus, the existence of multiple isotypes and isoforms of RAR/RXR genes expressed in differential, partially overlapping patterns during embryogenesis appears to reflect a combinatorial code that is important for RA signalling during embryonic differentiation.

In this study, we report on the characterization of the *Xenopus* homologue of the RA hydroxylase CYP26. XCYP26 is differentially expressed during early *Xenopus* development. Gastrula stage embryos exhibit the dorsal animal hemisphere and the marginal zone as primary expression domains; a region that includes the prospective hindbrain area does not express XCYP26. Ectopic expression of XCYP26 can rescue RA-induced morphological defects in *Xenopus* embryos and recovers expression of hindbrain markers and anterior neural markers in RA-treated *Xenopus* embryos. Ectopic expression of increasing amounts of XCYP26 in the absence of exogenous RA results in a gradual posterior shift of Krox20 and Pax6 expression in the hindbrain by one or two rhombomeric units, respectively. N-tubulin expression in XCYP26-injected embryos reveals a duplication of the trigeminus, indicative of segmental duplication in the rostral hindbrain. In contrast, the position of anterior neural markers and of markers for the mid-/hindbrain and hindbrain/spinal cord boundaries appears largely unaffected. Within the neuroectoderm, the only marked effect outside of the hindbrain was loss of Sox3 expression in the lens placode.

Results

Predicted protein sequence and expression characteristics of *Xenopus* CYP26

Screening of a *Xenopus* cDNA library prepared from activin-induced animal caps resulted in the identification of a plethora of clones with differential expression characteristics during gastrulation and neurulation. One of these was found to encode a protein that is a member of the cytochrome P450 (CYP) superfamily. The 492 amino acid predicted protein sequence is most closely related to that one of an RA-metabolizing enzyme, termed CYP26, which previously had been characterized in zebrafish (White *et al.*, 1996), mouse (Fujii *et al.*, 1997; Ray *et al.*, 1997) and humans (White *et al.*, 1997). The *Xenopus* sequence is 63–67% identical to these CYP26 sequence from different vertebrates (Figure 1). This degree of primary sequence conservation is comparable with the 68% amino acid identity that had been reported in a comparison of the human and zebrafish CYP26 sequences (White *et al.*, 1997). We therefore refer to the *Xenopus* protein as XCYP26.

Spatial and temporal expression characteristics of XCYP26 during *Xenopus* development were established by RT-PCR analysis and whole-mount *in situ* hybridization (Figure 2). During embryogenesis, XCYP26 transcripts were detected in all stages analysed (Figure 2D); the RT-PCR analysis suggests a slight increase in transcript levels during gastrulation/neurulation. Whole-mount *in situ* hybridization with staged oocytes and morula/blastula stage embryos did not reveal a differential distribution of XCYP26-encoding mRNA (data not shown). At the onset of gastrulation, XCYP26-specific signals define two primary domains of expression. The first one is within the marginal zone as defined by a circumblastoporal ring and the second is within the dorsal animal hemisphere (Figure 2A, panels 1 and 8). During further progress of gastrulation and also during neurulation, XCYP26 mRNA persists in those cells surrounding the blastoporus, with the notable exception of the dorsal midline area (Figure 2A, panels 2–7). In advanced gastrulae (stage 13), anterior XCYP26 transcripts are not only detected in the prospective neuroectoderm, but also in the underlying involuted mesoderm, in an area that corresponds to the prechordal plate; in the circumblastoporal area, XCYP26 gene transcription appears to be restricted to the more superficial cell layers and is extending into the prospective neuroectoderm (Figure 2A, panel 16). Expression in the second primary domain, that develops from the dorsal animal hemisphere of the embryo into the anterior neural plate, is found to be highly dynamic (Figure 2A, panels 9–14). An initially mostly homogeneous group of XCYP26-expressing cells that is maintained up to stage 12 develops into three elements clearly separated at stage 14. These correspond to the cement gland anlage, the mid-/hindbrain boundary and the auditory placodes. The latter structures were identified by double staining *in situ* hybridization with either En2- or Krox20-specific antisense probes in addition to XCYP26 (Figure 2B, panels 1–6). Expression of En2, that marks the midbrain/hindbrain boundary, co-localizes with XCYP26 in stage 14 and 18 embryos. Xkrox20 identifies rhombomeres 3 (r3) and 5 (r5), as well as migrating neural crest cells originating from r5. Early

CYP26	x1	1	MDLYTLTSLCTLALPLLLLLTAAKLWEVYCLRRKDAACANPLPPGTMLGFFFGETLQMVLRQRRLQVQRKSOY		
hs			-G-PA--A-----FV-----F-A-I---DL--VSGR-RS--L-----F-----K--M--RK-		
mm			-G-PA--A-----FV-----F-A-L---DL--VSSR-RS--L-----F-----K--M--RK-		
dr			-G---MVF---IV--V--F-A-V---MLMI--V-PN-RS-----I-----LI---K--RM--QK-		
		76	GRIYKTHLFGSPTVRVTGAENVRQILMGEHKLVSVHWPAVSVRTILGAGCLSNLHDNEHKYTKKVIAQAQFSREALA		
			-F-----R-----M--D--R--L--DDR-----S-----SS--QR---MR-----E		
			-F-----R-----M--D--R--L--R-----R-----SS--QR---M-----Q		
			-C-----N-----M--D-----L-----Q-----SDT--V-GVQ--NK--A-MR---D--E		
		151	NYVPQMEEVRCVSNLWQSGP.CVLVYPAIKRMMFRIAMRLLLGCDPQRMDR.EQEETLLEAFEMSRLNLSLP		
			C---VIT---GS-LEQ--SC-ERGL---EV--L-----I---E--LAGDGD--QQ-V-----T-----		
			C--LVIA---SSCLEQ--SC-ERGL---EV--L-----I---E--GPAGGG--D-QQ-V-----T-----		
			H-I-VIQQ--KSAIQE---KDS-----EM-KL-----I---FE-EQIKT..D-QE-V-----IK-----		
		224	IDVPFSGLYRGLRARNLIHAQIEENIKEKLQR...EPDEHCKDALQLLIDYSRRNGEPINLQALKESATELLFG		
			-----MK-----R--Q--RA-ICGLRAS-AGQG-----EH-WER--RLDM---Q-S-----		
			-----VK-----R-----RA-IR-LQAT---GG-----EH-WER--RLDM---Q-S-----		
			-----F--SK-----RK-I-D,DDN-NEQKY-----EN--SD--FS--M--A-----		
		295	GHGTASAATSLTSFLALHKDVLEKVRKELETQGLLSTKPEEKELSIEVLQQLKYTSCVIKETLRLSPVAGGF		
			--E-----ITY-G-YPH--Q---E--KSK---CKSNQDN-,DM-I-E---IG-----N---P---		
			--E-----ITY-G-YPH--Q---E--KSK---CKSNQDN-,DM-T-E---IG-----N---P---		
			--E---T---VM--G-NTE-VQ---E-VQEKVEMGMYTPG-G-,M-L-D---G-----IN---P---		
		370	RVALKTFVLNGYQIPKGNWVIYSIADTHGEADLFPTDKFNPDRLFTPLPRDSSRFGFIPIFGGVRCCIGKEFAK		
			-----E-----C---DV-EI--TNKEE-----ML-H-E-A---S-----L-S-V-----		
			-----E-----C---DV--I--TNKEE-----IV-H-E-A---S-----L-S-V-----		
			-----E-----C---DV--V--NKEE-Q-E--MSKGLG-G---NY-----S-M-V-----		
		445	ILLKVFVVELCRNCDWELLNGSPAMTTSPICPDVNLPAKFKPFSSSI	492	ID[%]
			---I-T---A-H---Q---P-T-K---TVY-----R-TH-HGE-	497	67
			---I-T---A-H---Q---P-T-K---TVY-----R-TY-QGD-	497	68
			V---I-L---TQH-N-I-S---P-T-K-G-T-Y-----T--TSYVRN	492	63

Fig. 1. Predicted primary sequence of *Xenopus* CYP26 and comparison with CYP26 from other species. The *Xenopus* CYP26 amino acid sequence as predicted from the corresponding *Xenopus* cDNA is displayed in comparison with the CYP26 sequences from human (hs), mouse (mm) and zebrafish (dr). The DDBJ/EMBL/GenBank accession No. for XCYP26 is AF057566. Dots represent gaps introduced into the amino acid sequence in order to obtain optimal alignment. Identical amino acids are represented by hyphens. The percentage of amino acid identity (ID) of XCYP26 in a comparison with the other three sequences shown here is indicated at the end of each individual sequence.

Xkrox20 expression in the open neural plate (stage 14) is specific to r3; a XCYP26-positive group of cells is located at the lateral tips of this early Xkrox20 stripe, slightly shifted to the posterior pole of the embryo (Figure 2B, panel 1). At the neural groove stage (stage 18), the r5 and neural crest cell expression of Xkrox20 becomes evident. According to the position of these marker cells, XCYP26 is expressed anterior to r5, on the same lateral level as the Xkrox20-positive neural crest cells (Figure 2B, panel 2). Thus, the position of these XCYP26-expressing cells co-localizes with the auditory placode. At tadpole stages of development (Figure 2B, panel 3), anterior expression of XCYP26b is most prominent in the first, second and third branchial arch (with gradually increasing signal intensity from anterior to posterior), in the lens epithelium (Figure 2C, panels 1 and 2) and in the posterior dorsal fin, as well as in the posterior wall of the tail tip (Figure 2C, panels 1 and 3). It is interesting to note that CYP26 was first isolated as an RA-inducible gene in regenerating caudal fin of zebrafish embryos (White *et al.*, 1996). RT-PCR analysis with RNA preparations from adult *Xenopus* organs and tissues reveals that, in addition to the ovary, XCYP26 is expressed primarily in brain and eyes (Figure 2D).

Differential regulation of XCYP26 gene expression in *Xenopus* embryos

In situ hybridization analysis with gastrula stage embryos had defined the dorsal animal hemisphere and the circumblastoporal marginal zone as the two primary XCYP26 expression domains. As a first step towards an understanding of XCYP26 transcription regulation, UV-ventralized and LiCl-dorsalized embryos were stained for XCYP26 expression (Figure 3A). Both treatments have very specific and clearly distinct effects. LiCl treatment completely ablates XCYP26 gene transcription in the marginal zone,

while maintaining apparently normal expression in the dorsal animal hemisphere. In contrast, UV treatment maintains normal expression in the marginal zone, while grossly inhibiting XCYP26 gene transcription in the dorsal animal hemisphere. Corresponding results were obtained with mesoderm-inducing factors, making use of the animal cap assay system (Figure 3B). Control animal caps exhibit a low level expression of XCYP26, which is inhibited in animal caps from UV-treated embryos and which appears unaffected in animal caps from LiCl-treated embryos. All mesoderm-inducing peptide growth factors tested [bone morphogenetic protein 4 (BMP4), activin and basic fibroblast growth factor (bFGF)] increase XCYP26 mRNA levels.

Previous studies in different vertebrate systems had revealed that CYP26 gene transcription can be induced by RA in tissue culture cells (White *et al.*, 1996; Fujii *et al.*, 1997; Ray *et al.*, 1997; Abu-Abad *et al.*, 1998). We therefore tested for the effect of RA on XCYP26 transcription in both animal cap explants and in whole embryos. Comparable with the results obtained with the different vertebrate cell lines, RA treatment greatly increases XCYP26 transcription in animal caps (Figure 3D). XCYP26 transcript levels were also found to be increased in the corresponding region of whole embryos, i.e. the animal hemisphere at the gastrula stage of development, at all RA concentrations tested (Figure 3C). However, the opposite effect was observed in the marginal zone; here, XCYP26 transcript levels were significantly reduced, already at a low concentration of RA. A further increase in the dose of RA results in an expansion of the expression domain in the animal hemisphere which eventually culminates in an apparently uniform expression in the entire ectoderm. Thus, RA appears to exert opposing effects on XCYP26 gene transcription in different areas of gastrula stage *Xenopus* embryos; XCYP26 expression

Fig. 2. XCYP26 is differentially expressed during *Xenopus* development. **(A)** Whole-mount *in situ* hybridization analysis using XCYP26 antisense RNA and staged *Xenopus* embryos. Nieuwkoop–Faber stages of embryogenesis are indicated above each column: (1–7) dorsal view (top, anterior; bottom, posterior); (8) lateral view (top, anterior; bottom, posterior); and (9–14) anterior view (top, dorsal; bottom, ventral). (15) Sagittal section of a stage 11 embryo; (15a) detail from (15), as indicated. (16) Sagittal section of a stage 13 embryo; (16a) and (16b) details from (16), as indicated. (17) Horizontal section of a stage 15 embryo. Abbreviations: sne, sensorial layer of the neuroectoderm; ene, epithelial layer of the neuroectoderm; pcpl, prechordal plate; dbi, dorsal blastopore lip. **(B)** Double staining whole-mount *in situ* hybridization of XCYP26 and Krox20/En2 expression. Staged embryos were stained for (1–3) XCYP26 (purple) and Krox20 (red) expression, or (4–6) for XCYP26 (purple) and En2 (red) expression. **(C)** Whole-mount *in situ* hybridization of a stage 34 *Xenopus* tadpole. (1) Entire embryo, (2) frontal section, detail from the region that includes the eye, (3) sagittal section, detail that includes the tail tip region. Abbreviations: lee, lens epithelium; dfn, dorsal fin; sc, spinal cord; nc, notochord; nec, neuroenteric canal. **(D)** RT–PCR analysis with RNA preparations from staged embryos (embryonic stages indicated according to Nieuwkoop and Faber) and from adult tissues (abbreviations: te, testis; ov, ovary; sc, spinal cord; br, brain; ey, eye; fa, fat body; bl, bladder; lu, lung; ki, kidney; li, liver; sp, spleen; in, intestine; st, stomach; gu, gut; mu, muscle; he, heart; sk, skin).

increases and expands in the epidermal and neuroectodermal cells, whereas it is inhibited within the marginal zone, already at a relatively low RA concentration. These effects are only transient. In neurula stage embryos, a comparable dramatic expansion of the anterior neural expression domain is no longer observed. One does, however, detect a loss of boundaries between the individual anterior neural signals that results in a rather homogeneous

area of expression within the anterior neural plate at the highest doses of RA tested (Figure 3C).

Ectopic expression of XCYP26 anteriorizes the developing hindbrain

RA treatment of *Xenopus* embryos has profound effects on the development of the central nervous system. We therefore investigated the effect of ectopic XCYP26 in

Fig. 3. Regulation of XCYP26 gene transcription in whole embryos and animal cap explants. **(A)** Whole-mount *in situ* hybridization using XCYP26 antisense RNA and gastrula (stage 10.5) *Xenopus* embryos that were either dorsalized by LiCl treatment (LiCl) or ventralized by UV irradiation (UV). **(B)** RT-PCR analysis with RNA preparations from animal cap explants (or whole embryos) which were either derived from UV- or LiCl-treated embryos, or treated with different peptide growth factors (as indicated). **(C)** Whole-mount *in situ* hybridization using XCYP26 antisense RNA and stage 12/15 *Xenopus* embryos that had been treated with different doses of all-*trans*-retinoic acid (ATRA, as indicated) at stage 11. **(D)** RT-PCR analysis with RNA preparations from animal cap explants (or total embryos) which were cultivated in the absence or presence of 1.8×10^{-7} M ATRA.

Xenopus embryos on the expression of various molecular markers that reflect different aspects of neural development (Figure 4). Krox20 expression identifies the prospective r3 and r5 in the developing hindbrain (Figure 4A, panel 1). Overexpression of XCYP26 was achieved by injection of synthetic mRNA into one cell of two-cell stage *Xenopus* embryos. Ectopic XCYP26 results in a posterior shift of these two signals in a dose-dependent manner by either one or two rhombomeric units, such that the anterior Krox20 stripe in the injected half of the embryo is at the level of either r4 or r5 in the control half, and the posterior stripe is next to r6 or r7 (Figure 4A, panels 2–4; Table I).

Similar to Krox20, Pax6 is also expressed in r3 and r5, but also strongly in mid- and hindbrain, as well as more weakly in the entire spinal cord (Figure 4A, panel 5). At a low dose of XCYP26, the r3/r5 expression is shifted posteriorly by one rhombomeric unit each; at a high dose, the anterior stripe is shifted to the level of r5, whereas the posterior stripe (former r5) is lost or condensed with the former r3 (Figure 4A, panels 6–8; Table I). This situation is also reflected in a double staining analysis, where the Krox20 probe identifies the shifted posterior stripe on the injected side of the embryo that is negative

for Pax6, whereas both genes are co-expressed in the anterior stripe (Figure 4A, panels 9–11). Expression of Pax6 outside the future hindbrain region was not significantly affected. Effects correlating with those described for Krox20 and Pax6 were observed with Hoxb3. Early Hoxb3 expression also identifies r5 (Figure 4A, panel 12); XCYP26 overexpression results in a posterior shift of the Hoxb3 r5 signal by what appears to correspond to two rhombomeric units. Thus, the consistent effect of ectopic XCYP26 on the expression of three different molecular markers for the developing hindbrain is a posterior shift that occurs in a concentration-dependent manner in steps of individual rhombomeric units.

En2 gene transcription marks the prospective mid-/hindbrain boundary within the open neural plate (Figure 4B, panel 1); ectopic XCYP26 does not affect the position of this marker, but it does result in a slight expansion of the En2-positive area (Figure 4B, panels 2 and 3). Double staining with probes for En2 and Krox20 reveals a significant increase in the distance between the mid-/hindbrain boundary marker En2 and the anterior Krox20 signal (Figure 4B, panels 4 and 5). Conversely, the anterior boundary of the spinal cord, which is marked

by expression of *Hoxb9* and which is also not significantly shifted upon *XCYP26* overexpression, comes to lie in close proximity to the posterior *Krox20* stripe on the injected side of the embryo (Figure 4B, panels 6 and 7). These observations indicate that anterior, as well as posterior boundaries of the developing hindbrain are maintained in *XCYP26*-injected embryos, and that the shifted position of different molecular markers within individual hindbrain segments may reflect an altered

molecular identity rather than an altered extension of individual rhombomeric units.

We also analysed the expression of anterior neural markers in embryos that had been injected with *XCYP26*. The position of *Otx2* expression domains in fore- and midbrain was not found to be out of register; however, similar to what was observed with *En2*, the *Otx2*-positive area was found to be slightly extended on the injected side of the embryo (Figure 4C, panels 1–3). *Sox3* gene

transcription occurs in the entire neural plate, as well as in the lens placodes. Ectopic XCYP26 results in a specific inhibition of Sox3 expression within this latter area, while leaving expression in the central nervous system unaffected (Figure 4C, panels 4–6). Thus, in addition to its signalling function in the hindbrain, RA also appears to function in the context of lens differentiation.

As a final neural marker, we analysed N-tubulin gene transcription as a marker for neuronal differentiation. At the open neural plate stage, no significant alterations in the pattern generated were detected (data not shown). After closure of the neural tube, N-tubulin expression identifies the trigeminal ganglion (with the projecting ophthalmic and mandibular nerves) that originates from r2 (Figure 4D, panels 1 and 4). Ectopic expression of XCYP26 results in a duplication of the trigeminal ganglion or even in the formation of three separate columns of cells that express N-tubulin (Figure 4D, panels 2, 3 and 5; data not shown). This observation indicates that, whereas the identity of caudal rhombomeres is shifted posteriorly upon application of ectopic XCYP26, the same treatment can result in a duplication of rostral rhombomeric identity.

XCYP26 overexpression rescues RA-induced developmental defects

The effects obtained upon overexpression of XCYP26 and described so far could be a result of a direct inactivation of the RA signalling activity, but they could also reflect the generation of a novel signalling activity coming from those RA metabolites which are produced by ectopic XCYP26. In order to distinguish between these two alternatives, RA treatment was performed on embryos that were injected with XCYP26-encoding mRNA and on non-

injected embryos. If XCYP26 simply inactivates RA, a rescue of RA-induced developmental defects could occur.

In the absence of RA, ectopic XCYP26 results in no gross morphological alterations, except for a compression of the anteroposterior body axis, that is most evident from a shortening of the trunk and tail structure (Figure 5A, panel 1). Anterior neural structures appear not to be affected. Conversely, RA-treated embryos exhibit massive defects in the development of anterior neural structures (Figure 5A, panels 2 and 4). These RA-induced defects in head formation can be rescued by ectopic expression of XCYP26, as judged from a statistical analysis of the dorso-anterior index (Table II). Low concentrations of RA lead to cyclopia, which can be rescued by ectopic XCYP26 (Figure 5A, panels 2 and 3). High doses of RA result in a dramatic reduction of anterior neural structures; again, ectopic XCYP26 rescues these defects to a significant extent, as is obvious from regained, and in some cases cyclopic, eye pigmentation in most embryos (Figure 5A, panels 4 and 5).

We wished to investigate whether ectopic XCYP26 can also rescue the effect of RA on the expression pattern of early neural marker genes such as Pax6, Krox20 and Sox3. The effect of ectopic XCYP26 on these markers was described above and is shown again for reference in Figure 5B, panels 1, 6 and 11. RA treatment has marked inhibitory effects on Pax6 and Krox20. The anterior Pax6 expression domain, in particular in the area corresponding to the evaginating optic vesicles, is severely reduced, and the rhombomeric expression appears to be condensed to one stripe (Figure 5B, panels 2 and 3). However, XCYP26 restores an almost regular pattern without resolving the r3 and r5 signals (Figure 5B, 4 and 5). RA application

Table I. Posterior shift of Krox20 and Pax6 expression in rhombomeres 3 and 5 with XCYP26-injected *Xenopus* embryos

XCYP26 (ng)	Krox20				Pax6			
	Normal	1×R ^a	2×R ^b	(n)	Normal	1×R ^a	2×R ^c	(n)
0.125	50%	27%	23%	(44)	46%	36%	18%	(56)
0.5	26%	40%	33%	(42)	26%	33%	40%	(42)
2.0	14%	26%	60%	(105)	19%	27%	54%	(102)

^aPosterior shift of both stripes by one rhombomeric unit.

^bPosterior shift of both stripes by two rhombomeric units.

^cPosterior shift of the r3 signal by two rhombomeric units and loss of the r5 signal.

Fig. 4. Effect of ectopic XCYP26 on the expression of various marker genes reflecting neural development. Embryos were injected with different doses of XCYP26-encoding mRNA (as indicated) into one blastomere at the two-cell-stage. β -gal-encoding mRNA was co-injected as a lineage tracer (light blue staining); the expression of the various marker genes (as indicated) was analysed by whole-mount *in situ* hybridization analysis (purple staining). (A) Posterior shift of hindbrain marker gene expression upon XCYP26 overexpression. (1–4) Krox20 identifies r3 and r5 in neurula (stage 18) *Xenopus* embryos. (5–8) Pax6 similarly identifies r3 and r5, as well as elements of mid- and forebrain, and the evaginating optic vesicle. (9–11) Double staining whole-mount *in situ* hybridization of XCYP26-injected embryos with Pax6 (purple) and Krox20 (red). (12–14) Hoxb3 identifies r5 in neurula (stage 18) *Xenopus* embryos. (B) The anterior and posterior boundaries of the hindbrain are maintained in XCYP26-injected embryos. (1–3) En2 expression demarcates the mid-/hindbrain boundary in neurula (stage 18) embryos. (4 and 5) Whole-mount *in situ* hybridization analysis of Krox20 and En2 expression (both in purple) in XCYP26-injected *Xenopus* embryos. The En2 signal is marked by an arrowhead. (6 and 7) Whole-mount *in situ* hybridization analysis of Krox20 and Hoxb9 (both in purple) in XCYP26-injected *Xenopus* embryos. The anterior boundary of Hoxb9 expression identifies the hindbrain/spinal cord boundary [indicated by an arrowhead in stage 18 (6) and stage 23 (7) *Xenopus* embryos]. (C) Lens placode-specific gene expression is inhibited in XCYP26-injected embryos. Otx2 (1–3) is an anterior neural marker, Sox3 (4–6) stains the entire central nervous system and the lens placodes; the arrowhead marks the position of the missing Sox3 lens placode signal on the injected side of the embryo. (D) Overexpression of XCYP26 results in a duplication of rostral hindbrain-derived structures. N-tubulin is a molecular marker for neuronal differentiation. The anteriormost staining identifies the trigeminal, which is duplicated in XCYP26-injected embryos. (1–3) Whole-mount *in situ* hybridization analysis with an N-tubulin-specific probe and XCYP26-injected embryos. (4 and 5) Details from (1) and (3), respectively, displaying the trigeminal-specific signals.

Fig. 5. Ectopic expression of XCYP26 inactivates RA-specific signalling activity. (A) Rescue of RA-induced morphological defects. (1) Collection of XCYP26-injected embryos along with a control embryo (top). (2 and 4) *Xenopus* embryos treated with two different concentrations of all-*trans*-retinoic acid (ATRA, as indicated) during gastrula stages of development. (3 and 5) Same treatment as in (2) and (4), but with XCYP26-injected embryos. Red arrows indicate cyclopic eyes. (B) Rescue of RA-induced effects on marker gene expression: reference Pax6 (1), Krox20 (6), En2 (11, arrowhead) and Sox3 (11) expression of XCYP26-injected embryos (as also illustrated in Figure 4). Pax6 (2–5), Krox20 (7–10), En2 (12–15, arrowhead) and Sox3 (12–15) expression in ATRA-treated embryos that had or had not been injected with XCYP26 (as indicated).

Table II. Rescue of RA-induced developmental defects in XCYP26-injected *Xenopus* embryos

Effector	Embryos (<i>n</i>)	DAI ^d					
		6 ^a	5 ^b	4.5	4	3 ^c	2.5
XCYP26 (4 ng)	537	82%	18%	0	0	0	0
ATRA (2×10 ⁻⁷ M)	154	0	0	0	9%	16%	75%
XCYP26 (4 ng)	126	0	12%	13%	24%	24%	27%

+ ATRA (2×10⁻⁷ M)

^aShortened axis; ^bnormal; ^ccyclopic; ^dDorso-Anterior-Index (Kao and Elinson, 1989).

further results in the condensation of the Krox20 signals corresponding to r3 and r5 into one stripe of r3 identity and ablates the En2 signal (Figure 5B, panels 7 and 8). Ectopic XCYP26 rescues the En2 stripe at the prospective mid-/hindbrain boundary and also partially restores Krox20 expression, as evident from the neural crest staining and an extended Krox20 expression domain, which as already observed with Pax6, fails to resolve into the two r3/r5 corresponding stripes (Figure 5B, panels 9 and 10). Finally, RA treatment also has an effect on Sox3

expression; it results in an extension of the lens placode signals, which, in the extreme case, can result in a fusion of the two lateral signals (Figure 5B, panels 12 and 13). Ectopic XCYP26 restores the anterior gap of Sox3 expression and exerts an additional, negative regulatory effect (Figure 5B, panels 14 and 15).

Taken together, the XCYP26-mediated rescue of RA effects on *Xenopus* embryos, as observed at the level of both tadpole morphology and molecular markers in neurula stage embryos, indicates that XCYP26 inhibits RA signal-

ling activity without producing developmentally relevant novel signalling information. The failure of ectopic XCYP26 to resolve the proper segmental pattern of gene expression within the hindbrain in the presence of excess RA is in line with the idea that such a segmental pattern would require an RA gradient along the anteroposterior axis of the hindbrain, which cannot be restored under these experimental conditions.

Discussion

Ectopic expression of XCYP26 can rescue developmental defects with respect to neural marker gene expression and morphology of RA-treated embryos, suggesting that XCYP26 inactivates RA without producing novel signalling activity. Ectopic XCYP26 has major effects on the expression of molecular markers that reflect hindbrain development; in a dose-dependent manner, the molecular identity of individual caudal rhombomeric segments is shifted posteriorly by either one or two rhombomeric units, whereas rostral rhombomere identity is partially duplicated. Anterior and posterior hindbrain boundaries are maintained. During gastrulation, XCYP26 is not expressed in the prospective hindbrain area but is expressed strongly within the prospective anterior neural plate, where it may serve to reduce RA signalling activity.

Metabolic inhibition of retinoid signalling

Overexpression of XCYP26 inhibits the teratogenic effects of RA on *Xenopus* embryos and rescues the normal expression pattern of hindbrain and anterior neural markers in RA-treated neurulae. A previous study on murine CYP26, making use of the effect of RA-induced differentiation of P19 cells, which were found to become ATRA hyposensitive upon CYP26 transfection, similarly had arrived at the conclusion that CYP26 inactivates ATRA signalling activity (Fujii *et al.*, 1997). Our *in vivo* results obtained with whole embryos further indicate that the metabolites produced by XCYP26 from the exogenously added ATRA have no significant additional signalling activity that would influence embryonic differentiation. Even though not all derivatives created by CYP26 from RA, including 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,8-epoxy-RA, have been identified (White *et al.*, 1996, 1997; Fujii *et al.*, 1997; Abu-Abed *et al.*, 1998), at least one of these, namely 4-oxo-RA, appears to be an equally potent teratogenic agent for *Xenopus* embryos (Pijnappel *et al.*, 1993). The finding of XCYP26-induced protection of *Xenopus* embryos against exogenous RA thus favours the idea that the primary function of this enzyme is to bring RA into a degradative pathway without producing significant quantities of teratogenic derivatives.

RA signalling in hindbrain development

In the early embryo, ectopic XCYP26 exclusively affected expression of hindbrain-specific molecular markers, without altering anterior neural gene expression patterns in particular. The hindbrain effects observed are compatible with the idea of an altered identity of individual rhombomeres. r3- and r5-specific gene expression was shifted posteriorly, in a dose-dependent manner, by either one or two rhombomeric units. On the other hand, the anteriormost rhombomeric units r1/r2 appeared to be

duplicated in at least some of the XCYP26-injected embryos. These effects are compatible with a scenario in which the identity of the different rhombomeres along the anterior–posterior axis of the central nervous system would, at least in part, be determined by an RA gradient with a high concentration at the posterior and a low concentration at the anterior end of the embryo. Direct experimental evidence for the existence of such a gradient in *Xenopus* gastrulae/neurulae has indeed been provided by Chen *et al.* (1994). Similarly, in the chicken embryo, Hensen's node has been reported to be a rich source of RA (Chen *et al.*, 1992). Overexpression of XCYP26 in *Xenopus* embryos would be predicted to shift posteriorly the correlating gradient of RA signalling activity in a dose-dependent manner, thus resulting in a graded anteriorization of the hindbrain. The opposite effect would be expected to be the result of application of exogenous RA. This has indeed been demonstrated to be the case with RA-treated mouse embryos; RA induces changes in the hindbrain Hox code which result in the homeotic transformation of r2/r3 to an r4/r5 identity (Marshall *et al.*, 1992). In analogy, our results obtained with XCYP26-treated *Xenopus* embryos suggest a homeotic transformation of r4/r6 (low dose) or r5/r7 (high dose) to an r3/r5 identity. It should be noted, though, that r7, after transformation to r5 at high doses of XCYP26, does not recapitulate the full spectrum of r5-specific gene activities, since, albeit that it expresses Krox20, it fails to express Pax6. This observation also indicates that r3/r5-specific expression of these two genes must, at least in part, be regulated via different molecular mechanisms.

Correlation of RA inhibition in *Xenopus* embryos by dominant-negative RA receptors and by ectopic expression of XCYP26

A number of recent studies report on the use of dominant-negative RA receptor variants for the analysis of the importance of RA signalling in the process of early neural development in *Xenopus*. With respect to the absence of major effects on fore-/midbrain and on spinocaudal-specific molecular markers, there is fairly good agreement with the results reported in this study. Kolm *et al.* (1997) did not observe effects on anterior neural or spinocaudal markers upon overexpression of dominant-negative RAR α 2.2, and van der Wees *et al.* (1998), making use of dominant-negative RAR β , describe normal fore- and midbrain patterns of gene expression. The moderate expansion of the Otx2 expression domain, as reported by Blumberg *et al.* (1997) with dominant-negative RAR α 1, was also achieved with ectopic XCYP26; however, the posterior shift of En-2 was not obtained. All three studies with dominant-negative receptors also agree with respect to the observation of an altered pattern of gene expression within the hindbrain, but the effects observed are quite different from those described here. Kolm *et al.* (1997) and Blumberg *et al.* (1997) describe loss of Krox20 expression in r5, or condensation of the two Krox 20 stripes into one that appears to correspond to r3; van der Wees *et al.* (1998) observed frequent expansion of Krox20 expression into the area covering r6–r8 and, less frequently, expansion into r1 and r2. Thus, the use of different experimental strategies to inhibit RA signalling in *Xenopus* embryos, such as use of dominant-negative RA receptors

and metabolic inactivation of RA by ectopic XCYP26, primarily affected hindbrain development, clearly and consistently arguing for an important function of RA signalling in the same process. The dose-dependent, step-wise anteriorization of individual rhombomeric units by overexpression of XCYP26, as described in this study, is a unique effect that is inversely correlated to what has been described as a result of RA treatment of mouse embryos, i.e. homeotic transformation of individual rhombomeric units to a more posterior character (see above).

Function of XCYP26 in RA signalling

The view that XCYP26 inactivates the signalling activity of RA would suggest that cells expressing XCYP26 in the living embryo are less responsive to RA, even though they may express the corresponding receptor(s). Early embryonic expression of *Xenopus* CYP26 would thus define areas of reduced responsiveness to RA. Interestingly, during gastrulation, XCYP26 is not expressed in the area of the prospective neural plate that will develop into hindbrain, whereas the prospective anterior neural plate as well as the underlying mesodermal prechordal plate are primary sites for XCYP26 expression. Thus, RA is not inactivated in a group of cells which appear to require RA in order to develop into a properly segmented hindbrain, whereas cells which will form anterior neural structures, a process which is prevented by application of exogenous RA, possess significant levels of RA-metabolizing activity. It is conceivable, therefore, that XCYP26 constitutes an activity that defines borders of RA signalling by reducing the intracellular RA concentration to critical threshold levels in *Xenopus* gastrulae.

We further note that the anterior XCYP26 expression domain can be expanded upon application of exogenous RA, until it will eventually include the entire ectodermal mantle of gastrula stage embryos, whereas posterior expression is inhibited already at relatively low RA concentrations. A similar situation has been encountered with murine embryos (Fujii *et al.*, 1997). Such differential regional regulatory mechanisms, as revealed upon application of ectopic RA, may reflect a physiologically relevant process that further protects one group of cells from RA signalling while making another set of cells more responsive. Taken together, results obtained in this study support the idea that XCYP26 constitutes an important regulatory activity in the context of early *Xenopus* embryogenesis that helps to establish boundaries of RA signalling by regionalized inactivation of this compound.

Materials and methods

Isolation of XCYP26 cDNA

A λ ZAP Express phage cDNA library was constructed with RNA from artificial notochord tissue, that was obtained by treating disaggregated animal cap cells from stage 8 *Xenopus* embryos with 20 ng/ml recombinant human activin A (kindly provided by Professor Asashima, Japan). Part of the randomly primed phage cDNA library was converted into a plasmid library by *in vivo* excision of the pBK-CMV phagemid from the λ ZAP Express vector following the protocol provided by the manufacturer (Stratagene). A large-scale whole-mount *in situ* hybridization method was used for screening this plasmid library, in principle as described by Hollemann *et al.* (1998b). Briefly, single colonies were grown in 96-well miniplates. Fluorescein-labelled antisense RNA probes were transcribed from templates obtained by PCR amplification of the insert region from these single colonies. Four sets of a special 24-well

device for simultaneous *in situ* hybridization were used per round. Colonies with a suggestive expression pattern were sequenced and matched with the DDBJ/EMBL/GenBank sequence information. After four rounds of screening, 120 interesting cDNA clones were selected. XCYP26 is one of these.

Embryos and whole-mount *in situ* hybridization

Wild-type and albino *Xenopus laevis* embryos were obtained by hormone-induced egg laying and *in vitro* fertilization using standard methods. Whole-mount *in situ* hybridization was done in principle as described by Harland (1991), with modifications as reported in Hollemann *et al.* (1998a). For double staining analysis, digoxigenin-UTP- and fluorescein-UTP-labelled RNA probes were used. After the first staining with NBT/BCIP, the enzyme reaction was stopped by heating the embryos for 20 min in 0.1× MBS supplemented with 10 M EDTA. The staining for the second transcript was as for the first one, but using Fast Red (Boehringer Mannheim) as a dye. Probes were prepared using the digoxigenin or fluorescein RNA-labelling mixes (Boehringer Mannheim) and subsequently purified using the RNA Easy Kit (Qiagen). The probes used were: XCYP26, cut with *EcoRI*, transcribed with T7 RNA polymerase; En2, cut with *XhoI*, transcribed with T7 RNA polymerase (Hemmati Brivanlou *et al.*, 1991); Otx2, cut with *NotI*, transcribed with T7 RNA polymerase (Lamb *et al.*, 1993); Krox20, cut with *EcoRI*, transcribed with T7 RNA polymerase (Bradley *et al.*, 1993); N-tubulin, cut with *BamHI*, transcribed with T3 RNA polymerase (Oschwald *et al.*, 1991; Chitnis *et al.*, 1995); Sox3, cut with *EcoRI*, transcribed with T7 RNA polymerase (Penzel *et al.*, 1997); Pax6, cut with *PstI*, transcribed with T7 RNA polymerase (Hollemann *et al.*, 1998a); Hoxb3, cut with *EcoRI*, transcribed with Sp6 RNA polymerase (Godsave *et al.*, 1994); and Hoxb9, cut with *EcoRI*, transcribed with T7 RNA polymerase (Cho *et al.*, 1988).

RNA injection and animal cap explants

The entire XCYP26 coding region was subcloned into the pCS2+ vector (Turner and Weintraub, 1994). After linearization with *NotI*, the DNA template was transcribed *in vitro* with SP6 RNA polymerase in the presence of m7GpppG to produce capped XCYP26 transcripts. Up to 2 ng of XCYP26 RNA, either alone or together with 20 pg of LacZ RNA (Chitnis *et al.*, 1995), was injected in a volume of 10 nl into one or two blastomeres of two-cell or four-cell stage *Xenopus* embryo. As a negative control, embryos were injected with LacZ RNA alone. At various stages, the injected embryos were either fixed for normal histological analysis, or fixed, stained with X-gal to reveal the distribution of the LacZ tracer and then analysed by whole-mount *in situ* hybridization.

For the animal cap assay, embryos were injected at the two-cell stage into the animal pole of both blastomeres with the following mRNAs: 50 pg of zebrafish activin β B (Wittbrodt and Rosa, 1994); 5–10 pg of eFGF (Isaacs *et al.*, 1992); and 1–2 ng of BMP4 (Köster *et al.*, 1991). Animal caps were dissected from stage 10 embryos, cultured in 0.5× MBS and harvested until control siblings had reached stage 13 or 18, respectively.

RT-PCR

Total RNA from embryos and tissues was isolated with phenol/chloroform extraction and LiCl precipitation (Döring and Stick, 1990). The Qiagen RNeasy Kit was used for RNA isolation from animal caps. All RNA preparations were treated with DNase I (Boehringer Mannheim) and checked with 35 cycles of histone H4-specific PCR for DNA contamination. RT-PCR was carried out using the Gene Amp RNA PCR kit from Perkin-Elmer. The manufacturer's protocol was followed except that 1 μ Ci of [α - 32 P]dCTP was included in each PCR. One-tenth of the PCR products was separated on 6% polyacrylamide gels under denaturing conditions. Dried gels were analysed using a PhosphorImager and the ImageQuant 2.0 program (Molecular Dynamics). The following primer oligonucleotides were utilized: XCYP26, forward (F) 5'-GCTGCC-ACGTCCTCACCCTCTT-3' and reverse (R) 5'-GCCGATGCAGCA-CCTCACTCCA-3'; histone H4, F 5'-CGGGATAACATTCAGGGTA-TCACT and R 5'-ATCCATGGCGGTAACCTGCTCTTCT (Niehrs *et al.*, 1994); Xbra, F 5'-TCTCTGGAGTAATGAGTG and R 5'-ACAAAGT-CCAGCAGAACCGTA (Smith *et al.*, 1991).

UV, LiCl and ATRA treatment

Fertilized eggs were dejellied 30 min after insemination, distributed in Petriperm™ Petri dishes with a UV-permeable bottom (Heraeus) and treated with UV transilluminator (Appligene) for 1 min. LiCl treatment was performed by treating embryos with 0.3 M LiCl in 0.1× MBS for 8–10 min at the 32-cell stage (Kao and Elinson, 1988). ATRA treatment

was performed as described in Sive *et al.* (1990) for animal caps and Durston *et al.* (1989) for whole embryos.

Histological procedures

Vibratome sections were prepared as described previously (Hollemann *et al.*, 1996).

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