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Retinoic Acid Mediated Gene Expression in Transgenic Reporter Zebrafish

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Retinoic acid-mediated gene activation is important for normal vertebrate development. The size and nature of retinoic acid make it difficult to identify the precise cellular location of this signaling molecule throughout an embryo. Additionally, retinoic acid (RA) signaling is regulated by a complex combination of receptors, coactivators, and antagonizing proteins. Thus, in order to integrate these signals and identify regions within a whole developing embryo where cells can respond transcriptionally to retinoic acid, we have used a reporter transgenic approach. We have generated several stable lines of transgenic zebrafish which use retinoic acid response elements to drive fluorescent protein expression. In these zebrafish lines, transgene expression is localized to regions of the neural tube, retina, notochord, somites, heart, pronephric ducts, branchial arches, and jaw muscles in embryos and larvae. Transgene expression can be induced in additional regions of the neural tube and retina as well as the immature notochord, hatching gland, enveloping cell layer, and fin by exposing embryos to retinoic acid. Treatment with retinoic acid synthase inhibitors, citral and diethylaminobenzaldehyde (DEAB), during neurulation, greatly reduces transgene expression. DEAB treatment of embryos at gastrulation phenocopies the embryonic effects of vitamin A deprivation or targeted disruption of the RA synthase retinaldehyde dehydrogenase-2 in other vertebrates. Together these data suggest that the reporter expression we see in zebrafish is dependent upon conserved vertebrate pathways of RA synthesis.

Key Words: **retinoic acid; RARE; zebrafish; transgenic; neural tube; retina; heart; and diethylaminobenzaldehyde.**

INTRODUCTION

Nutritional studies in vertebrates have implicated retinoic acid (RA), a metabolite of vitamin A, in the normal development of the central nervous system, eye, heart, limb, ear, and kidney (Dowling and Wald, 1960; Kalter and Warkany, 1959; Zile, 1998). The cloning of transcription factors that are activated by RA suggests that it mediates its developmental effects by altering gene expression (Giguere, 1994; Gudas, 1994; Linney and LaMantia, 1994; Underhill *et al.*, 1995). The importance of the regulated expression of RA-responsive genes is confirmed by the sensitivity of nearly all embryonic organ systems to excessive doses of RA at critical times (Shenefelt, 1972).

The molecular biology of retinoic acid-mediated gene activation has been examined extensively using cultured cell lines to define and characterize the proteins involved. There are two multigene families of nuclear hormone receptors that respond to retinoic acid, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR) (Glass, 1994; Green and Chambon, 1988; Leid *et al.*, 1992). Retinoid receptors function most commonly as RAR/RXR heterodimers by binding retinoic acid response elements (RARE) in genes and recruiting coactivator molecules to enhance transcription when RA is available (Chambon, 1994; Pfahl, 1994; Xu *et al.*, 1999). Retinoid receptor activity may be limited by RA and receptor availability and by competition from other steroid hormone receptors that also dimerize with RXR, as well as by negative regulators of RARs and RAREs (Glass, 1994; Pfahl, 1994). Thus, the complex cast of players makes defining the location and timing of retinoic acid-mediated gene activation during vertebrate embryogenesis difficult. Instead it is informative to integrate the influence of all potential RA signaling effectors on RAREs in the context of the whole animal.

Previously this laboratory and others have used a RARE-driven reporter transgene approach to study RA-mediated gene activation in mice (Balkan *et al.*, 1992; Mendelsohn *et al.*, 1991; Reynolds *et al.*, 1991; Rossant *et al.*, 1991). Because mice develop *in utero* and β-galactosidase visual-

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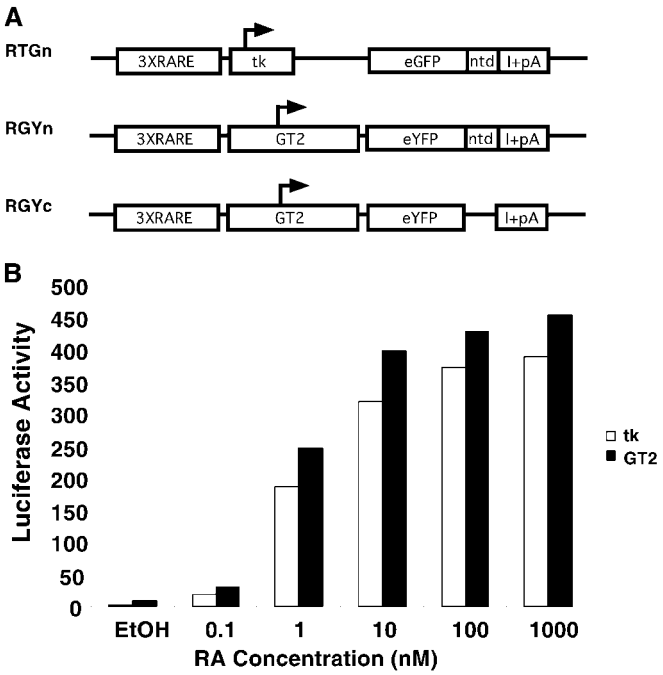

FIG. 1. Characterization of RARE-driven reporter constructs. (A) Schematic depiction of three RA-responsive reporter transgenes. All constructs contain three copies of the RARE from the mouse *rarβ* gene (3XRARE) and an SV40 polyadenylation signal and small t intron (I+pA). In RTGn the RAREs are upstream of a viral thymidine kinase basal promoter (tk), and the reporter is an enhanced green fluorescent protein gene (eGFP) with a nuclear translocation domain (ntd). The constructs RGYn and RGYc each contain the zebrafish GATA-2 basal promoter (GT2) and an enhanced yellow fluorescent protein (eYFP). RGYn has a ntd fused to the reporter protein, while RGYc lacks a ntd and localizes to the cytoplasm. (B) Comparison of the responsiveness to RA of RARE-tk (white bars) and RARE-GT2 (black bars) regulatory sequences in zebrafish liver cell line (ZFL). Both regulatory sequences were cloned upstream of firefly luciferase and transfected into ZFL cells along with an expression plasmid for zebrafish retinoic acid receptor-α2.B. Luciferase activity was used to quantitate the transcriptional responsiveness of RARE-tk and RARE-GT2 to RA. When RA was added, both constructs showed a dose-dependent increase in luciferase activity. The luciferase activity of RARE-GT2 was slightly higher than that of RARE-tk at all RA concentrations.

ization requires fixation and permeabilization, it is possible to use them to observe reporter expression only at static time points. In this paper we describe similar lines of transgenic zebrafish that contain RAREs driving green or yellow fluorescent protein genes. The optical clarity, rapid development, and *ex utero* culturing of zebrafish, combined with a fluorescent signal, allows reporter analysis in live zebrafish embryos (Amsterdam *et al.*, 1995; Peters *et al.*, 1995). Presumably, throughout the embryo cells that contain the requisite assortment of positive RARE regulators and lack competitors or repressors will express fluorescent protein.

For this study we have successfully made transgenic zebrafish using isolated *cis*-regulatory elements coupled to a basal promoter to drive fluorescent protein reporter expression. We have also compared two basal promoters for their ability to mediate a reporter response in zebrafish. These lines display transgene expression that is both RA dependent and inducible *in vivo*. We also present an aldehyde dehydrogenase inhibitor, diethylaminobenzaldehyde, that abolishes transgene expression and phenocopies RA deprivation observed in several vertebrate species. These data provide regional information about where active RA signaling occurs during embryonic and larval stages and demonstrate the morphological effects caused by loss of RA signaling in zebrafish. These lines should be useful for studying regional retinoic acid signaling during embryonic development, particularly in the context of available zebrafish mutants. Additionally they will be useful for targeted genetic screens to identify additional mutations in pathways upstream of RA. Finally, these lines may be useful as environmental biosensors for toxicants that alter retinoid signaling.

MATERIALS AND METHODS

Recombinant DNA Constructs

Two RA-responsive regulatory elements were made. The first, RARE-tk (tk is the thymidine kinase basal promoter), was described in Balkan *et al.* (1992). The second, RARE-GT2, used the same 3XRARE (three copies of the retinoic acid response element) and the basal promoter from the zebrafish GATA-2 gene (GT2), P5-GM2, described in Meng *et al.* (1997). For testing the RA inducibility of the regulatory elements in zebrafish cell lines, we used a RARE-tk-firefly luciferase construct (RTF) (Balkan *et al.*, 1992) or added firefly luciferase reporter gene (de Wet *et al.*, 1987)

TABLE 1

Comparison of Transgene Expression Patterns in Different RARE Reporter Transgenic Zebrafish Lines

	Transgenic lines			
Expression	RTGn3	RTGn32	RGYn2	RGYc1
Notochord	−	−	+	+
Neural tube	+++	++	++++	+++
Ventral retina	++	+	+++	++
Dorsal retina	+	−	++	+
Pronephric ducts	−	−	+	+
Somites	−	−	+	+
Heart	−	−	++	+/−
Branchial arches	−	−	+	−
Forebrain	−	−	+	−
Jaw muscles	−	−	+	−

Note. Regions are listed according to order of expression onset. Levels of expression are indicated by "+" and absence of expression by "−".

to RARE-GT2 (RGF). For transgenic zebrafish, the lacZ reporter gene of 3XRARE-TKpr-β-gal (Balkan *et al.*, 1992) was replaced with an enhanced green fluorescent protein (eGFP) gene with a nuclear translocation domain (ntd) (Linney *et al.*, 1999). The resulting plasmid is referred to as RTGn. We also coupled the RARE-GT2 element to a GFP mutant with a yellow-shifted emission spectra (YFP; Topaz-Gold from Packard Instrument Company) with or without a ntd to test the difference in signal between nuclear and cytoplasmic localization (RGYn and RGYc, respectively). For transfection assays, we also made an expression construct for zRARα2.B (GenBank Accession No. L03399), driven by *Xenopus* Ef1α promoter and enhancer (Johnson and Krieg, 1995).

Transient Transfections

The RA inducibility of the RARE-tk and RARE-GT2 regulatory sequences was quantified by transient transfection of RTF or RGF into zebrafish liver cells (ZFL) cultured at 28.5°C without CO_2 in LDF medium (Ghosh *et al.*, 1994), supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 0.5 mg/ml gentamicin. For transfections, 2×10^6 cells were plated per 60-mm dish and incubated at 28.5°C for 15–30 min, and then charcoal-dextran stripped serum was added to 5%. Cells were transfected after 24 h. Per transfection, 5 μg of RTF or RGF, 1 μg of SV40-*Renilla* luciferase plasmid (Promega), 1 μg Ef1α/RARα2.B, 8 μl Lipofectamine Plus reagent, and 12 μl Lipofectamine (Gibco BRL) were used following the manufacturer's protocols. After 3–5 h, transfections were washed with LDF containing 5% charcoal-dextran stripped serum and 2 mM L-glutamine. At this time either all-*trans*-RA dissolved in ethanol or ethanol only was added to the medium. (The final ethanol concentration was always 0.1%.) After 24 h, plates were washed with cold PBS and cells were scraped from plates in a volume of 400 μl PBS. Cells were subjected to two rounds of freezing in liquid nitrogen and thawing at 37°C. The Dual Luciferase Reporter Assay System (Promega) was used to quantitate both firefly and *Renilla* luciferase activity of 20-μl aliquots from cell extracts. Transfections were performed in duplicate.

Embryo Injections and Screening

DNA was prepared and injected into one-cell embryos at 100 ng/μl as previously described (Linney *et al.*, 1999). After 24 h postfertilization, injected embryos were screened by fluorescence microscopy for expression in the anterior trunk as described by Marsh-Armstrong *et al.* (1995). Expressing embryos were cultured and grown to adulthood as described in Westerfield (1993). The F1 offspring of injection-derived fish were screened for germ-line transmission of the transgene by PCR analysis 5–7 days postfertilization as described in Linney *et al.* (1999).

Transgenic Embryo Analysis

Founder transgene-expressing lines were bred to homozygosity for both the transgene and the *albino* (*alb*) mutation (Streisinger *et al.*, 1986). Embryos for photographs were derived from male transgenics crossed with nontransgenic *alb/alb* females and staged as described by Kimmel *et al.* (1995). Photographs were made using a Zeiss Axiovert microscope equipped with Chroma filters and a Princeton Instruments cooled-CCD camera. Images were captured and pseudo-colored using IPLab Spectrum software. Confocal sections were acquired using an Atto CARV, Nipkow disk confocal scanner. Background autofluorescence was distinguished from fluorescent protein signal by comparing transgenic embryos to nontransgenic embryos at identical stages and exposure times. In order to provide visual landmarks, bright-field/fluorescent overlay images were created using Adobe PhotoShop software.

RA, Citral, and Diethylaminobenzaldehyde (DEAB) Treatments

All-*trans*-RA stocks were made in ethanol at 10 mM concentration. Citral and DEAB stocks were made in DMSO at 10 mM. Embryos were cultured in RA, citral, or DEAB-treated egg water (Westerfield, 1993) and incubated at 28.5°C. Ethanol and DMSO were always kept to 0.1% final concentration in egg water and controls were cultured in 0.1% ethanol or DMSO.

RESULTS

Promoter Construct Characterization

To make retinoic acid-responsive transgenic zebrafish we used the RARE-tk regulatory sequence that was engineered originally to make transgenic mice (Fig. 1A) (Balkan *et al.*, 1992). It contains three copies of the retinoic acid response element from the mouse *rarβ* gene and the thymidine kinase basal promoter from herpes simplex virus. Because cell culture data suggested that viral basal promoters like tk were less active in fish cell lines than in mammalian cell lines (Sharps *et al.*, 1992), we also made transgenic zebrafish lines using the same 3XRARE coupled to a basal promoter from the zebrafish GATA-2 gene (Meng *et al.*, 1997).

The RA inducibility of the RARE-containing regulatory sequences in ZFL was tested, using a firefly luciferase reporter gene. In transient transfections, RA induced a dose-dependent increase in luciferase activity from both regulatory sequences. Strong induction began at 1 nM RA. As predicted, there was a slightly higher induction from the RARE-GT2 construct compared to the RARE-tk construct (Fig. 1B).

Generation and Isolation of Transgenic Lines

Having shown that both regulatory elements are RA-inducible in a zebrafish cell line, we made three different fluorescent protein reporter constructs for transgenesis. All three used the same 3XRARE and had an SV40 polyadenylation signal and intron downstream of the reporter. One contained the RARE-tk and an enhanced GFP with a ntd. It is referred to as RARE-tk-GFPntd (RTGn). The other two used the RARE-GT2 basal promoter and YFP either with or without the ntd. These transgenes were called RARE-GT2-YFPntd (RGYn) and RARE-GT2-YFPcytoplasmic (RGYc) for nuclear and cytoplasmic localization, respectively.

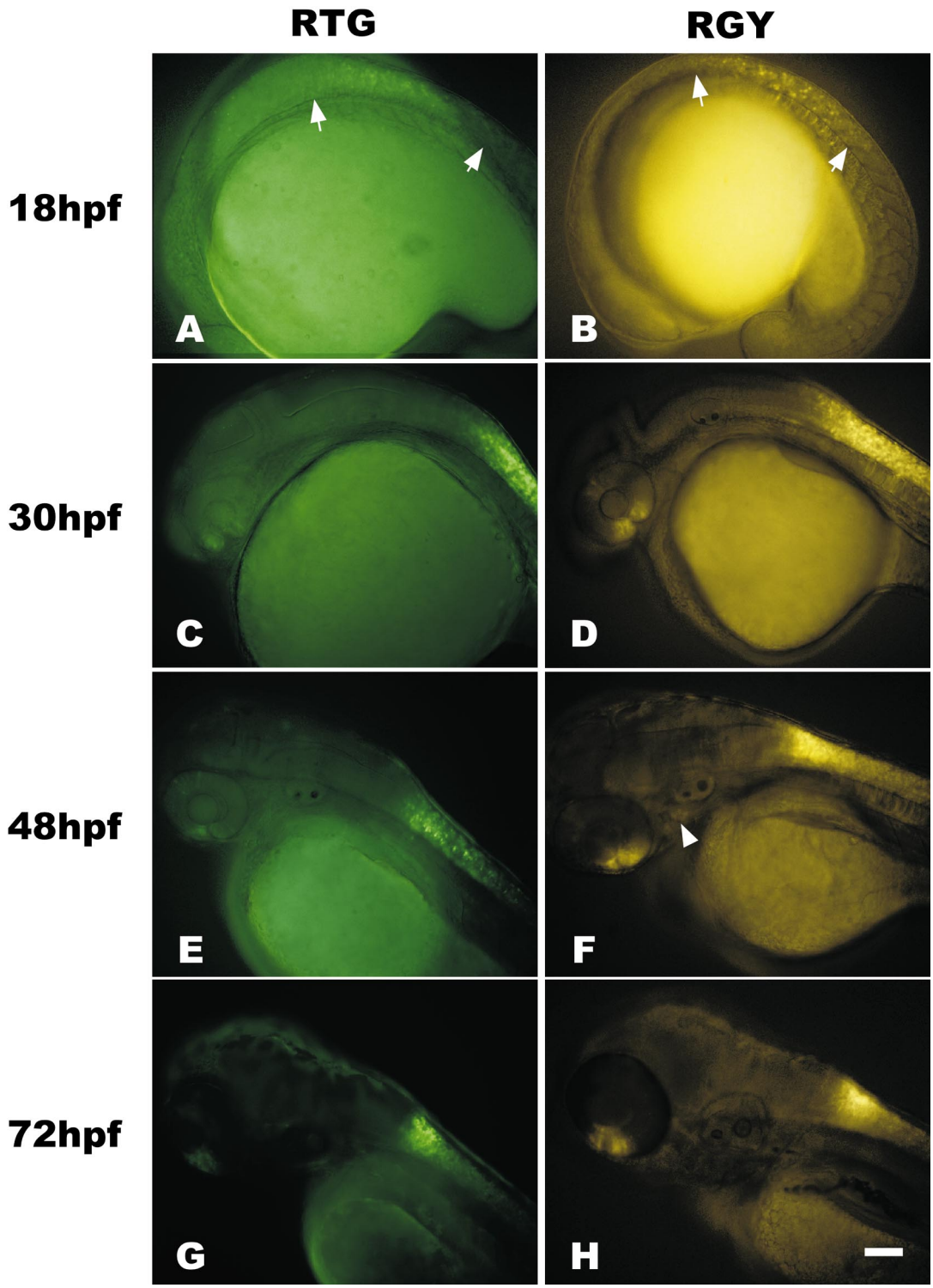

FIG. 2. Time course comparison of fluorescent protein expression in transgenic lines that utilize different minimal promoters and fluorescent reporter genes. (A and B) Eighteen-somite stage (18 hpf) RTGn3 (A) and RGYn2 (B) embryos have neural tube expression, extending from just anterior to somite 1 to about somite 8 (arrows). RGY lines have additional expression in the underlying notochord. (C and D) At 30 hpf neural tube expression extends anteriorly into the caudal hindbrain and is initiated weakly in the dorsal retina and strongly in the ventral retina. (E and F) By 48 hpf, expression continues to strengthen in the retina and neural tube. Expression in the neural tube

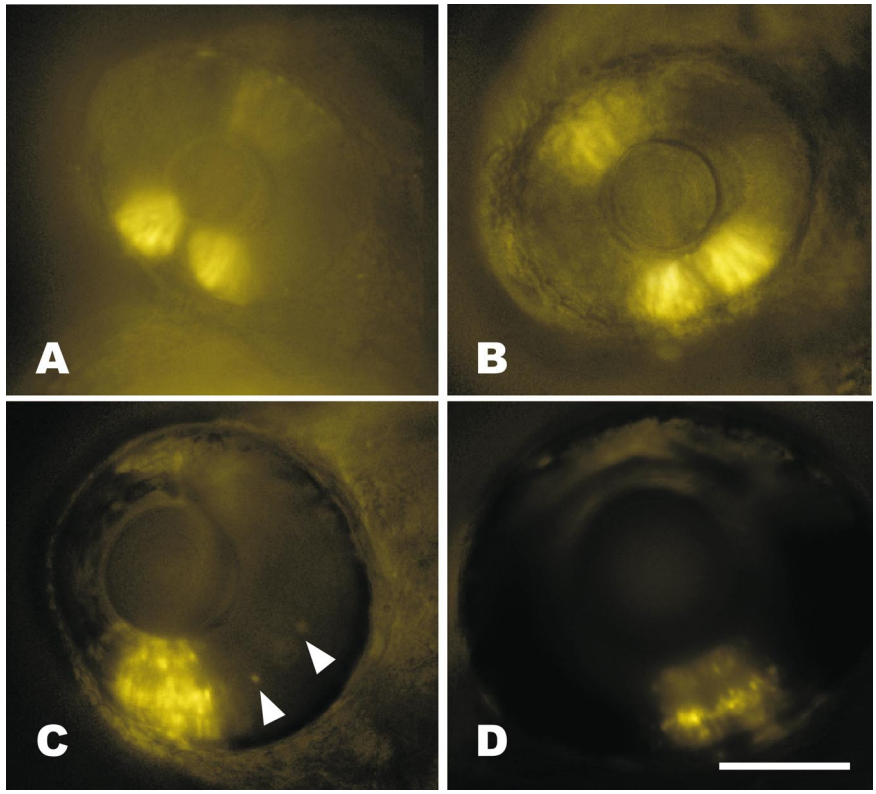

FIG. 3. Time course of retinal expression in RGYn2. (A) Expression in the ventral retina patch is broader and stronger at 30 hpf than dorsal. Exposure time was 1 s. (B) Expression continues in similar dorsal and ventral patches by 48 hpf, but is much stronger. Exposure time was 0.25 s. (C) By 72 hpf the neural retina has differentiated into three distinct layers, all of which have transgene expression in RGY lines. A few expressing cells outside the ventral patch are visible in the inner nuclear layer (arrowheads). The dorsal retina is out of focus, but continues weak expression at this time. Exposure time was 0.1 s. (D) The layered expression continues in the ventral retina at 5 days postfertilization and occurs primarily in the inner and outer nuclear layers of the dorsal retina (out of focus). Exposure time was 0.5 s. Bar, 100 μm. All fish are oriented with rostral left and dorsal up.

From the three constructs we derived four transgenic lines, summarized in Table 1. All founders were chimeric with transgene transmission in 9–39% of the F1 generation. Selection and breeding of transgene-positive F1 embryos produced adults that transmitted the transgene to their offspring in a Mendelian manner. These lines have been bred to homozygosity for the *albino* mutation (Streisinger *et al.*, 1986) to aid in fluorescent signal detection. We have bred the RGYn2 line to the F7 generation without loss or reduction of the transgene expression. Male transgenics were bred to derive embryos for all photographs because there is a maternal effect (i.e., transgene expression in oocytes prior to fertilization that carries over into the embryo).

All lines express fluorescent protein in the neural tube and retina, although the levels of expression vary among the different lines. The expression patterns of both lines derived from RARE-GT2 transgenes are more complex than that of the RARE-tk transgene (Table 1), even though levels of expression in the neural tube and retina are roughly equivalent in RGYc1 and RTGn3. For this reason and because it had the strongest signal we will focus on RGYn2 and note differences with the other lines where they occur.

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of RTG lines is less dense than in RGY lines. RGY also has weak expression in branchial arches (arrowhead). (G and H) By 72 hpf neural tube expression has retreated from the hindbrain and tail, leaving a region of strong expression in the spinal cord adjacent to the pectoral fins. Embryos and larvae are oriented with rostral left and dorsal up and photographs are bright-field/fluorescent overlays. Exposure times for fluorescent images were 5 s for A–D, 1.5 s for E and F, and 0.5 s for G and H. Bar, 100 μm. RTG embryos were larger than RGY embryos.

Expression Pattern during Embryonic and Early Larval Development

We followed the transgene expression throughout embryogenesis and into larval development. The earliest expression we see is at the 13-somite (S) stage in notochord cells of RYGn2 embryos (data not shown). All lines have detectable expression in the neural tube by 18 S. The expression begins just anterior to the first somite. Moving posteriorly it strengthens, then fades in the region of somite 8 (Figs. 2A and 2B and corresponding bright-field micrographs, not shown). RARE-GT2 lines both have expression in notochord cells, underlying the cells of the neural tube that express the transgene (Figs. 2B and 3A). The long exposure times required to capture these signals result in the detection of high levels of background autofluorescence, particularly in the yolk. Use of a different filter set to detect YFP reduces the autofluorescence, making the transgene signal more distinct from background (compare transgenic embryos in Figs. 2A and 2B to nontransgenic embryos in Figs. 4B and 4C).

Between 18 and 30 hpf the neural tube expression strengthens and extends anteriorly into the caudal hindbrain (Figs. 2A–2D and 4F) and posteriorly toward the tail (Figs. 5A and 5B). By 30 hpf the neural tube expression extends into the posterior hindbrain in a wedge shape such that the dorsal expression extends farther rostrally than the ventral. Moving posteriorly, expression spans the dorsal–ventral extent of the neural tube (Figs. 2C and 2D). Strong expression is retained posteriorly in the neural tube until the level of the yolk extension where it trails off such that it is expressed diffusely at the level of the tail (Figs. 5A and 5B).

Around 24–30 hpf expression appears in the retina (Figs. 2C, 2D, and 3A). The undifferentiated cells of the neural retina expressing the transgene have columnar-shaped nuclei that span the distance from the outer edge of the retina, inward to the lens (Fig. 3A). Transgene expression in the retina follows the same temporal sequence as differentiating neural epithelium by starting in the ventronasal patch (Schmitt and Dowling, 1996; Schmitt and Dowling, 1999), so it often appears brighter in the nasal than in the temporal ventral retina between 24 and 48 hpf. Starting after the onset of ventral retina expression, there is also a smaller patch of weaker expression in the central dorsal retina in all lines except RTGn32 (Figs. 2C–2E, 3A, and 3B).

By 30 hpf both RGY lines display additional regions of expression in the pronephric duct and in muscle cells at the surface of the somites (Figs. 4D and 4E). In RGYn2 it is possible to detect one or two fluorescent cells in the beating heart tube (data not shown). At this time we can detect lingering expression in a subset of cells in the notochord in the anterior trunk (Fig. 4D).

By 48 hpf fluorescent protein expression strengthens in the neural tube and retina of all lines and in the heart of RGYn2 (Figs. 2E, 2F, and 3B and data not shown). There also appears to be weak expression in the branchial arches of RGYn2 (Fig. 2F). At this time expression is waning in the notochord and pronephric duct, but unchanged in the somites in both RGY lines (data not shown). In the neural tube the expression appears to be retreating from the caudal hindbrain and tail, relative to the strengthening signal at the anterior spinal cord. The spinal cord expression is strongest just posterior to the hindbrain ventricle to about the third or fourth somite and then begins to diminish posteriorly (Figs. 2E and 2F). In the anterior trunk fluorescent axons of neurons project ventrally (Fig. 4G).

At 72 hpf the neural tube expression is stronger, but begins in the spinal cord just caudal to the hindbrain ventricle and is limited to the region adjacent to the third and fourth myotomes (Figs. 2G, 2H, and 4I). The ventral retina expression is also stronger while weak dorsal expression continues (Fig. 3C). There also appears to be a ring of expressing nuclei in RGY lines all around the retina in the inner nuclear layer (Figs. 3C and 4H). In RGY lines we can see expression in all three layers of the differentiated retina (Figs. 3C and 3D). In contrast in RTG lines, the ventral retina expression is limited to rounded nuclei in the ganglion cell layer and inner nuclear layer to the exclusion of the outer nuclear layer. By 96 hpf these lines will begin to express GFP in columnar-shaped photoreceptor cells of the outer nuclear layer (data not shown).

By 5 days postfertilization, larvae have expression in the retina, dispersed cells in the dorsal forebrain, ventral jaw muscles, and heart (Figs. 3D and 4J–4L). The heart expression is in a dispersed population of cells on the surface of both the atrium and the ventricle, but excluded from the outflow tract (Fig. 4L). The expression in the anterior spinal cord is limited to cells on the dorsal margin (data not shown). By 3 weeks postfertilization the anterior dorsal spinal cord, heart and ventral retina continue to express the transgene, but the density of expressing cells is reduced. During this time, pelvic fin development has occurred, but a region of spinal cord expression comparable to that seen at the level of the pectoral fins does not develop at the level of the pelvic fins.

RA Inducibility and Dependence of Transgene

We tested the RA inducibility of the transgene *in vivo*. Treatment of late neurula stage embryos (18 hpf) with 1 μM RA induced expression throughout the nervous system, pronephric ducts, caudal notochord, neural retinal epithelia, enveloping cell layer, and hatching gland by 24 hpf (Figs. 5A and 5D). Treatment at the same stage with 10-fold lower concentration of RA (0.1 μM) demonstrates the variable response of the rostral neural tube. For example, the rostral forebrain, caudal midbrain, and alternate rhombomeres of the hindbrain are less responsive than other regions to the induction. Treatment of zebrafish blastulae with 0.01 μM RA induces transgene expression in the fin ectoderm, enveloping cell layer, and pronephric duct by 40 hpf (data not shown). We also treated embryos with retinoic acid at gastrulation to induce teratogenic defects in the brain

(Holder and Hill, 1991). Transgene expression is expanded into regions that are morphologically altered by teratogenesis, e.g., the anterior nervous system and retina (data not shown). Treatment after 22 hpf only slightly induces the transgene expression.

Both basal promoters had been previously tested without RAREs in reporter transgenics and showed negligible levels of activity in zebrafish embryos (Marsh-Armstrong *et al.*, 1995; Meng *et al.*, 1997). Nonetheless, we tested the RA specificity of the transgenic expression patterns by culturing embryos in RA synthesis inhibitors, citral and DEAB, at 18 hpf. Compared to an untreated transgenic embryo, citral or DEAB cause a significant reduction in the fluorescent protein expression by 24 hpf (Fig. 5B and data not shown). The signal remains at only low levels in the neural tube and at high levels in large cells, possibly primary neurons (data not shown).

DEAB Teratogenizes Embryos

We tested the ability of DEAB to teratogenize zebrafish embryos during gastrulation. Embryos treated with 10 μM DEAB prior to 65% epiboly lack fin buds, touch response, functioning heart tube, circulation, and transgene expression (Figs. 6A and 6C). They have reduced hindbrains, neural retinae, otic vesicles, and faces (Figs. 6C–6E). In 20% of treated embryos the choroid fissure of the ventral retina is absent (data not shown). Additionally, embryos display mild cardiac edema, slightly rounded somites, shortened and curved body axes, enlarged blood islands, and laterally distended yolks (Figs. 6C and 6D).

Using *in situ* probes for hindbrain markers, hoxb1-α and krox-20, we determined that rhombomeres 3 and 4 are present, but rhombomere 5 (r5) expression of krox-20 is variably altered. DEAB treatment prior to 65% epiboly can cause complete, partial, or no loss of krox-20 expression in r5 (data not shown).

DISCUSSION

We have generated stable transgenic zebrafish lines carrying RA-inducible reporter genes in order to localize regions of RA-mediated gene activation during zebrafish development. In this paper we describe four independent transgenic lines that express fluorescent proteins under the regulation of retinoic acid response elements. Embryos from all lines exhibit reporter expression in the posterior hindbrain, anterior spinal cord, and retina. Lines made using a zebrafish (GT2) rather than viral (tk) basal promoter have additional expression in the notochord, pronephric ducts, somites, branchial arches, heart tube, ventral jaws, and forebrain. The transgene expression is both RA dependent and RA inducible. We also present an aldehyde dehydrogenase inhibitor that eliminates transgene expression and appears to phenocopy malformations associated with RA deprivation.

The accuracy with which these lines report RA-mediated gene activation in the neural tube and retina is supported by published analyses of RA and RA synthases in zebrafish embryos. For example, at 48 hpf both RA and RA synthases appear at highest levels in the anterior trunk region associated with the first three somites, dropping progressively toward the posterior (Marsh-Armstrong *et al.*, 1995). The transgenic fish described here have strong expression in the neural tube, which by 72 hpf has retreated from the caudal hindbrain and level of the yolk extension to the anterior spinal cord, adjacent to the first three somites. Likewise, the different levels of transgene expression in the dorsal and ventral retinae are supported by a similar analysis of the RA-synthesizing activity in retina. Marsh-Armstrong *et al.* (1994) showed that there are distinct aldehyde dehydrogenases in these two retinal regions and that the activity of the ventral enzyme appears earlier and is stronger than that of the dorsal retina. As predicted from that study, we detect both earlier onset and stronger transgene expression in the ventral retina compared to the dorsal.

A number of roles for RA in the ventral retina have been suggested by studies in zebrafish. For example, exogenously applied RA has been shown to induce differentiation of ventral retina at embryonic stages (Hyatt *et al.*, 1992, 1996b; Li *et al.*, 2000) and RA-synthase inhibitor studies suggest that RA is required for normal ventral patterning (Marsh-Armstrong *et al.*, 1994). Additionally, experiments in cultured cells and embryos suggest that RA may drive differentiation of rod photoreceptors, which are particularly abundant in the zebrafish ventral retina (Hyatt *et al.*, 1996a; Kelley *et al.*, 1994, 1995; Kljavin, 1987). During larval development the high level of transgene expression in the ventral retina, including rod-shaped cells in the outer nuclear layer by 72 hpf, correlates with the timing of photoreceptor differentiation (Kljavin, 1987; Raymond *et al.*, 1995; Schmitt and Dowling, 1996).

The polarized transgene expression in the heart tube, neural tube, and retina appears to be required for normal development of these organs. Exposure of zebrafish embryos to teratogenic doses of RA causes malformations of the anterior heart tube, rostral neural tube, and retina (Hill *et al.*, 1995; Holder and Hill, 1991; Hyatt *et al.*, 1992, 1996b; Stainier and Fishman, 1992). These are all regions in which transgene expression is absent but inducible by exogenously applied RA. This suggests that normal development of these regions proceeds only in the absence of RA signaling.

The importance of RA-mediated signaling in the regions of transgene expression has been illustrated by the defects seen in the posterior heart tube, posterior hindbrain, ventral retina, and pronephric ducts in vertebrate models of RA deficiency generated by dietary elimination of vitamin A or mutation of an RA-synthesizing enzyme, retinaldehyde dehydrogenase-2 (RALDH2), or retinoid receptors (Dickman *et al.*, 1997; Gale *et al.*, 1999; Ghatpande *et al.*, 2000; Kastner *et al.*, 1995; Kostetskii *et al.*, 1999; Maden *et al.*, 1996, 1997; Niederreither *et al.*, 1999, 2000; White *et al.*,

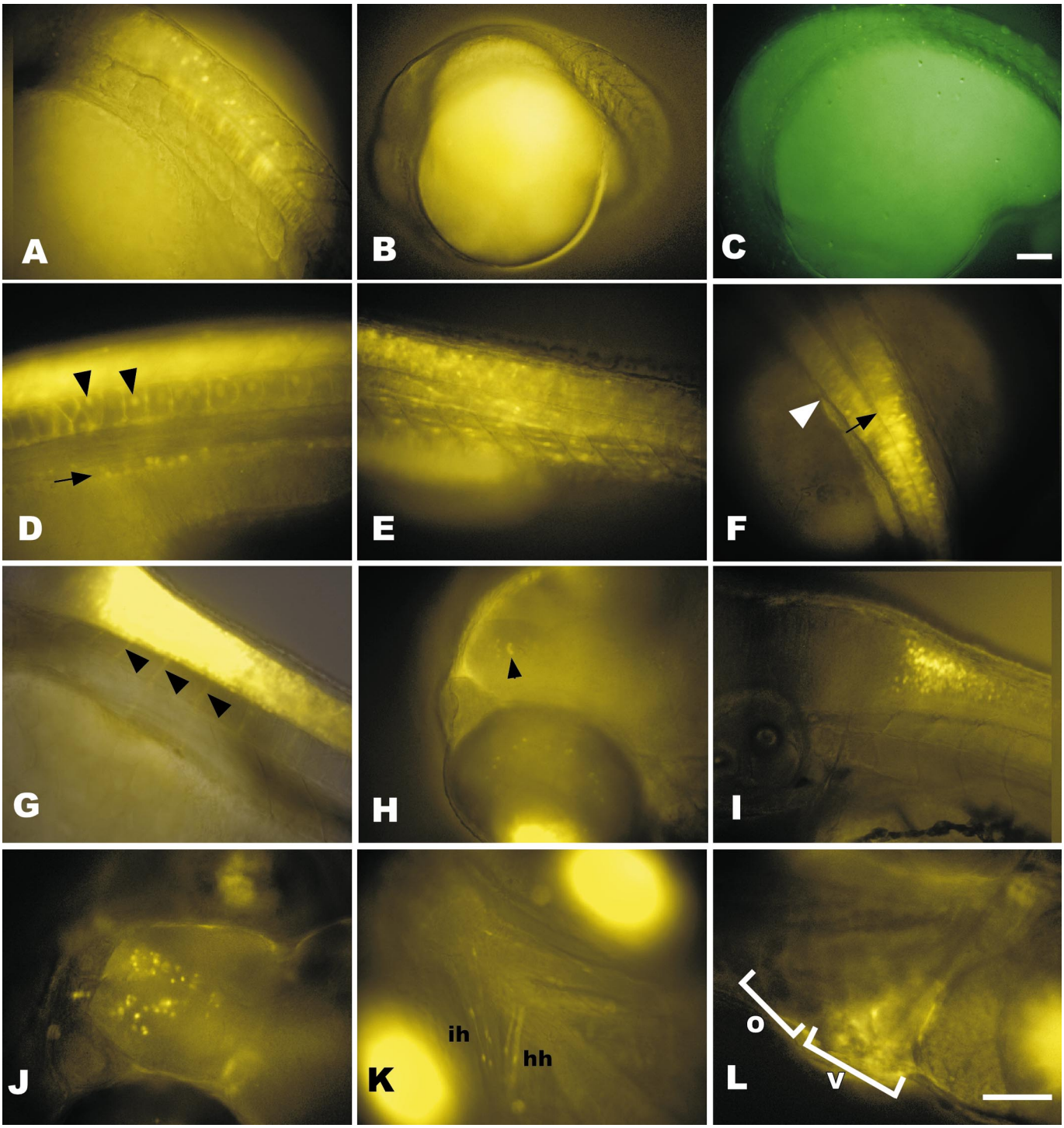

FIG. 4. RGY expression patterns are more complex than RTG expression. (A) Notochord and neural tube expression in RGY at 18 hpf is in a subset of cells in each tissue. (B and C) Fluorescent images of nontransgenic embryos at 18 hpf to demonstrate typical autofluorescence when using long exposure times required to capture early transgene expression shown in Figs. 2A and 2B with yellow (B) or green (C) filter sets. Exposure times were 5 s. (D–F) Transgene expression in 30 hpf RGY embryos in the pronephric ducts (arrow) and vacuolated notochord (arrowheads) at the level of the junction between yolk and yolk extension (D). Expression also occurs in the somites (E). In the neural tube (shown in dorsal view in F) expression extends rostrally into the caudal hindbrain beyond the posterior tip of the hindbrain ventricle (black arrow) and first myotome (white arrowhead). (G) At 48 hpf neural tube expression extends ventrally in axon-like projections to the somites

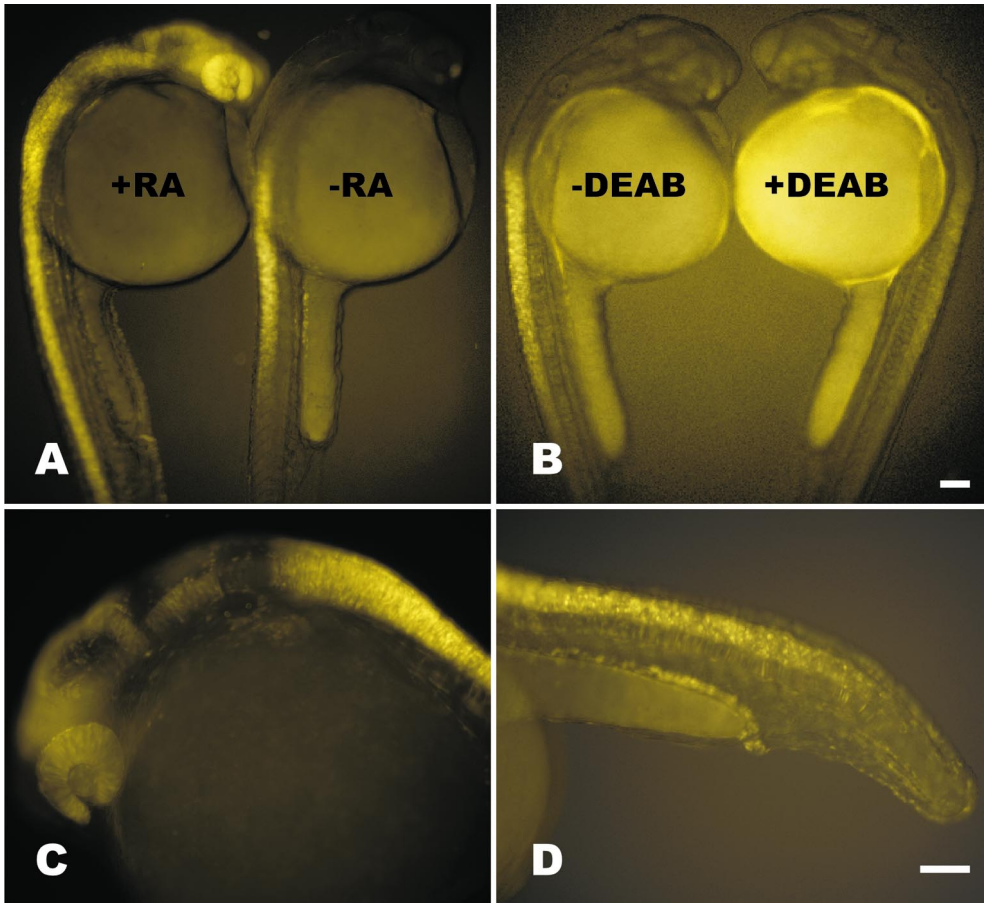

FIG. 5. Transgene signal is RA inducible and dependent. (A) Treatment of embryos with 1 μM RA at 18 hpf induces transgene expression throughout the neural tube and neural retina by 24 hpf (+RA, left) compared to an ethanol-treated control (−RA, right). (B) Treatment of embryos with 10 μM DEAB, an RA synthase inhibitor, at 18 hpf significantly reduces transgene signal in the neural tube and abolishes it in all other regions of expression by 24 hpf (+DEAB, right) compared to DMSO-treated control (−DEAB, left). (C) Expression in the anterior nervous system responds to RA induction at varying levels when treated with 0.1 μM RA at 18 hpf. (D) Details of posterior induction, including the pronephric ducts, immature notochord cells, neural tube, and enveloping layer when embryos are treated at 1 μM RA at 18 hpf. Bars, 100 μm. Scale for A is same as in B. Scale for C is same as in D.

1998, 2000; Wilson *et al.*, 1953). Interestingly, treatment of gastrulating embryos with DEAB, a retinaldehyde dehydrogenase inhibitor (Mahmoud *et al.*, 1993; Russo *et al.*, 1988), abolishes transgene expression and confers phenotypes in the hindbrain, heart, otic vesicles, eyes, limb buds, somites, and face similar to those generated by retinoic acid deprivation in other vertebrates (Dersch and Zile, 1993; Dickman *et al.*, 1997; Maden *et al.*, 1996; Niederreither *et al.*, 1999; White *et al.*, 2000). From these data it appears that DEAB can inhibit the enzymes that synthesize RA required

(arrowheads). (H) Dorsolateral view of rostral end of 72 hpf RGY larvae showing an arch of expression in cells of the inner nuclear layer of the dorsal retina and a small patch of forebrain expression (arrowhead). Bright fluorescence at the bottom of the image is the ventral retina. (I) Lateral view of 72-hpf hindbrain/spinal cord junction imaged as a confocal slice shows higher density of dorsal cells expressing the transgene than ventral. (J) Dorsal view of expression in the forebrain at 5 days postfertilization. (K) Ventral view of transgene expression in cranial skeletal muscles, hyohyoideus (hh) and interhyodoideus (ih). Bright bilateral fluorescence is expression in the ventral retina. (L) Lateral view of 5-day heart showing blurred expression in the beating ventricle (V) that is excluded from the outflow tract (O). A–E, G–I, and L are lateral views with rostral left and dorsal up. F is a dorsal view with rostral up. J and K are dorsal and ventral views, respectively, with rostral left. Bars, 100 μm. Scale for A and D–K is the same as in L. Scale for B is same as in C.

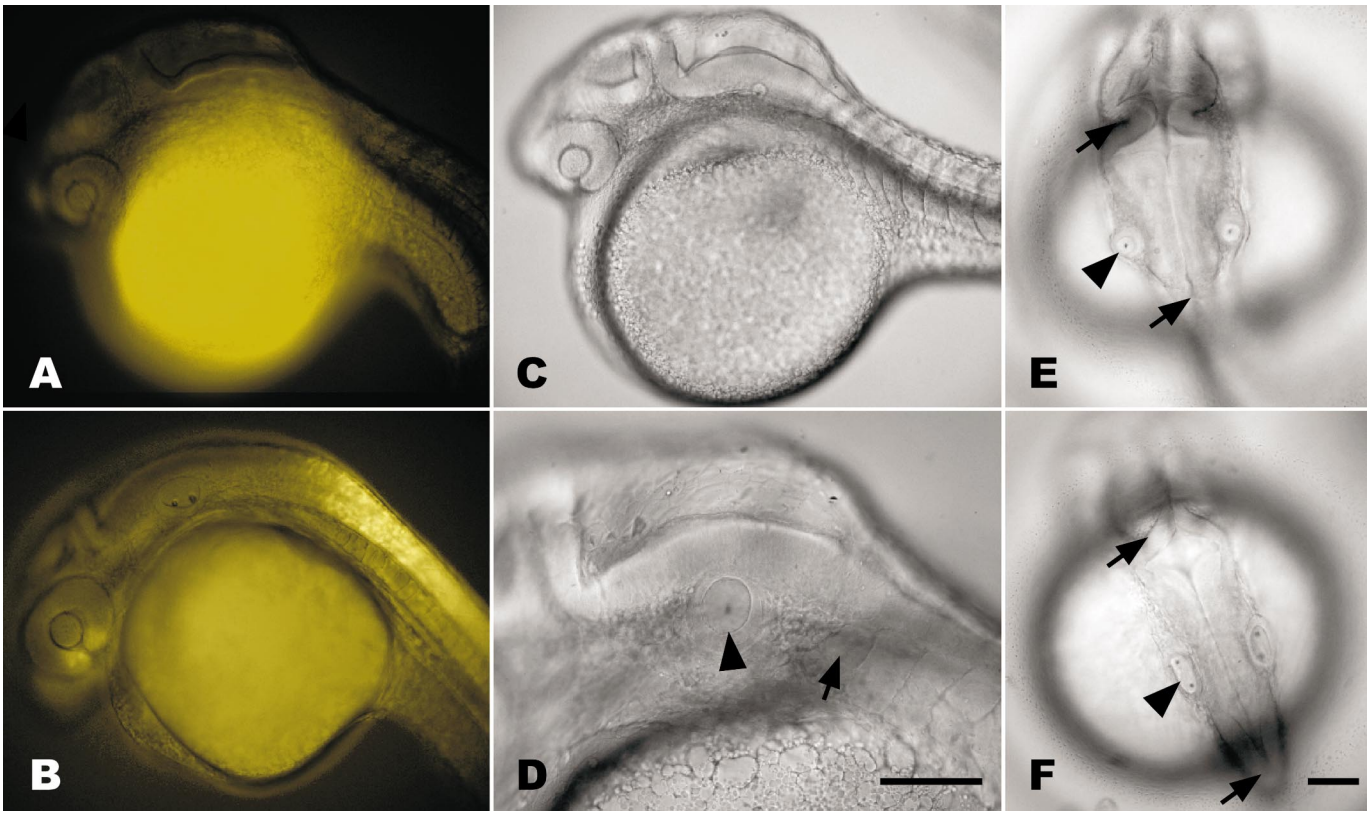

FIG. 6. DEAB treatment at gastrulation abolishes transgene signal and teratogenizes zebrafish embryos by 24 hpf. (A) Treatment of transgenic embryo (RGYn2) with10 μM DEAB at shield stage (6 hpf) completely abolishes YFP expression by 24 hpf compared to DMSO-treated control (B). (C) DEAB treatment reduces frontonasal region, retina, hindbrain, and otic vesicle while inhibiting development of heart tube and fin bud. (D) Lateral view of the hindbrain demonstrates the significant reduction in the distance between the otic vesicle (arrowhead) and first myotome (arrow) in DEAB-treated embryo. (E and F) Dorsal views of DEAB-treated (E) and DMSO-treated (F) embryos demonstrate that the hindbrain is slightly reduced between the isthmus (top arrows) and the otic vesicles (arrowheads) while the caudal hindbrain between the otic vesicle and the termination of the ventricle (bottom arrows) is significantly reduced. The overall width of the hindbrain is also increased as a result of DEAB treatment. All embryos are oriented with rostral left and dorsal up, except for dorsal views in E and F, which have rostral up. Bars, 100 μm. Scale for A–C and E is the same as F.

for transgene response. Similar results have not been noted for citral (our unpublished data), perhaps owing to its short half-life. The similarity between DEAB treatment in zebrafish to targeted disruption of RALDH2 in mice (Niedereither *et al.*, 1999, 2000) and the ability of DEAB to abolish transgene expression support a role for an RALDH2-like enzyme in zebrafish morphogenesis.

The data from the transgenic lines and DEAB treatment support the story of RA in patterning the posterior hindbrain. A recent review by Gavalas and Krumlauf (2000) suggests that the hindbrain is patterned by antagonizing signals from the posterior hindbrain and the midbrain–hindbrain boundary or isthmus. This model predicts a gradient of RA starting at the anterior spinal cord, diminishing rostrally toward the isthmus. Our transgenic zebrafish lines show a graded response to RA in the posterior hindbrain with the highest levels of activity in the anterior spinal cord that extend anteriorly, diminishing in the caudal hindbrain. DEAB treatment shows both a complete loss of transgene signal and that RA is essential for the hindbrain posterior to rhombomere 4 or 5. At the anterior end of the hindbrain, the isthmus expresses FGF8 and extends its influence caudally, inhibiting Hox gene expression in the anterior hindbrain (Gavalas and Krumlauf, 2000). Antagonism between FGF8 and RA in the hindbrain is supported by the loss of the isthmus caused by RA teratogenesis (Hill *et al.*, 1995; Holder and Hill, 1991) or mutation of *Fgf8* in *acerebellar* (*ace*) zebrafish mutants (Reifers *et al.*, 1998). A more recent analysis in limb development suggests that FGF8 can antagonize RA signaling by inhibiting the expression of RALDH-2, thereby preventing RA synthesis in regions of FGF8 influence (Mercader *et al.*, 2000). If the same is true in the zebrafish hindbrain, it may restrict the response of the transgene to the posterior hindbrain.

All regions of fluorescent protein expression in the zebrafish lines are confirmed by transgenic mouse lines made

with the same or similar RARE-driven promoters and lacZ reporters with the exception of the notochord expression (Balkan *et al.*, 1992; Rossant *et al.*, 1991). However, an identical RARE from the Hoxa-1 gene in mouse has been shown to be necessary for notochord expression of that gene (Frasch *et al.*, 1995).

It is interesting to compare the mouse and zebrafish transgenics made with the identical RARE-tk transcriptional regulatory element (Balkan *et al.*, 1992). The RTG fish lines have expression limited to the neural tube and retina, while the mouse line has a more complicated expression pattern. This may indicate species-specific differences in RARE-mediated gene activation or the reduced sensitivity of GFP compared to an enzymatic reporter like lacZ. On the other hand, the transgene expression may be more limited in zebrafish because the viral tk basal promoter has been shown to be less efficient in zebrafish cell lines than in mammalian cell lines (Sharps *et al.*, 1992). This is likely to be the case because we saw that with a zebrafish basal promoter (GT2) coupled to the 3XRARE there was an increase in the complexity of the transgene signal that was dependent on RA and distinct from the activity of the promoter alone. The original characterization of the GT2 minimal promoter showed that it does not confer tissue-specific activity related to our transgenic expression patterns (Meng *et al.*, 1997). This suggests that promoter choice is important when designing zebrafish transgenics and should not be based solely on activity on other species.

The regulation of genes during embryogenesis by retinoic acid depends upon the promoter context of RAREs and the overlapping transcription factor binding sites (e.g., Frasch *et al.*, 1995). Thus, isolated RAREs and a basal promoter may not represent the full complexity of RA signaling that occurs in a developing embryo. Additionally, DEAB treatments and teratogenesis studies suggest that embryonic requirement/sensitivity to RA begins as early as gastrulation. However, we are unable to detect transgene expression until midsomitogenesis (13 somites). This is likely due to the long folding time and need for GFP to accumulate before fluorescence is visible. Nonetheless, the transgenic lines presented here express fluorescent proteins in an RA-specific manner, and the regional expression patterns appear to represent at least a subset of regions in which RA is available along with receptors and other necessary cofactors for activating transcription.

There are several advantages to making RARE-responsive transgenics in zebrafish using fluorescent reporters. The stable transgenic lines and nuclear-localized fluorescent proteins enable a more precise identification of specific cells within a region that have localized sources of RA. An additional benefit of the RARE reporter approach is that it goes beyond localizing RA sources or individual receptors and integrates the signals required for RA-mediated gene activation, thus providing information about where RA can function as a transcriptional activator. Likewise, because embryos do not have to be fixed to visualize GFP, it is possible to follow reporter gene expression in living embryos (Amsterdam *et al.*, 1995; Higashijima *et al.*, 1997, 2000; Linney *et al.*, 1999; Long *et al.*, 1997; Meng *et al.*, 1997). The rapid *ex utero* development and optical clarity of zebrafish embryos make them an ideal vertebrate system to follow live fluorescent reporter expression. The RARE-driven transgenic lines will be useful to explore the retinoid signaling system in developing embryos, including mutants; to create targets for mutagenesis to screen for mutants that disrupt the transgenic expression patterns; and to detect contaminants in the environment that alter retinoid signaling.

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