A Novel Cytochrome P450, Zebrafish Cyp26D1, Is Involved in Metabolism of All-trans Retinoic Acid

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Retinoic signaling is essential for development of vertebrate embryos, and its action is mainly through retinoic acid (RA) binding to its RA receptors and retinoid-X receptors, while the critical concentration and localization of RA in embryos are determined by the presence and activity of retinal dehydrogenases (for RA synthesis) and cytochrome P450 RAs (Cyp26s) (for degradation of RA). Previously, we identified a novel cyp26 gene (cyp26d1) in zebrafish that is expressed in hindbrain during early development. Using reverse-phase HPLC analyses, we show here that zebrafish Cyp26D1 expressed in 293T cells could metabolize all-trans RA, 9-cis RA, and 13-cis RA, but could not metabolize retinol or retinal. The metabolites of all-trans RA produced by Cyp26D1 were the same as that produced by Cyp26A1, which are mainly 4-hydroxy-all-trans-RA and 4-oxo-all-trans-RA. Performing mRNA microinjection into zebrafish embryos, we demonstrated that overexpression of Cyp26D1 in embryos not only caused the distance between rhombomere 5 and the first somite of the injected embryos to be shorter than control embryos but also resulted in left-right asymmetry of somitogenesis in the injected embryos. These alterations were similar to those caused by the overexpression of cyp26a1 in zebrafish embryos and to that which resulted from treating embryos with 1 μM 4-diethylamino-benzaldehyde (retinal dehydrogenase inhibitor), implying that cyp26d1 can antagonize RA activity in vivo. Together, our in vitro and in vivo results provided direct evidence that zebrafish Cyp26D1 is involved in RA metabolism. (Molecular Endocrinology 20: 1661–1672, 2006)

Retinoic signaling has long been recognized to be crucial to embryonic development. It was first found in the 1930s that farm animals fed with a vitamin A-deficient diet produced abnormal embryos with widespread defects in the eye, craniofacial, limb, heart, and urogenital systems (1). On the other hand, overexposure of retinoid signaling to embryos also disrupted the development of embryos (2). It is known that retinoid signaling is transduced through the ligand, retinoic acid (RA), binding to the heterodimers of RA receptors (RARs) and retinoid-X receptors to regulate expression of target genes containing RA response elements (3). However, gene knockout studies in mice have shown that RARs and retinoid-X receptors can function in a partly redundant manner during development (4). Therefore, the temporal and spatial regulation of RA presence plays determining roles in retinoid signaling.

RA cannot be synthesized de novo in vertebrates. Animals take dietary vitamin A and convert it into RA in vivo through two dehydrogenation steps, in which the absorbed vitamin A is first converted into all-trans retinaldehyde by retinol dehydrogenase, and retinaldehyde is then oxidized into all-trans RA by the retinal dehydrogenase (Raldh) (5). On the other hand, specific cytochrome P450 RAs (Cyp26s) degrade RA into inactive polar forms, such as 4-hydroxy-all-trans-RA (4-OH-RA), 4-oxo-all-trans-RA (4-oxo-RA), and 18-hydroxy-all-trans-RA (18-OH-RA) (5, 6). Because the reaction catalyzed by retinol dehydrogenase is reversible, RA homeostasis is maintained by regulation of its rate of synthesis from retinal and by control of its rate of degradation through oxidative pathways (5).

Three Raldhs (Raldh1, Raldh2, and Raldh3) have been identified in vertebrates. Although genetic ablation of Raldh1 had no apparent effects on mouse embryo development (7), the phenotypes of mouse embryos with Raldh2 or Raldh3 gene disrupted were similar to those observed in vitamin A-deficient fetuses. Raldh2-null mice died at midgestation with a shorter anterior-posterior axis, open neural tube, absence of limb buds (8), and asymmetric somitogenesis (9, 10). Similarly, zebrafish raldh2 mutants (nls, nof...
were characterized with a truncation of the anterior-posterior axis anteriorly to the somites, absence of pectoral fins (11, 12), and asymmetric somitogenesis (13). Raldh3 knockout in mice caused malformations in ocular and nasal regions, notably resulting in choanal atresia, which is responsible for respiratory distress and death of Raldh3-null mutants at birth (14).

Three Cyp26s (Cyp26A1, Cyp26B1, and Cyp26C1) have been characterized in vertebrates (15–22). Although loss of function of Cyp26C1 during vertebrate development has not been characterized yet, loss of Cyp26A1 function caused mouse embryonic lethality (23, 24), whereas the Cyp26B1-null mouse died after birth (25). Cyp26A1-null embryos died from caudal regression associated with exencephaly, spina bifida, agenesis of the caudal portions of the digestive and urogenital tracts, malformed lumbosacral skeletal elements, and lack of caudal tail vertebrae (23, 24). In zebrafish, embryos with cyp26a1 mutation, giraff, displayed a phenotype just opposite to that of the raldh2 mutant, exhibiting expanded rostral spinal cord territory (26). Additionally, when Cyp26A1+-/- homoygous mice were bred into a Raldh2-/- background, the Cyp26Aa1-null phenotypes were suppressed, suggesting that a function of Cyp26A1 could be to titrate the local level of RA to prevent inappropriate RA signaling (6). Cyp26B1-/- mice manifested various malformations, including meromelia, micrognathia, and open eyes at birth, and they died immediately after birth as a result of respiratory distress (25).

Previously, we described a novel cyp26, cyp26d1, in zebrafish (27). Here, we provide in vitro and in vivo evidence to demonstrate that, like other Cyp26 members, zebrafish cyp26d1 is involved in the metabolism of RA.

RESULTS

Zebrafish Cyp26D1 Contains the Conserved Functional Domains Found in Other Cyp26s

Previously, we had cloned a novel cyp26 (cyp26d1) in zebrafish (27). Bioinformatic analysis suggested that cyp26d1 defines a new Cyp26 subfamily rather than being an ortholog of any known mammalian Cyp26 member (27). By comparing its predicted amino acid sequence to mouse Cyp26A1 and zebrafish Cyp26A1, we found that the predicted functional domains in Cyp26, including anchor domain, proline domain, oxygen binding domain, retinoid domain, and heme-binding domain are all conserved in Cyp26D1 (28) (Fig. 1). The sequence characteristics of Cyp26D1 suggest that it would be involved in the metabolism of RA.

Zebrafish Cyp26D1 Specifically Metabolizes RA, But Not Retinol or Retinal

To examine whether zebrafish Cyp26D1 can metabolize RA, we first overexpressed cyp26d1 in 293T cells. Because Cyp26D1 possesses a transmembrane domain typical of microsome-associated P450s (anchor region; Fig. 1), microsome fractions of the cells overexpressing Cyp26D1 were collected and then incu-
bated with various retinoids to allow the expressed Cyp26D1 to metabolize retinoids. After reaction, the retinoids and their metabolites from the reaction mixtures were extracted and then analyzed by reverse-phase HPLC. As shown in Fig. 2B, in addition to a peak corresponding to unmetabolized all-trans RA (Table 1), two major peaks along with two small peaks were displayed after the retinoid was incubated with microsomes of cyp26d1-expressing cells. In contrast, extraction of the reaction mixture incubated with microsomes of cells transfected with vehicle vector only yielded one peak that corresponded to the unmetabolized all-trans RA (Fig. 2A and Table 1), indicating that microsomes from 293T cells themselves do not have detectable activity to metabolize all-trans RA. Therefore, our results revealed that zebrafish Cyp26D1 could metabolize all-trans RA into at least four different metabolites. When 9-cis RA was used as a substrate, extraction of the reaction mixture incubated with microsomes of cyp26d1-expressing cells exhibited one extra major peak along with one small peak (Fig. 2D), in addition to the peak corresponding to unmetabolized 9-cis RA (Fig. 2D and Table 1). When 13-cis RA was used as a substrate, in addition to the peak of unmetabolized 13-cis RA (Fig. 2F and Table 1), extraction of the reaction mixture after incubation with microsomes from cyp26d1-expressing cells produced two additional major peaks (Fig. 2F). In contrast, extraction of the reaction mixture of 9-cis RA and 13-cis RA incubated with microsomes of cells transfected with vehicle vector only yielded one peak that corresponded to the unmetabolized 9-cis RA and 13-cis RA, respectively (Fig. 2, C and E, and Table 1), indicating that microsomes from 293T cells themselves do not have detectable activity to metabolize 9-cis RA or 13-cis RA. Furthermore, when retinol or all-trans retinal were used as substrates, extraction of reaction mixtures gave only one peak (Fig. 2, G and H) that corresponded to the unmetabolized retinol or all-trans retinal, respectively (Table 1), indicating that Cyp26D1 could not metabolize retinol or retinal. Together, these results indicated that Cyp26D1 could metabolize RA including all-trans RA, 9-cis RA and 13-cis RA, but not retinol or all-trans retinal.

The Major Metabolites of All-trans RA Produced by Zebrafish Cyp26D1 Include 4-oxo-RA and 4-OH-RA

As shown in Fig. 2B, there were at least four different metabolites (peak 1 and peak 2, p3 and p4, as shown in Fig. 3A) of all-trans RA produced by Cyp26D1. To identify them, we compared the metabolites produced by Cyp26D1 with those produced by zebrafish Cyp26A1, because the major metabolites of all-trans RA produced by Cyp26A1 had been previously identified as 4-oxo-RA and 4-OH-RA (15). To perform this assay, we added all-trans RA as a substrate to react with microsomes isolated from 293T cells expressing zebrafish cyp26a1 and performed reverse-phase HPLC with Hypersil BDS C18 column on the reaction mixture. In addition to the peak corresponding to unmetabolized all-trans RA, the HPLC analysis also yielded two major peaks (peak 1 and peak 2) along with two small peaks (p3 and p4), which were the same as that exhibited by the cyp26d1 metabolites (Fig. 3, A and B). Most interestingly, the retention time of each peak from the metabolites produced by Cyp26A1 was similar to those produced by Cyp26D1 (Table 2 and Fig. 3, A and B). We then mixed the extracts from Cyp26D1 reaction mixture with those from Cyp26A1 reaction mixture and performed HPLC analyses using the same column and the same elution conditions and found that all of the peaks including the four different metabolites’ peaks and unmetabolized substrate peak were comigrating, respectively (Fig. 3C). This result is consistent with the conclusion that the metabolites produced by zebrafish Cyp26D1 are the same as those produced by zebrafish Cyp26A1.

To further confirm this, we performed reverse-phase HPLC on the metabolites with a different type of column (SB-phenyl column), using a different elution condition. And that produced the same result. As with the C18 column, HPLC analyses using the SB-phenyl column on reaction mixture metabolized by Cyp26D1 or Cyp26A1 both exhibited two major peaks along with two small peaks, respectively, in additional to one peak of unmetabolized all-trans RA (Fig. 3, D and E, and Table 2). Also, the four peaks from Cyp26D1 reaction and Cyp26A1 reaction have similar retention times (Fig. 3, D and E, and Table 2). When two sources of the metabolites were mixed together to run the SB-phenyl column, all five peaks including the four metabolites’ peaks and one unmetabolized all-trans RA peaks were comigrating, respectively (Fig. 3F), further proving that the metabolites produced by Cyp26D1 are the same as the metabolites produced by Cyp26A1. Because the major metabolites of all-trans RA produced by zebrafish cyp26a1 were previously identified as 4-oxo-RA and 4-OH-RA (15), therefore our results indicate that like Cyp26A1, the major metabolites of all-trans RA produced by Cyp26D1 could also be 4-oxo-RA and 4-OH-RA. When 9-cis-RA or 13-cis-RA was used as a substrate, our results showed that the peaks of metabolites produced by Cyp26D1 were also comigrating with those produced by Cyp26A1, respectively (Table 2 and data not shown). Together, these results demonstrated that the metabolites of RA produced by Cyp26D1 are the same as that produced by Cyp26A1.

Cyp26D1 Can Inactivate RA in Vivo

It has been reported that the distance between rhombomere 5 (r5) and first somite (s1) in raldh2 mutant (nls) embryos is much shorter than that of wild-type zebrafish embryos (11). Because raldh2 is the major source for RA synthesis, it would be predicted that the distance between r5 and s1 would be shorter if embryos have reduced endogenous RA activity. To ex-
amine this hypothesis, we treated embryos with 1 \textmu M 4-diethylamino-benzaldehyde (DEAB) (Raldh inhibitor) until the 11-somite stage (11S). As expected, whole-
mount in situ hybridization on the treated embryos with krox20 (the molecular marker for r3 and r5) (29) and myod (the marker for somites) probes revealed

**Fig. 2.** HPLC Analysis Showing that Zebrafish Cyp26D1 Can Metabolize RA Including All-trans-RA, 9-cis-RA, and 13-cis-RA

All-trans-RA (A and B), 9-cis-RA (C and D), 13-cis-RA (E and F), retinol (G), and all-trans retinal (H) were added as substrates to react with microsomes isolated from 293T cells transfected with vehicle vector or zebrafish cyp26d1, respectively. By performing reverse-phase HPLC with a Hypersil BDS C18 column, retinoids extracted from the reaction mixture of all-trans RA, 9-cis-RA, and 13-cis-RA incubated with microsomes of cells transfected with zebrafish cyp26d1 were separated into two major peaks plus two small peaks (B), one major peak plus a small peak (D), and two major peaks (F), respectively. In contrast, extraction of the reaction mixture of all-trans RA, 9-cis-RA, and 13-cis-RA incubated with microsomes of cells transfected with vehicle vector only yielded one peak that corresponded to the unmetabolized all-trans RA, 9-cis-RA, and 13-cis-RA, respectively (A, C, and E). Furthermore, no metabolite was detected from the reaction mixtures when retinol (G) or all-trans retinal (H) was used as a substrate. Unmetabolized substrates were marked as judged by the retention time of standard all-trans RA, 9-cis-RA, 13-cis-RA, retinol, and all-trans retinal (Table 1). x-axis, Retention time (in minutes) of retinoids; y-axis, absorbance value at 350 nm (mAU).
that the distance between r5 and s1 (Fig. 4, C and D; 0.88 ± 0.04 mm, n = 33; Table 3) was shorter than (P < 0.01) that of control embryos (Fig. 4, A and B; 1.28 ± 0.03 mm, n = 33; Table 3). We then microinjected zebrafish cyp26a1 mRNA into zebrafish embryos, and the result showed that, like the DEAB-treated embryos (Fig. 4, C and D), the distance between r5 and s1 of the microinjected embryos (Fig. 4, E and F; 0.93 ± 0.29 mm, n = 27; Table 3) was also shorter than (P < 0.01) that of control embryos (Fig. 4, A and B; 1.28 ± 0.03 mm, n = 33; Table 3). When microinjecting cyp26d1 mRNA into zebrafish embryos, we obtained the same result. The distance between r5 and s1 of the injected embryos (Fig. 4, G and H; 0.97 ± 0.23, n = 30; Table 3) was also shorter than (P < 0.01) that of the control (Fig. 4, A and B; 1.28 ± 0.03 mm, n = 33; Table 3) but was approximately equal to (P = 0.60) that of embryos injected with cyp26a1 (Fig. 4, E and F; 0.93 ± 0.29, n = 27; Table 3). This result suggested that, like cyp26a1, zebrafish cyp26d1 was involved in reducing RA activity in vivo.

Additionally, a recent report demonstrated that zebrafish nls embryos also have defects in left-right symmetry of somitogenesis during 6S–13S stages (13). Because raldh2 is the major source for RA synthesis, we hypothesized that the embryos would have asymmetric somite development if their endogenous RA activities were reduced. To test this, we first examined 1 μM DEAB-treated embryos during 6S–13S stages. We found that 28 of 179 of treated embryos exhibited asymmetric somites, with 67.8% of them (19 of 28) having one to three extra somites on the right side (Fig. 5A and Table 4). The rate (15.6%; 28 of 179) of the treated embryos with asymmetric somites was significantly higher than (P < 0.01) that of control embryos (0 of 180). This is consistent with the role of cyp26a1 in reducing RA activity in vivo. When microinjected with cyp26d1, 29 of 196 embryos showed asymmetric somites, of which 72.4% (21 of 29) had one to three extra somites on the right side (Fig. 5C and Table 3). The rate (14.8%; 29 of 196) of the cyp26d1-injected embryos with asymmetric somites was significantly higher than (P < 0.01) that of control embryos (0 of 180). The result further demonstrated that like cyp26a1, cyp26d1 is also involved in reducing RA activity in vivo.

**Tissue Distributions of the cyp26d1 Gene**

Previously, we have reported that cyp26d1 has a unique dynamic expression pattern during early development of zebrafish (27). By performing RT-PCR analyses, we showed that zebrafish cyp26d1 was also widely expressed in different adult tissues examined including brain, eye, heart, gill, liver, intestine, swimming bladder, ovary, muscle, caudal fin, and pectoral fin (Fig. 6A). However, the expression levels of cyp26d1 in these tissues were different. Using Kodak ID Image Analysis software, we found that the relative expression levels in different tissues (calculated as the intensity of the amplified cyp26d1 product divided by the intensity of amplified β-actin control product for each sample, and averaged over three independent experiments) were 0.028 ± 0.007 (brain), 0.050 ± 0.019 (caudal fin), 0.109 ± 0.021 (eye), 0.431 ± 0.101 (gill), 0.029 ± 0.002 (heart), 0.052 ± 0.013 (intestine), 0.023 ± 0.009 (liver), 0.309 ± 0.014 (muscle), 0.047 ± 0.019 (ovary), 0.124 ± 0.047 (pectoral fin), and 0.045 ± 0.017 (swimming bladder), respectively. Statistical tests showed that the abundance of cyp26d1 message in different tissues were in this order: gill and muscle > eye and pectoral fin > brain, caudal fin, heart, intestine, liver, ovary, and swimming bladder (Fig. 6B).

**DISCUSSION**

Cyp26 family genes are known to encode enzymes that can specifically inactivate RA, and the first member, cyp26a1, was initially found in zebrafish (cyp26a1) (15). Until now, three different Cyp26 genes including Cyp26A1, Cyp26B1, and Cyp26C1 have been identified in mammals. Of the three genes, orthologs of Cyp26A1 and Cyp26B1 genes have been found in zebrafish (15, 22). Previously, we cloned a novel cyp26 gene in zebrafish and named it cyp26d1 (27). While our results (AY920470) were in press (27), Kawakami et al. (13) published a paper reporting a zebrafish "Cyp26C1" sequence (AY904301). The authors used the "cyp26c1" as a probe for whole-mount in situ hybridization as part of the paper linking RA and left-right patterning without performing any functional analysis on it. However, analysis on this cyp26c1
cDNA revealed that the AY904031 was an incomplete coding sequence without stop codon. When aligning AY904031 sequence with our cyp26d1 cDNA, we found that it was only the 5' part of our full-length coding cDNA. In our previous paper, we demonstrated that our cloned cyp26d1 gene is not an ortholog of mammalian Cyp26C1 genes (27). First, unlike zebrafish Cyp26A1 and Cyp26B1, which share more than 66 and 75% amino acid identities with their mammalian orthologs, respectively, zebrafish Cyp26D1 shares only 39–40% identity with mammalian Cyp26A1, 51% identity with mammalian Cyp26B1, and 51–54% identity with mammalian Cyp26C1, respectively (27). This lower amino acid identity (<60%) suggests that zebrafish Cyp26D1 may define a new subfamily instead of being a member of the Cyp26C1 subfamily. Second, phylogenetic analyses revealed that evolutionarily this protein is closer to Cyp26B1 subfamily than to Cyp26C1 subfamily (27), which is consistent with the conclusion that this gene is not an ortholog of the mammalian Cyp26C1 gene. Third, the physical locus of Cyp26C1 is adjacent to Cyp26A1 in chromosome 10q23 in humans (http://www.ncbi.nlm.nih.gov). The physical distance between the two genes.

**Fig. 3.** HPLC Analysis Showing that Metabolites of All-trans RA Produced by Zebrafish Cyp26D1 Are Comigrating with Those Produced by Zebrafish Cyp26A1, Respectively

All-trans-RA was incubated with the microsomes isolated from 293T cells transfected with zebrafish cyp26d1 and cyp26a1, respectively. By performing reverse-phase HPLC with Hypersil BDS C18 column (A and B) and SB-phenyl column (D and E), retinoids extracted from the reaction mixtures of Cyp26D1 (A and D) and Cyp26A1 (B and E) all displayed three major peaks plus two small peaks. Of the three major peaks in each chromatography, one peak had similar retention time to that of standard all-trans RA (Table 2) and therefore was recognized as the peak of unmetabolized substrate (as marked). When run by either Hypersil BDS C18 column (A and B) or SB-phenyl column (D and E), peak 1, peak 2, p3, and p4 from Cyp26D1 reaction mixture (A and D) had similar retention times to those from Cyp26A1 reaction mixture (B and E), respectively (Table 2). When the two sources of retinoids were mixed together, peak 1, peak 2, p3, p4, and the unmetabolized substrate were comigrating, respectively, either in Hypersil BDS C18 column (C) or in SB-phenyl column (F). Unmetabolized substrates were marked as judged by the retention time of standard all-trans RA (Table 2). x-axis, Retention time (in minutes) of retinoids; y-axis, absorbance value at 350 nm (mAU).
Table 2. Retention Time of RA Metabolites Produced by Cyp26A1 and Cyp26D1 in Chromatographic Columns

<table>
<thead>
<tr>
<th>Source of Retinoids</th>
<th>Hypersil BDS C18 Column</th>
<th>SB-Phenyl Column</th>
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<tr>
<td></td>
<td>Standard (min)</td>
<td>D1 (min)</td>
</tr>
<tr>
<td>Peak 1</td>
<td>8.765</td>
<td>8.598</td>
</tr>
<tr>
<td>Peak 2</td>
<td>14.539</td>
<td>14.293</td>
</tr>
<tr>
<td>p3</td>
<td>15.377</td>
<td>15.128</td>
</tr>
<tr>
<td>p4</td>
<td>16.093</td>
<td>15.841</td>
</tr>
<tr>
<td>Peak 1</td>
<td>8.714</td>
<td>8.637</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>13.904</td>
<td>14.486</td>
</tr>
<tr>
<td>Peak 1</td>
<td>8.778</td>
<td>8.374</td>
</tr>
</tbody>
</table>

Reverse-phase HPLC was performed using a Hypersil BDS C18 column and 20RBAX SB-Phenyl column. Listed are the retention times in the column of standard RA including all-trans-RA, 9-cis-RA, and 13-cis-RA, and the metabolites or unmetabolized substrates after RAs were incubated with microsomes isolated from the cells transfected with zebrafish cyp26d1 expression vector (D1) or cyp26a1 expression vector (A1), or the retinoids from the reaction mixtures of cyp26d1 reaction and cyp26a1 reaction (A1 + D1).

is 4778 bp long. However, cyp26d1 is located in linkage group 17 (27) and cyp26a1 is located in linkage group 12 in zebrafish (http://zfin.org). The facts further support the idea that cyp26d1 is not an ortholog of the human Cyp26C7 gene.

However, zebrafish Cyp26D1 contains all the predicted conserved functional domains in Cyp26s including anchor domain, praline-rich domain, oxygen binding domain, ligand binding domain, and heme-binding domain, implying that it is involved in RA metabolism. Through reverse HPLC analyses, we have demonstrated directly that, like other Cyp26 members (15–18, 20, 30), zebrafish Cyp26D1 specifically metabolized RA including all-trans RA, 9-cis-RA, and 13-cis-RA examined, but not retinol and retinal. The metabolites of all-trans RA produced by Cyp26D1 were found to be the same as that metabolized by zebrafish Cyp26A1, which are mainly 4-oxo-RA, 4-OH-RA, and 4,6-epoxy-RA (17). As suggested by Petkovich and coworkers (30), these discrepancies may arise from differences in metabolites extraction and characterization techniques. In this study, the HPLC analyses on metabolites of all-trans RA produced by Cyp26D1 and Cyp26A1 revealed that, in addition to the two major peaks (peak 1 and peak 2), there were two other small peaks (p3 and p4) present (Fig. 4), which is consistent with the idea that metabolites of all-trans RA metabolized by Cyp26s include four different retinoids.

A number of studies have shown that metabolites of all-trans RA including 4-oxo-RA, 4,6-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA are all biologically active. For example, 4-oxo-RA was shown to be a highly active modulator in embryogenesis (31), and 4-oxo-RA, 4,6-epoxy-RA, and 5,6-epoxy-all-trans-RA (5,6-epoxy-RA) could fully rescue the vitamin A-deficient quail embryo (32), while 4-oxo-, 4-OH-, 18-OH-, and 5,6-epoxy-RA could induce granulocytic differentiation of NB4 acute promyelocytic leukemia cells (33). Moreover, like all-trans RA, in vitro transcription assays had demonstrated that all four retinoids function through binding the RA response element of regulated genes (34). While using selective agonists against RAR, the metabolites were shown to regulate the Cyps through the RARα receptor in quail embryos (32). However, when Cyp26A1<sup>+/−</sup> homozygous mice were introduced into a Raldh<sup>2<sup>+/−</sup></sup> background, the Cyp26A1-null phenotypes were suppressed. The Cyp26A1<sup>−/−</sup>/Raldh<sup>2<sup>−/−</sup></sup> mouse could survive to adulthood without any phenotypic defect apart from their abnormal tails, and both males and females of the surviving Cyp26A1<sup>−/−</sup>/Raldh<sup>2<sup>−/−</sup></sup> mice were fertile (6). These findings provide direct genetic evidence that the normal function of Cyp26A1 is opposite to that of Raldh2, and Cyp26A1 may play a role in preventing inappropriate RA signaling, rather than producing signaling by bioactive RA metabolites such as 4-oxo-RA, 4,6-oxo-RA, and 5,6-epoxy-RA. These data are consistent with the fact that overexpression of Cyp26A1 in Xenopus laevis embryos can rescue the developmental defects induced by exogenous excess RA (35). In zebrafish, nls embryos with raldh2 mutation were characterized by a truncation of the anteroposterior axis anterior to the somites (11), whereas giraffe embryos with cyp26a1 mutation had an expanded rostral spinal cord territory (26). The opposite phenotypes of nls embryos and giraffe embryos again suggested that raldh and cyp26 work together to establish local embryonic RA levels that must be finely tuned to allow normal development of embryos. For example, the development of the anteroposterior axis anterior to the somites seemed to largely depend on the endogenous RA activity. And the length of anteroposterior axis appeared to corre-
late positively with the amount of RA activity, that is, lower RA activity would result in a shorter axis and higher RA activity would result in a longer axis. By treating embryos with DEAB, which can specifically inhibit endogenous RA synthesis (36), we revealed that, like n/s embryos, the DEAB-treated embryos had a short “neck,” that is, the distance between r5 and s1 was shorter than that of untreated embryos. Based on this observation, we next examined the in vivo activity of cyp26d1 by microinjecting embryos with cyp26d1.

**Table 3. Distances between r5 and the s1 of 11S Zebrafish Embryos after Different Treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (mm)</th>
<th>1 μM DEAB (mm)</th>
<th>Cyp26A1 (mm)</th>
<th>Cyp26D1 (mm)</th>
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<tr>
<td>Experiment 1</td>
<td>1.29 ± 0.02 (n = 11)</td>
<td>0.89 ± 0.03 (n = 11)</td>
<td>0.94 ± 0.39 (n = 8)</td>
<td>1.01 ± 0.20 (n = 14)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1.27 ± 0.03 (n = 11)</td>
<td>0.86 ± 0.05 (n = 11)</td>
<td>1.04 ± 0.22 (n = 8)</td>
<td>0.95 ± 0.27 (n = 8)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>1.27 ± 0.04 (n = 11)</td>
<td>0.87 ± 0.03 (n = 11)</td>
<td>0.85 ± 0.26 (n = 11)</td>
<td>0.91 ± 0.27 (n = 8)</td>
</tr>
<tr>
<td>Total</td>
<td>1.28 ± 0.03 (n = 33)</td>
<td>0.88 ± 0.04 (n = 33)</td>
<td>0.93 ± 0.29 (n = 27)</td>
<td>0.97 ± 0.23 (n = 30)</td>
</tr>
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</table>

The distances between r5 and s1 of 11S were measured on the whole-mount embryos that were in situ hybridized with krox20 (the molecular marker for r3 and r5) and myod (the marker for somites). The distance is shown as the average value ± SD in millimeters. n is the number of the embryos measured.
mRNA, and then measured the distance between r5 and s1. We showed that overexpression of cyp26d1 genes in embryos caused a shortened distance between r5 and s1, similar to the cyp26a1 overexpression phenotype in zebrafish embryos and to the phenotype of the DEAB-treated embryos. This result is consistent with our hypothesis that the in vivo role of cyp26d1 is to reduce RA activity and the metabolites of all-trans RA produced by Cyp26D1 are biologically inactive. Moreover, recent discoveries have revealed that blocking endogenous RA activities can disrupt the bilateral symmetry of somite formation in vertebrates (9, 10, 13). We demonstrated that treating embryos with 1 μM DEAB disrupted the left-right symmetry in somite development. Because 1 μM DEAB can only partly inhibit endogenous RA synthesis (36), the result suggested that partial reduction of endogenous RA activity can also cause asymmetric somite development. We also observed this somite asymmetry phenotype in zebrafish embryos microinjected with cyp26a1 or cyp26d1 mRNA. Together, our study provided in vivo evidence that cyp26d1 gene is responsible for the degradation of RA.

Previously, we have described that cyp26d1 has a dynamic expression during the early development of zebrafish (27). Here, we found that cyp26d1 is also widely expressed in adults but with different individual expression levels, suggesting that cyp26d1 could play important roles not only in embryogenesis but also in the growth of adult organs.

**MATERIALS AND METHODS**

**Experimental Animals**

Research on the zebrafish used in this study was conducted in accordance with accepted standards of humane animal care.

**Detection of cyp26d1 Expression in Adult Zebrafish Organs**

The distributions of cyp26d1 in adult organs were detected by RT-PCR method. Different organs from adult zebrafish
were collected to extract total RNA using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Reverse transcription of RNA was carried out with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The sequences of primers used to detect presence of cyp26d1 cDNA were 5'-HG1032-AAGTCACTCACCTTC-GGC-3' (forward) and 5'-HG1032-GAAACGGTCTGGGTCAAA-3' (reverse). PCR was performed using Taq DNA polymerase (Promega) and the conditions were 94°C for 2 min; 40 cycles of 94°C, 30 sec; 54°C, 45 sec; 72°C, 1 min; followed by 10-min incubation at 72°C. The sensitivity of the RT-PCR was controlled by performing the amplification (20 cycles) of zebrafish H9252-actin using the same cDNA templates as described before (37). The amplified products were run in 0.8% native agarose gel, stained with ethidium bromide, and photographed by Kodak EDAS 290. The intensities of amplified products were measured using Kodak ID Image Analysis software on the ethidium bromide-stained gel. The relative expression levels were normalized by dividing the intensity of amplified cyp26d1 product by the intensity of amplified H9252-actin control product for each sample. The intensity of the amplified product was measured using Kodak ID Image Analysis software. Results were subjected to Student’s t test. **, The expression levels of cyp26d1 in gill and muscle are much higher than (P < 0.01) that in other organs. *, The expression levels of cyp26d1 in eye and pectoral fin are significantly higher than (P < 0.05) that in brain, caudal fin, heart, intestine, liver, ovary, and swimming bladder. Lane 1, cyp26d1 cDNA template as positive control; 2, brain; 3, caudal fin; 4, eye; 5, gill; 6, heart; 7, intestine; 8, liver; 9, muscle; 10, ovary; 11, pectoral fin; 12, swimming bladder.

**Fig. 6.** RT-PCR Analysis Showing Expression Pattern of Cyp26d1 Gene in Different Adult Organs

A, Amplified products were separated in 0.8% agarose gel, stained with ethidium bromide, and photographed by Kodak EDAS 290. An 837-bp fragment of cyp26d1 cDNA was detected from different adult organs. Expression of zebrafish β-actin was detected as a control of RT-PCR sensitivity using same RNA template as above. B, The expression levels of cyp26d1 varied in different adult organs. Data were normalized by dividing the intensity of amplified cyp26d1 product by the intensity of amplified β-actin control product for each sample. The intensity of the amplified product was measured using Kodak EDAS 290. The expression levels of cyp26d1 in gill and muscle are much higher than (P < 0.01) that in other organs. **, The expression levels of cyp26d1 in gill and muscle are much higher than (P < 0.01) that in other organs. *, The expression levels of cyp26d1 in eye and pectoral fin are significantly higher than (P < 0.05) that in brain, caudal fin, heart, intestine, liver, ovary, and swimming bladder. Lane 1, cyp26d1 cDNA template as positive control; 2, brain; 3, caudal fin; 4, eye; 5, gill; 6, heart; 7, intestine; 8, liver; 9, muscle; 10, ovary; 11, pectoral fin; 12, swimming bladder.

Expression of Zebrafish cyp26d1 and cyp26a1 Genes in 293T Cells by Transient Transfection

To express zebrafish cyp26d1 and cyp26a1 genes, we first cloned cyp26d1 and cyp26a1 coding sequences (GenBank nos. AY920470 and U68234). Expression vectors of the genes were constructed by recombining the coding sequences with a preceding Kozak sequence into pcDNA3.1 (Invitrogen). The inserts were sequenced to confirm their identities. Transient transfections of 293T cells (American Type Culture Collection, Manassas, VA) with the expression vectors or vehicle vector pcDNA3.1 were performed using Lipofectamine (Invitrogen) following the manufacturer’s manuals. Cells were harvested 48 h after transfection and microsome fractions were prepared from the cultured cells by centrifugation at 100,000 x g after the cells were sonicated.

**In Vitro RA-Metabolizing Assay**

The in vitro RA-metabolizing activities of Cyp26s were analyzed according to the method described previously (17). Briefly, the standard reaction mixture in 1 ml contains microsome fractions (from five 100-mm dishes) in a reaction buffer [10 mM NADP, 0.1 mM glucose-6-phosphate, 50 mM K2HPO4 (pH 7.5), 0.2 mM MgCl2, 1 U glucose-6-phosphate dehydrogenase] [chemicals were bought from Sigma (St. Louis, MO)]. After adding 1μg of each retinoid dissolved in 3 μl of dimethysulfoxide to the reaction, the mixture was incubated in a light-protected tube at 37°C for 2 h. The metabolites plus unmetabolized substrate from the reaction mixture were then extracted, concentrated, and analyzed as described previously (38). The resulting residues were dissolved in 80 μl of 2-propanol for reverse-phase HPLC analysis.
Reverse-Phase HPLC Analysis on Retinoids

The retinoids were detected by reverse-phase HPLC at a wavelength of 350 nm and at 35 °C of the column temperature with HP 1050 HPLC instrument (Hewlett Packard, Palo Alto, CA). Two different types of columns were used to separate retinoids. One of the columns is Hypersil BDS C18 (150 × 4.6 mm, 5 μm; Dalian Elite, Dalian, China). The solution and program of elution used in this column were described previously (17, 38). Briefly, the mobile phase consisted of two solvents (solvent A, methanol-acetonitrile, 8:2 (vol/vol); solvent B, 2% acetic acid). The flow rate of the elution was 1.0 ml/min.

And the gradient program was followed as such: 80:20 of solvent A to solvent B (vol/vol) was used to elute for first 8 min, followed by a second 8-min linear gradient up to 90:10 (vol/vol), then at the fixed ratio eluted for the third 8 min, and finally returned to 80:20 (vol/vol) and eluted for the last 2 min. A second column was 20RAX SB-phenyl (250 × 4.6 mm, 5 μm; Agilent Technologies, Kista, Sweden). The components of the mobile phase for eluting this column and the elution rate were the same as used in C18 column. However, the gradient program was changed such that 72:28 of solvent A to solvent B (vol/vol) was first used to elute for the first 10 min, followed by a second 10-min linear gradient up to 81:19 (vol/vol), and then at the fixed ratio eluted for another 4 min, and finally returned to 72:28 (vol/vol) and eluted for the last 2 min.

In Vivo RA-Metabolizing Assay

To test Cyp26D1 activities in vivo, mRNAs of cyp26d1 and cyp26a1 were synthesized in vitro. Briefly, full-length cDNA sequences of cyp26d1 (AY920470) and cyp26a1 (U68234) were introduced into pBluescript vector (Stratagene, La Jolla, CA) under T7 promoter direction. mRNAs of cyp26d1 and cyp26a1 were synthesized and capped in vitro using mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX). About 2–3 nl of 0.5 μg/μl mRNAs were injected into zebrafish embryos at the one-to four-cell stage. In the meantime, embryos treated with 1 μM DEAB (an inhibitor of Raldhs; Sigma) at the one-cell stage served as positive controls, and the embryos injected with the same amount of nanopure water (mock injection) served as negative controls. All of the embryos were then allowed to grow at 28.5 °C and fixed at about 11-somite stages to do double whole-mount in situ hybridization to detect the expressions of krox20 in hindbrain and myod in somites. Whole-mount in situ hybridization was performed as described previously (22). Both probes were labeled with 11-Dig-UTP (Roche, Basel, Switzerland). During hybridization, the two probes were added together and the concentrations of the RNA probes used in hybridization were 0.5 ng/μl. After whole-mount in situ hybridization, the distances between r5 and s1 of 11S embryos were measured and the number of embryos during 65–13S stages with asymmetric somites was recorded. The experiment was repeated three times independently. The data were subjected to Student’s t test (for distances between r5 and s1) or χ² test (for asymmetric somitogenesis). Images were captured using a digital camera.

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