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Developmental expression of *Xenopus* short-chain dehydrogenase/reductase 3

RICHARD K.T. KAM[1], YONGLONG CHEN[2], SUN-ON CHAN[1], WOOD-YEE CHAN[1], IGOR B. DAWID[3] and HUI ZHAO[1,*]

[1]School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, P.R. China, [2]Key Laboratory of Regenerative Biology, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, P.R. China and [3]Laboratory of Molecular Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, Maryland, USA

ABSTRACT During early embryonic development, the retinoic acid signaling pathway coordinates with other signaling pathways to regulate body axis patterning and organogenesis. The production of retinoic acid requires two enzymatic reactions, the first of which is the oxidization of vitamin A (all-*trans*-retinol) to all-*trans* -retinal, mediated in part by the short-chain dehydrogenase/reductase. Through DNA microarrays, we have identified a gene in *Xenopus laevis* which shares a high sequence similarity to human *short-chain dehydrogenase/reductase member 3*. We therefore annotated the gene *Xenopus short-chain dehydrogenase/reductase 3 (dhrs3)*. Expression of *dhrs3* was detected by whole mount *in situ* hybridization in the dorsal blastopore lip and axial mesoderm region in gastrula embryos. During neurulation, *dhrs3* transcripts were found in the notochord and neural ectoderm. Strong expression of *dhrs3* was mainly detected in the brain, spinal cord and pronephros region in tailbud and tadpole stages. Temporal expression tested by RT-PCR indicated that *dhrs3* was activated at the onset of gastrulation, and remained highly expressed at later stages of embryonic development. The distinct and highly regulated spatial and temporal expression of *dhrs3* highlights the complexity of retinoic acid regulation.

KEY WORDS: *dhrs3, retinoic acid, Xenopus*

Early embryonic development is regulated by coordination of multiple signaling pathways that include Wnt, TGF-β, FGF, as well as retinoic acid (RA) signaling. Among them, the retinoic acid (RA) and RA metabolites play essential roles for germ layer differentiation and the body axis formation (Chen *et al.*, 2001; Hollemann *et al.*, 1998; Sirbu and Duester, 2006; Strate *et al.*, 2009; Vermot and Pourquie, 2005). In addition, the RA signaling pathway has been implicated in organogenesis of the heart (Mic *et al.*, 2002; Niederreither *et al.*, 2001; Ryckebusch *et al.*, 2008), lung (Chen *et al.*, 2007; Esteban-Pretel *et al.*, 2009), kidney (Cartry *et al.*, 2006; Wingert *et al.*, 2007) and pancreas (Chen *et al.*, 2004). Deficiencies in the metabolism of retinoids lead to severe defects of vertebrate embryonic development (Abu-Abed *et al.*, 2001; Mendelsohn *et al.*, 1994; Sandell *et al.*, 2007). RA is synthesized from Vitamin A (all-*trans*-retinol) via two enzymatic steps. In the first step, all-*trans*-retinol is oxidized to all-*trans*-retinal either by the short-chain dehydrogenase/reductase (SDR) or medium-chain dehydrogenase/reductase (MDR). The second step is to convert all-*trans*-retinal to RA catalyzed by the retinaldehyde dehydrogenases that include RALDH1, RALDH2, and RALDH3. Retinoic acid directly regulates transcription via interaction with the heterodimer of retinoic acid receptor (RAR) and retinoid receptor (RXR) (Gronemeyer *et al.*, 2004). In this study, we identified a new member of short-chain dehydrogenase/reductase in *Xenopus laevis* and investigated its spatial and temporal expression during the embryonic development.

Abbreviations used in this paper: dhrs3, *Xenopus* short-chain dehydrogenase/reductase 3; MDR, medium-chain dehydrogenase/reductase; RA, retinoic acid; raldh, retinaldehyde dehydrogenase; rdh10, *Xenopus* retinol dehydrogenase 10; RT-PCR, reverse-transcription polymerase chain reaction; Xcyp26, *Xenopus* cytochrome P450 family 26; SDR, short-chain dehydrogenase/reductase.

***Address correspondence to:** Hui Zhao. School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, P. R. China. Fax: +852-2603-5031. e-mail: zhaohui@cuhk.edu.hk

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Results and Discussion

In an attempt to study genome-wide regional gene expression during embryogenesis of *Xenopus laevis*, we have investigated the spatial differences in gene expression in early gastrula embryos (Stage 10) by using DNA microarray. A number of novel genes have been identified (Tanegashima *et al.*, 2008; Zhao *et al.*, 2008) including one gene showing distinct dorsal expression at the onset of gastrulation, which encodes a protein with a sequence similar to the human short-chain dehydrogenase/reductase. This gene was therefore designated *Xenopus* short-chain dehydrogenase/reductase 3 (*dhrs3*). The full cDNA sequence has been deposited to GeneBank, and the accession numbers is FJ607948 (Fig. 1A).

dhrs3 encodes an open reading frame of 302 amino acids, with a predicated co-factor binding site (TGxxxGxG) and catalytic sites (YxxxK) (Fig. 1B), both of which are characteristic of the short-chain dehydrogenase/reductase family (Persson *et al.*, 2003). When compared with *Xenopus* retinol dehydrogenase 10 (rdh10), another member of short-chain dehydrogenase/reductase family, their functional domains were similar except rdh10 carries a co-factor binding site NNAG, instead of NNAA as found in dhrs3. Similar to rdh10, *Xenopus* dhrs3 have a signal peptide at the amino terminus, as predicted by SignalP3.0 (Bendtsen *et al.*, 2004) and Signal-3L (Shen and Chou, 2007). We created a phylogenetic tree showing the evolutionary relationship between dhrs3 and its orthologs in other vertebrates (Fig. 1C). The dhrs3 protein is 94.7% identical to its ortholog in *Xenopus tropicalis* (NM_001008431), 84.1% to chicken (XM_417636), 82.5% to human (BC002730), 81.8% to cow (NP_776605.2), 82.1% to rat (EF125189), 81.1% to mouse (NM_011303), 78.8% to zebrafish sdr3b (BC083252), and 79.1% to zebrafish sdr3a (BC078383) respectively. The high identity of amino acid sequences among short-chain dehydrogenase/reductases 3 from differ-

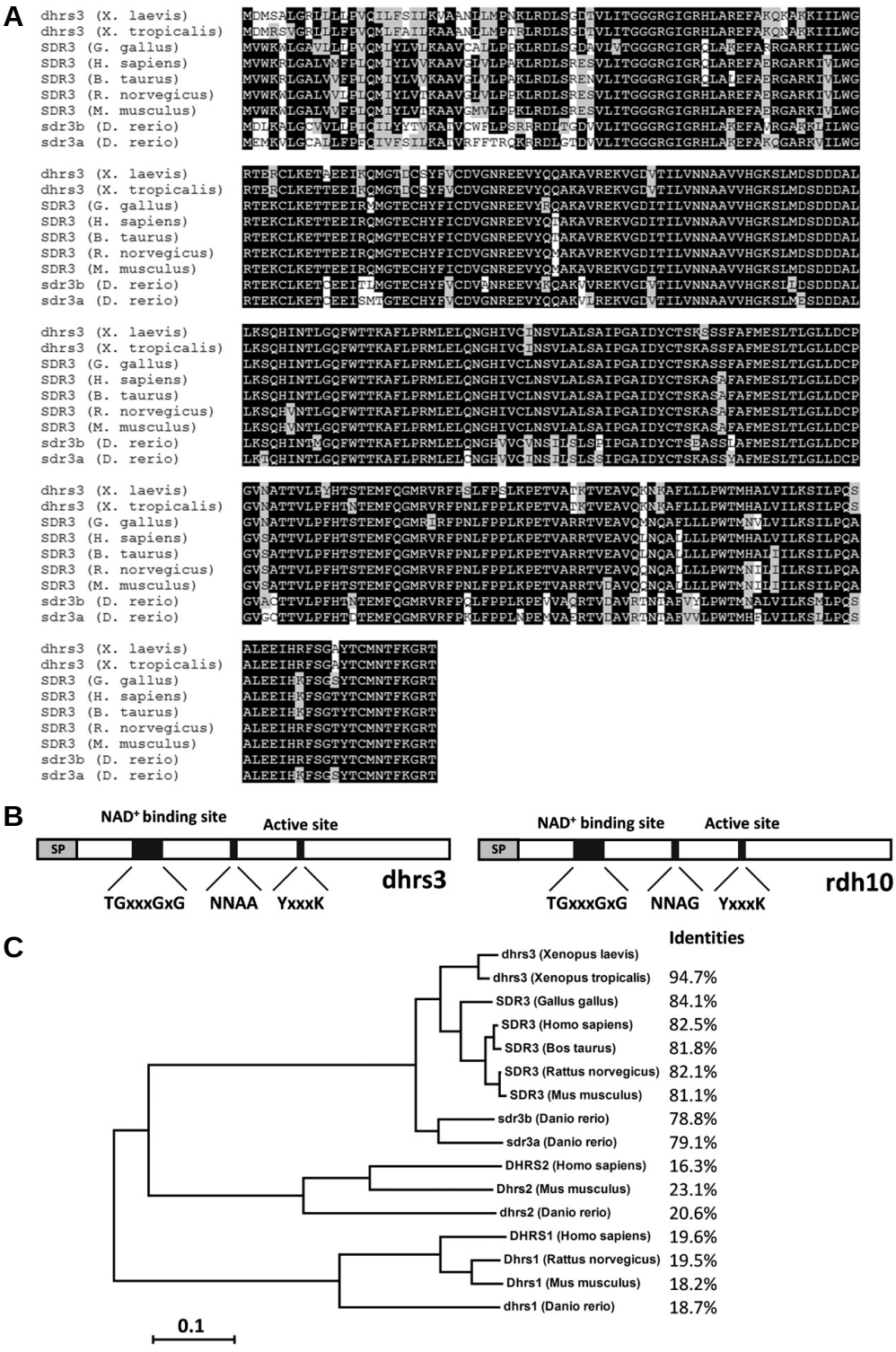

Fig. 1. Characterization of the *Xenopus dhrs3* gene. (A) *Protein sequence comparisons. Alignment of dhrs3 from* Xenopus laevis, Xenopus tropicalis, *chicken, human, cow, rat, mouse and zebrafish, respectiely. Identical amino acids are shaded in black and similar amino acids are shaded in grey.* **(B)** *Schematic drawing of the protein structure of dhrs3 and* Xenopus *rdh10. The conserved sequences, TGxxxGxG for the co-factor binding, NNAA and YxxxK for the active catalytic site, are indicated and the x represents any amino acid residue. SP, signal peptide.* **(C)** *Dendrogram tree of the dhrs1, 2 and 3 families. The identity of amino acid sequences between dhrs3 and its orthologs in other species is indicated as percentage.*

ent species suggests an evolutionarily conserved role for dhrs3 in the embryonic development. We have also compared the sequence similarities between *Xenopus* dhrs3 with dhrs1 and dhrs2 from other species. Their evolutionary relationship was showed in figure 1C.

The spatial expression pattern of *dhrs3* was examined by whole-mount *in situ* hybridization. While signals were observed at the animal pole, no obvious expression was detected in the vegetal hemisphere before midblastula transition (Fig. 2A). At the onset of gastrulation (stage 10), *dhrs3* expression became intensified in the dorsal blastopore lip (Fig. 2B) and continued to expand in the prospective neural plate. With advancing gastrulation, *dhrs3* expression was detected at the dorsal midline, appearing in two bilateral domains on the dorsal side, which progressively decreased anteriorly (Fig. 2C, 3A). Sections from stage 11 embryos confirmed that the expression of *dhrs3* was localized in the dorsal blastopore lip and axial mesoderm (Fig. 3B). In addition, the *dhrs3* signals formed a circumblastoporal ring (Fig. 2C, arrow), which is reminiscent of *Xcyp26a* expression pattern (Hollemann *et al.*, 1998). This expression pattern was similar to that of *rdh10,* which was also found surrounding the yolk plug (Fig. 2C', arrow).

In early neurula stages, signals were observed in the notochord, neural ectoderm, and paraxial mesoderm (Fig. 2D, 3 C-F). With the progression of neurulation, the bilateral expression domains of *dhrs3* in the neural plate gradually converged towards the midline, forming two signal strips extending posteriorly, corresponding to the dorsal ridge of the in-folding neural tube (Fig. 2 D,E,F). Further, V-shape domain at the inner perimeter of the frontal neural groove also stains for *dhrs3* (Fig. 2G, arrow). The anterior edge of neural plate, however, was devoid of discernible expression (Fig. 2 G-I). *rdh10,* on the other hand, was not expressed in the dorsal midline (Fig. 2D'). A dorsal-to-ventral strip of dhrs3 signals was found in the paraxial mesoderm and dorsal

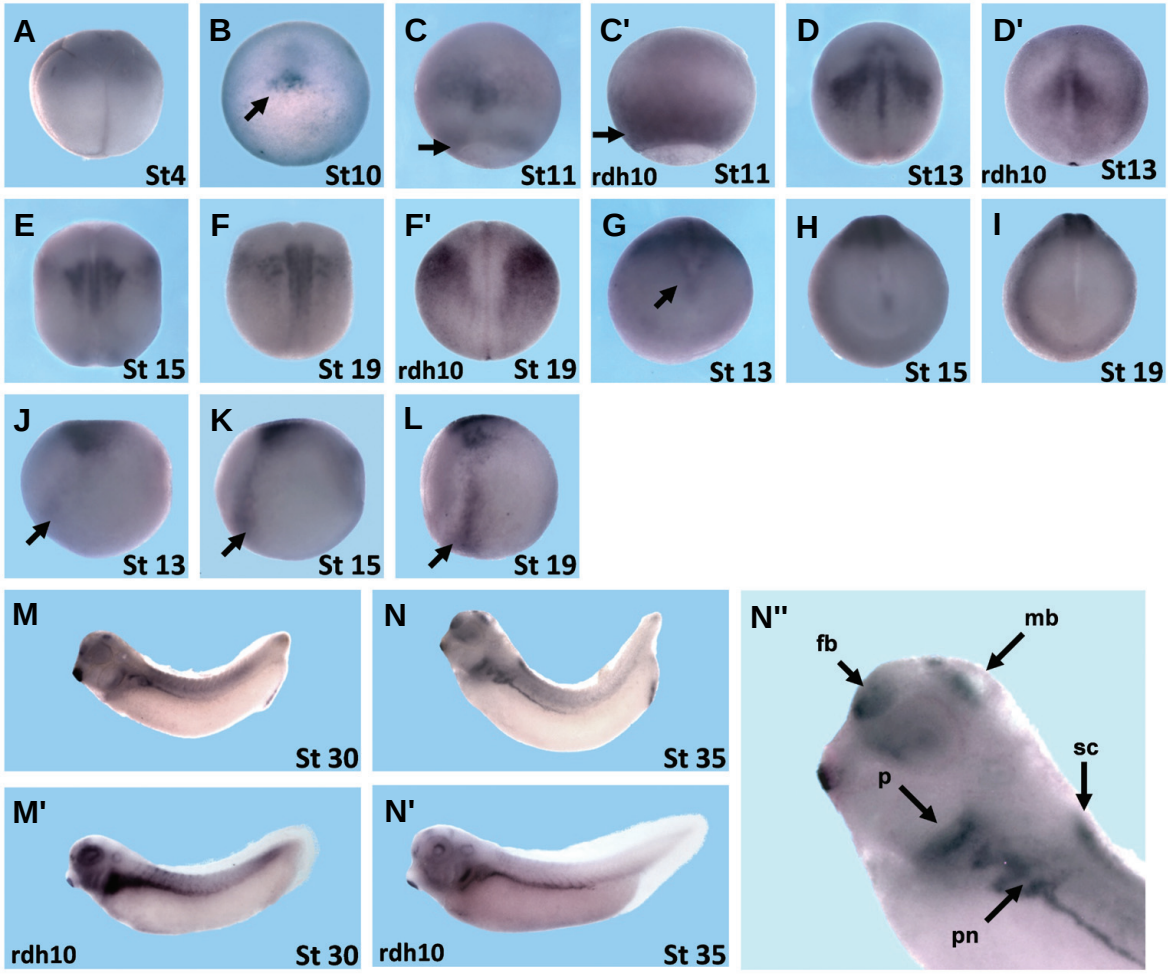

Fig. 2. Spatial expression pattern of *dhrs3*. *Whole mount* in situ *hybridization was employed to examine the spatial expression of* dhrs3 *at different developmental stages as indicated.* **(A)** *Lateral view of a four-cell stage embryo.* **(B-F')** *Dorsal view of early stage embryos.* dhrs3 *transcripts were localized in the dorsal blastopore lip and the ridge of neural fold. The expression of* rdh10 *in stage 11, 13 and 19 are shown in* **(C',D',F')** *for comparison.* **(G-I)** *Frontal view of stage 13, 15, 19 embryos, which showed that* dhrs3 *expression was absent in the head, but present in the posterior edge of the up-folding neural tube.* **(J-L)** *Lateral view of stage 13, 15, 19 embryos, showing* dhrs3 *expression in ventral bilateral regions (arrow).* **(M-N')** *Lateral views of tailbud and tadpole stage embryos, showing the expression pattern of* dhrs3 *and* rdh10. *The anterior region of a stage 35 embryo showing* dhrs3 *expression was magnified in* **(N'')**. dhrs3 *expression was detected in the forebrain, midbrain, pharynx, spinal cord and pronephros (arrow). In lateral views* **(J-N'')**, *anterior is to the left. Abbreviations: fb, forebrain; mb, midbrain; p, pharynx; sc, spinal cord; pn, pronephros.*

notochord (Fig. 2 E,F,J-L). *dhrs3* is also expressed in the dorsal notochord (Fig. 3 D,D'').

In tailbud and tadpole stages, *dhrs3* was expressed in the head region that covered prosencephalon, mesencephalon, lens, and otic vesicle (Fig. 2M, N, 3I, J-L). The expression in otic vesicles was not visible from the exterior, but embryo sections showed that *dhrs3* was expressed in the internal region of otic vesicles (Fig. 3 J-L, upper arrow). *dhrs3* was expressed in primary lens fibers and the lens epithelium (Fig. 3I). Interestingly, *dhrs3* positive cells were only observed in the roof plate and the adjacent region of the mesencephalic ventricle (Fig. 3I). The expression of *dhrs3* in the roof plate overlapped with that of *rdh10*. Abundant transcripts of *rdh10* were also localized in the floor plate (Fig. 3I'), where *dhrs3* was not expressed. Retinoic acid signalling in the neural tube was tightly regulated so that it is mostly localized in the interneuron region (Maden, 2006). The distinct expression of *dhrs3* and *rdh10* suggests that these two genes played a role in such regulation by adjusting the local level of retinoic acid. The expression of *dhrs3* in spinal cord appeared only in a more posterior region, but not in the region immediately adjacent to the hindbrain (Fig. 2N'', 3H). Besides the central nervous system, distinct expression domains were also observed in the pronephros including pronephric tubule and pronephric duct (Fig. 2N). These expression domains were confirmed by embryo section (Fig. 3M). The expression in pronephros regions overlap strongly with that of *rdh10* (Fig. 2 M-N', Fig. 3 M,M'). *dhrs3* expression was detected in the ventral but not dorsal pharynx (Fig. 3J), and expression was also detected in the following gut region, mainly in the medial part (Fig. 3 K,L, middle arrow).

We examined the temporal expression pattern of *dhrs3* by RT-PCR, using primers covering the 3' UTR region of *dhrs3*. The results showed that *dhrs3* is a maternal factor and is weakly expressed before gastrulation. *dhrs3* was up-regulated from stage 10. Peak expression of *dhrs3* was observed in stage 15, which was followed by a decline from stage 19 to stage 25, and an increase after stage 30 (Fig. 4).

Taken together, we have identified *dhrs3* in *Xenopus laevis* and we illustrate the developmental expression pattern of this gene in *Xenopus* embryos. We suggest that dhrs3 is involved in RA metabolism, as some evidence indicates that human Dhrs3 is involved in RA synthesis (Haeseleer *et al.*, 1998). We have also compared and contrasted the expression patterns of *dhrs3* and

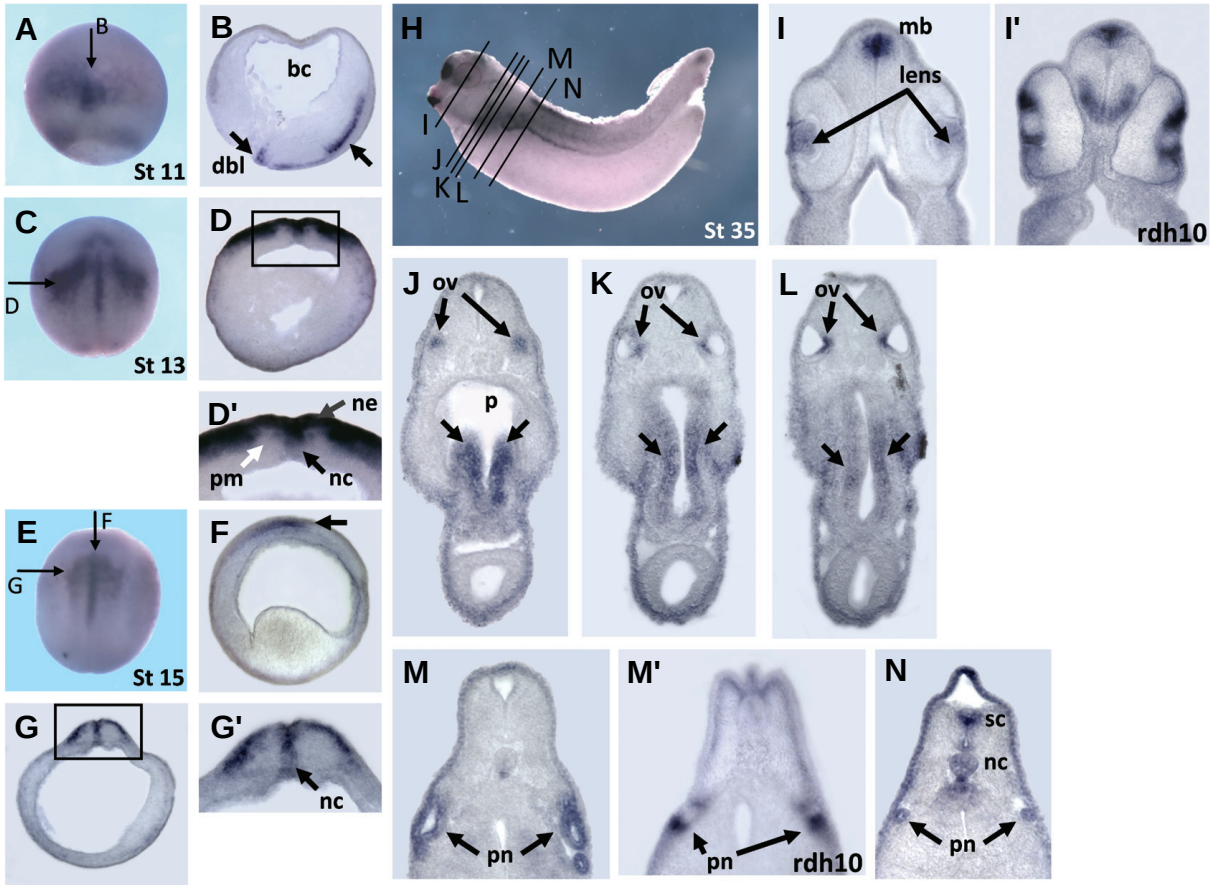

Fig. 3. Transverse and longitudinal sections of embryos showing *dhrs3* expression. *Stage 11 embryo* **(A)** *was sectioned showing the expression domain in dorsal blastopore lip and axial mesoderm* **(B)**. **(C-D')** *Transverse section from a stage 13 embryo* **(C)**, *indicating the expression in notochord and neuroectoderm. The higher magnification of framed region* **(D)** *is shown in* **(D')**. **(E-G')** *Longitudinal and transverse sections from a stage 15 embryo* **(E)** *indicated* dhrs3 *was expressed in notochord and neuroectoderm.* **(H-N)** *Transverse sections of a stage 35 embryo* **(H)** *illustrating the expression in the midbrain, lens, otic vesicles, ventral pharynx, gut, pronephros, dorsal spinal cord and notochord. Expression pattern of* rdh10 *is shown in* **(I',M')**. *Abbreviations: bc, blastocoel; bpl, blastopore lip; ne, neuroepithelium; pm, paraxial mesoderm; nc, notochord; mb, midbrain; ov, otic vesicle; p, pharynx; pn, pronephros; sc, spinal cord.*

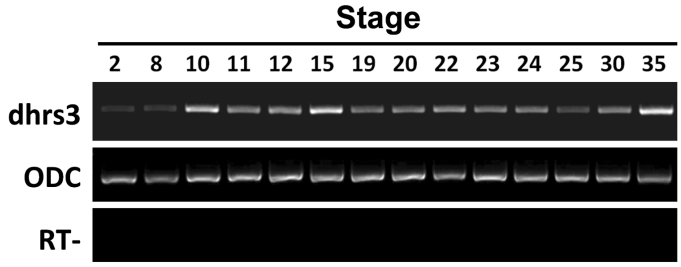

Fig. 4. Temporal expression of *dhrs3*. dhrs3 *expression in embryos of different developmental stages was examined by RT-PCR. A housekeeping gene* ornithine decarboxylase *(*ODC*) was used as the internal standard control. RT-, without reverse transcriptase.*

rdh10 (Strate *et al.*, 2009). *dhrs3* was expressed in the dorsal midline in early neurula stage, where *rdh10* was absent (Fig. 2 D,D'). *dhrs3* was expressed in the dorsal neural fold region in late neurula stages, and *rdh10* was expressed in the bilateral region outside of dorsal neural fold (Fig. 2 F,F'). *dhrs3* was only expressed in the roof plate in midbrain in tailbud stage, while *rdh10* was also expressed in the floor plate, and the adjacent region in ventral neural tube (Fig. 3 I,I'). On the other hand, both *dhrs3* and *rdh10* expression can be found in the circumblastoporal ring in early gastrulation (Fig. 2 C,C') and the pronephros region in tailbud stages (Fig. 2 N,N', 3 M,M'). These observations highlight the complexity of retinoic acid regulation. In order to fully elucidate the function of *dhrs3,* we attempt to carry out an enzymatic analysis to determine the substrate and co-factor specificities of the protein. The functional role of this enzyme in embryonic development will also be studied by gene knock-down and overexpression experiments. The action of *dhrs3* on midbrain development was particularly interesting since it was localized only in the roof plate, while *rdh10* was also localized in the floor plate. The relationship between these two enzymes should provide valuable information to the action of retinoid signaling on midbrain development. Further investigation of *dhrs3* function during embryonic development will expand our understanding of the retinoic acid signaling pathway and shed light on the mechanism by which retinoic acid regulates embryonic development.

Materials and Methods

Phylogenetic analysis

BLAST searches were performed on the NCBI website, using the blastx program with the non-redundant protein sequence database. Protein sequence alignment was done with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The dendrogram was constructed by using MEGA 4.0.2 (http://www.megasoftware.net) with the neighbor-joining method and p-distance model.

Whole-mount in situ *hybridization and vibratome sectioning*

Whole-mount *in situ* hybridization was performed according to the standard protocol (Harland, 1991) except that BM purple was used for developing signals. Vibratome sections were prepared as described elsewhere (Hollemann *et al.*, 1996). Briefly, after whole-mount *in situ* hybridization the embryos were embedded in gloop solution (5 g/L gelatin, 380 g/L chick egg albumin and 200 g/L sucrose in 0.1 M phosphate buffer, pH 7.4) mixed with 1/10 volume 25 % (v/v) glutaraldehyde, and sectioned at a thickness of 50 μm.

RNA extraction and RT-PCR

Total RNA was extracted from staged *Xenopus* embryos using Trizol (Invitrogen), precipitated with isopropanol, and purified with RNeasy (Qiagen) after DNase I treatment. cDNA was synthesized by using Hexamer random oligonucleotides (Roche) and Superscript III (Invitrogen) following the manufacturer's instruction. The primer for *dhrs3* was designed by Primer 3. *ODC* was employed as loading control. The primer sequences are listed as below.

dhrs3 Fw: TTCCTCAATCAGCACTTGAA
dhrs3 Re: AGGATGAATGACCTGGAAGA
ODC Fw: CAGCTAGCTGTGGTGTGG
ODC Re: CAACATGGAAACTCACACC

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