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Retinoic acid is necessary for development of the ventral retina in zebrafish

(citral/retinaldehyde/dehydrogenases/morphogenesis/pattern formation)

N. MARSH-ARMSTRONG*, P. MCCAFFERY†‡, W. GILBERT*§, J. E. DOWLING*§¶, AND U. C. DRÄGER*†‡

*Program in Neuroscience, Harvard University, Boston, MA 02115; †Department of Neurobiology, Harvard Medical School, Boston, MA 02115; ‡E. K. Shriver Center, Waltham, MA 02254; and §Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

Contributed by J. E. Dowling, April 18, 1994

ABSTRACT **In the embryonic zebrafish retina, as in other vertebrates, retinoic acid is synthesized from retinaldehyde by two different dehydrogenases, one localized dorsally, the other primarily ventrally. Early in eye development only the ventral enzyme is present. Citral competitively inhibits the ventral enzyme *in vitro* and decreases the production of retinoic acid in the ventral retina *in vivo*. Treatment of neurula-stage zebrafish embryos with citral during the formation of the eye primordia results in eyes lacking a ventral retina. This defect can be partially rescued by retinoic acid. The results demonstrate that synthesis of retinoic acid can be selectively inhibited *in vivo* and suggest that retinoic acid is necessary for the proper development of the ventral retina.**

Retinoids are likely to be involved in pattern formation of several vertebrate structures. In the chicken limb bud, retinoic acid induces a mirror-image duplication of digits (1, 2), presumably by altering the expression patterns of certain genes such as sonic hedgehog (3–5) and the Hox-d cluster (6–8). In *Xenopus* (9–11) and zebrafish (12), bath application of retinoic acid during gastrulation causes truncation of anterior neural and mesodermal structures, suggesting that retinoic acid plays a role in the specification of the embryonic anteroposterior axis. In the retina, retinoic acid may be involved in patterning along the dorsoventral axis. Retinoic acid synthetic enzymes and retinoic acid itself are distributed asymmetrically along the dorsoventral axis (13, 14), and exposure of zebrafish embryos to exogenous retinoic acid at the stage when the optic primordia begin to form results in duplication of the retina with the second retina arising in a ventral location (15).

We show here that the zebrafish embryo has retinaldehyde-oxidizing dehydrogenases in the right place and at the right time for retinoic acid synthesis to regulate the specification of the dorsoventral retinal axis. Furthermore, inhibition of retinoic acid synthesis at early stages of eye formation results in the development of eyes lacking a ventral retina.

MATERIALS AND METHODS

Zebrafish embryos were obtained from an outbred laboratory colony as described (15) and were raised between 22°C and 28.5°C. Embryos were dissected with fine insect needles in RPMI 1640 medium (GIBCO) containing 10% bovine calf serum. Tissues were transferred into microcentrifuge tubes by using siliconized glass capillary pipets connected to a Captroll III microdispenser (Drummond) and were frozen on dry ice. Retinaldehyde dehydrogenase activities were measured with a retinoic acid reporter cell line (16) in a zymography bioassay (13). This assay involves separating native protein by isoelectric focusing, slicing up the gel, and testing protein fractions eluted from the gel slices for retinoic acid synthesis from 50 nM retinaldehyde at 37°C for 3 hr in the presence of 1.2 mM NAD and 2 mM dithiothreitol. Enzyme activities, as reflected in intensity of the β-galactosidase reaction product in the reporter cells, were quantified as colorimetric readings by an ELISA reader. For the *in vitro* enzyme inhibition assays, adult zebrafish retinas were dissected, frozen on dry ice, and assayed as above, except that the gel slices were preincubated with various concentrations of citral (3,7-dimethyl-2,6-octadienal; Sigma) for 10–20 min at 4°C before retinaldehyde, NAD, and dithiothreitol were added and the reactions were run for 3 hr at 37°C. For measurements of retinoic acid levels, adult zebrafish retinas were quartered into dorsal, nasal, ventral, and temporal quadrants, and the dorsal and ventral quarters were further dissected into peripheral and central components. Only the peripheral components from dorsal and ventral quarters were used for the assay. The live tissue pieces were rinsed and preincubated for 30 min in Leibovitz' L15 medium and then incubated in L15 medium with or without 50 μM citral for 20 hr. The supernatants were titrated onto the reporter cells. The bars in the figures represent equivalent dilutions along the linear part of the dose–response curve.

For the citral treatments of live embryos, stock solutions of 100 mM citral were made up in dimethyl sulfoxide immediately prior to use and then directly diluted in 28.5°C tank water containing the staged embryos. An aqueous citral solution loses most of its potency within an hour (N.M.-A., unpublished data). Some embryos received mock dosages of dimethyl sulfoxide, which caused no discernible effects even at 10-fold higher concentrations than those used in the inhibitor treatments. For the phenotype-rescue experiments, all-*trans*-retinoic acid (Sigma) was prepared and administered as described (15): a 0.1 M stock solution was made in dimethyl sulfoxide, frozen, and then diluted in tank water immediately before use. For histological sections the embryos were embedded in Epon-Araldite, sectioned at 0.75 μm, and stained with a saturated 1:1 methylene blue/azure II stain made up in 1% borax buffer.

RESULTS

Dorsal and ventral halves of retinas from 2.5-day zebrafish embryos were assayed for retinaldehyde dehydrogenases by zymography bioassay. As described for other vertebrates (13, 14, 17), the zebrafish retina contains two different enzymes, distinguished by isoelectric focusing points, distributed asymmetrically along the dorsoventral retinal axis. Fig. 1*A* shows that a relatively more acidic enzyme activity (pI 5.2) is found in the dorsal retina (the D-enzyme) and a relatively more basic enzyme activity (pI 5.8) is found in the ventral retina (the V-enzyme). In the mouse, the D-enzyme

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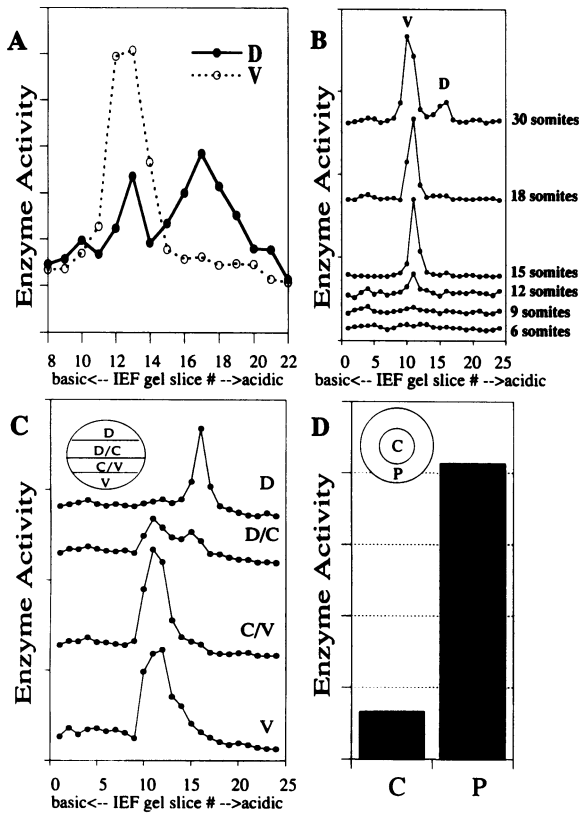

FIG. 1. Spatial and temporal patterns of expression of retinaldehyde dehydrogenases in the zebrafish retina. The two forms of the enzyme were separated by isoelectric focusing (IEF). (*A*) Retinaldehyde dehydrogenases in 20 ventral (V) and dorsal (D) retina halves from 2.5-day zebrafish embryos. (*B*) Temporal pattern of expression of V- and D-enzymes in the embryonic eye region in zebrafish: 6 somites, 9 somites, 12 somites, 15 somites, 18 somites, and 30 somites. One hundred retinas were dissected for each stage. D and V represent the D- and V-enzymes. (*C*) Distribution of V- and D-enzymes along the dorsoventral axis of an adult zebrafish retina: D, dorsal; C, central; V, ventral. (*D*) Distribution of enzymatic activities in center versus periphery of an adult zebrafish retina: C, center; P, periphery.

is identical to AHD-2, the major class I aldehyde dehydrogenase, and the V-enzyme is a novel dehydrogenase (13).

To study the temporal expression pattern of these enzymes in the zebrafish retina, 100 retinas were dissected at each stage and analyzed by isoelectric focusing and zymography bioassay using as substrate 50 nM retinaldehyde, a concentration which gives comparable activity levels of the V- and D-enzymes in a 2-day retina (data not shown). As has been found in the mouse (13), the V-enzyme becomes detectable before the D-enzyme (Fig. 1*B*). The zebrafish V-enzyme first becomes detectable between the 9- and 12-somite stages (13–15 hr postfertilization). Between the 15- and 18-somite stages (16–18 hr), the V-enzyme is clearly apparent, but there is no trace of the D-enzyme. By the 30-somite stage (24 hr), the D-enzyme is detectable. In the adult zebrafish retina, both enzymes persist and retain their asymmetrical distribution (Fig. 1*C*). When adult retinas are sectioned into four slices along the dorsoventral axis (see sketch in Fig. 1*D*), the ventral-most sector has only the V-enzyme, and the dorsal-most sector has only the D-enzyme. However, most of the retinoic acid synthetic activity is found in the periphery of the adult retina (Fig. 1*D*).

The drug citral, a competitive inhibitor of aldehyde dehydrogenases, has been used to inhibit the synthesis of retinoic acid in epithelial cells (18, 19) and in gastrulating *Xenopus* embryos (20). At low micromolar concentrations, citral inhibits the zebrafish V-enzyme more than the D-enzyme in our *in vitro* zymography bioassay (Fig. 2*A*). The inhibition is not

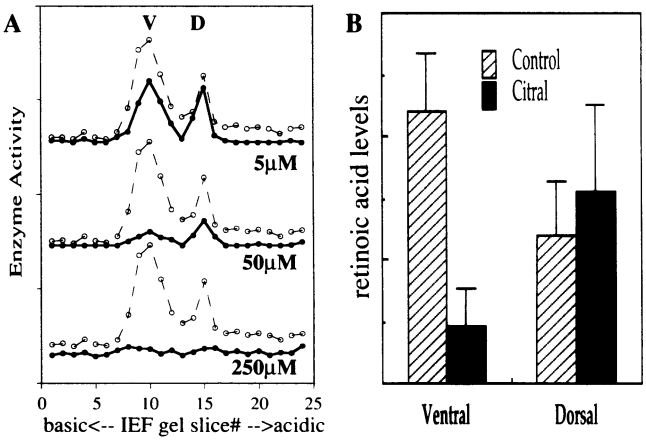

FIG. 2. Inhibition of retinoic acid synthesis by citral. (*A*) Effect of citral on retinaldehyde dehydrogenases of the adult zebrafish retina. The enzymatic activities in the absence of any drug are shown (○, dashed lines) for comparison. V and D represent the V- and D-enzymes, respectively. IEF, isoelectric focusing. (*B*) Effect of citral on endogenous retinoic acid levels in dorsal and ventral adult retina pieces. The values are averages of four experiments, and the error bars represent the standard deviation.

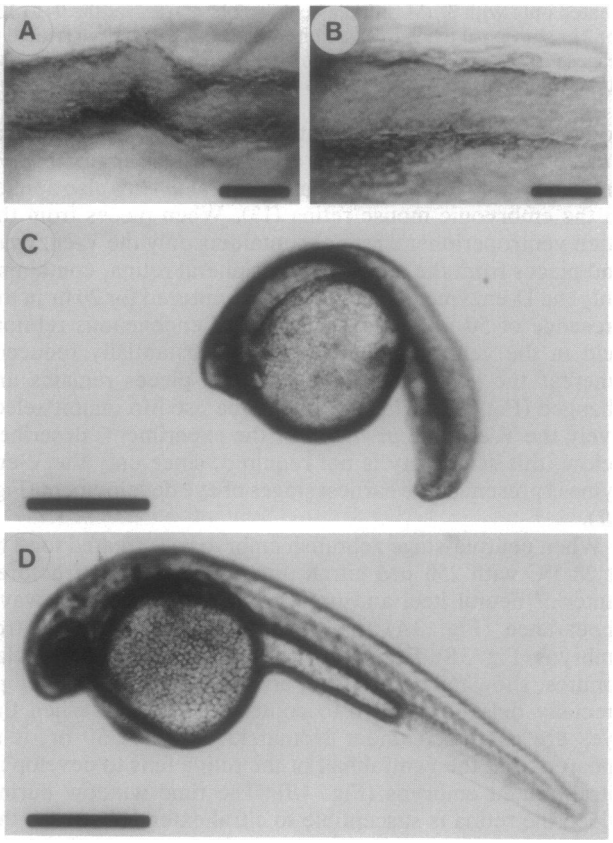

FIG. 3. Morphological effects caused by exposing zebrafish embryos, at the 6- to 7-somite stage, to 250 μM citral for 2 hr. (*A* and *B*) Dorsal views, obtained by Nomarski differential interference contrast optics, of anterior neuraxis at the level of the prospective midbrain and hindbrain of treated (*A*) and control (*B*) embryos immediately at the end of the 2-hr treatment. (*C* and *D*) Low-power lateral view of a 1.5-day embryo which had been treated for 2 hr with 250 μM citral at the 6- to 7-somite stage (*C*) and of a control embryo (*D*). Anterior is to the left. (Bars = 50 μm in *A* and *B* and 500 μm in *C* and *D*.)

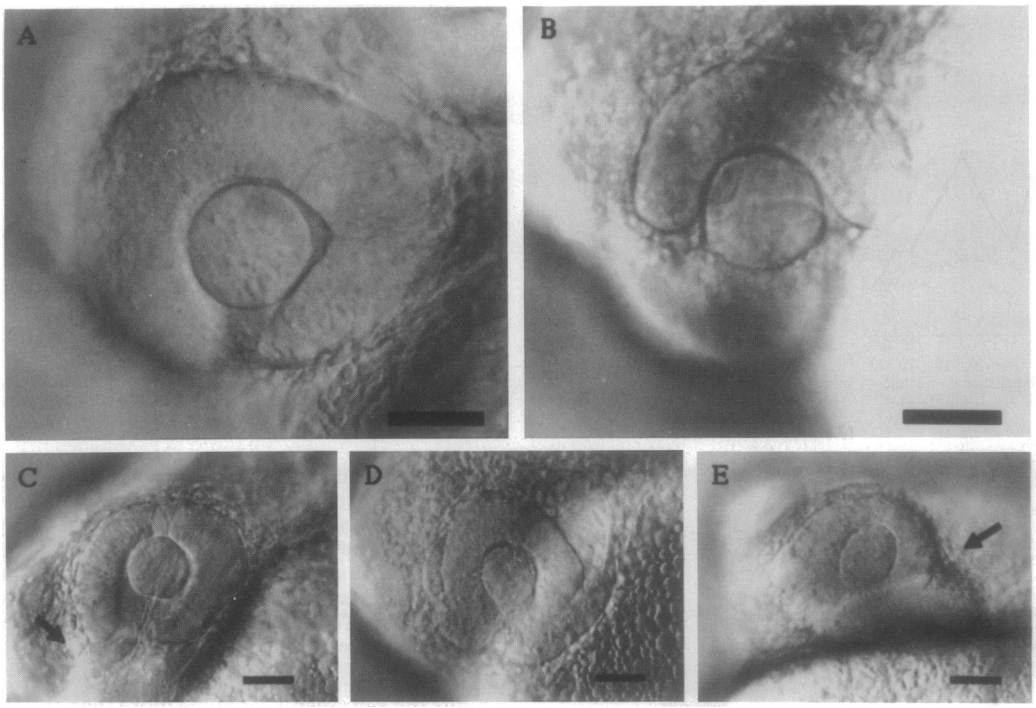

FIG. 4. Effect of citral on retinal morphogenesis. Lateral view, obtained by Nomarski differential interference contrast optics, of eyes from 1-day embryos: (*A*) control embryo; (*B*) embryo which had been treated with 250 μM citral for 2 hr starting at the 2- to 4-somite stage; (*C*) embryo treated only with 3 μM retinoic acid for 2 hr starting at the 9- to 12-somite stage; (*D*) embryo treated with 250 μM citral and 1 μM retinoic acid for 2 hr starting at the 9- to 12-somite stage; and (*E*) embryo treated with 250 μM citral and 3 μM retinoic acid for 2 hr starting at the 9- to 12-somite stage (see Table 2). Arrow in *C* shows a thickened optic stalk; arrow in *E* shows the pigmented bifurcation that is indicative of a retinal duplication (15). Anterior is to the left. (Bars = 50 μm.)

completely selective, since at high micromolar concentrations both V- and D-enzymes are affected. A similar preferential inhibition of the V-enzyme by citral *in vitro* also applies to the embryonic mouse retina (13). When pieces from the adult ventroperipheral retina, containing only the V-enzyme, and pieces from the adult dorsoperipheral retina, containing only the D-enzyme (see Fig. 1*C*), are cultured for 20 hr in the presence of 50 μM citral, the level of endogenous retinoic acid in the ventral retinal pieces is substantially reduced, whereas the level in the dorsal retinal pieces remains unchanged (Fig. 2*B*). Thus, citral can be used to inhibit selectively the V-enzyme *in vivo*. For the experiments described below, this selectivity is not required, since only the V-enzyme is present at the earliest stages of eye development (Fig. 1*B*).

When neurula-stage zebrafish embryos are treated for 2 hr at 28.5°C with 250 μM citral, anterior neuraxial structures (anterior neural keel and optic primordia) take on a wavy appearance (Fig. 3*A*) that is never observed in control embryos (Fig. 3*B*). By 24 hr (Fig. 3*C*), they develop irregular somites, show tail deformities, and are shorter and developmentally delayed relative to controls (Fig. 3*D*). When the eyes are examined under Nomarski optics at 30 hr, it is apparent that the ventral half of the retina fails to develop in citral-treated embryos (Fig. 4*B*). The time window during which the retina is susceptible to citral extends between the 2- and 11-somite stages (Table 1). However, at the early somite stages, extensive embryo mortality is observed, and for this reason, the optimal stage for treatment is around the 9- to 12-somite stage.

The citral-induced retinal halving can be partially rescued by retinoic acid (Table 2). For example, when 9- to 12-somite stage embryos are treated with 250 μM citral, virtually every surviving embryo fails to develop a ventral retina (see Tables 1 and 2 and Fig. 4*B*). If they are treated simultaneously with 1 μM retinoic acid, only about a third of surviving embryos show the retinal halving, whereas nearly half appear to have normal eyes as judged through a dissecting microscope (Table 2). Under Nomarski optics, however, these "rescued" embryos are not completely normal, as they usually have too much or too little ventral retinal tissue, and the pigment epithelial layer is often separated from the the neural retina (for an example, see Fig. 4*D*). Curiously, following concurrent treatment with citral and retinoic acid, many 9- to 12-somite-stage treated embryos develop a retinal duplication (15) (for an example, see Fig. 4*E*). Previously, we have found that a retinal duplication consistently happens when 1- to 6-somite-stage zebrafish embryos are treated with retinoic acid, and it is not observed in embryos treated at this later, 9- to 12-somite stage (Fig. 5*B* and Table 2), where normally only a thickening of the optic stalk is observed (G. A. Hyatt, E. A. Schmitt, and J.E.D., unpublished work). Thus, citral treatment seems to extend the retinoic acid-sensitive period in eye development.

To analyze the effects of citral in more detail, we sectioned the retinas of citral-treated and control embryos for histological examination. In control 5-day embryos, the retina is divided by the optic disk, the optic nerve exit point, into a dorsal two-thirds and a ventral one-third (Fig. 5*A*). In about

Table 1. Stage dependence of citral-induced retinal halving (sum of four experiments)

Stage, somite no.	Half-eye, no. (% of live)	Normal, no. (% of live)	Dead,* no.
0	6 (24)	19 (76)	75
2–4	35 (90)	4 (10)	61
6–8	61 (69)	27 (31)	12
9–11	55 (98)	1 (2)	44
12–14	31 (38)	50 (62)	19
16–18	13 (16)	68 (84)	19

*Or monsters (i.e., too deformed to analyze).

Table 2. Retinoic acid rescue of citral induced retinal halving (sum of four experiments)

Citral, μM	Retinoic acid, μM	Half-eye, no. (% of live)	Normal, no. (% of live)	Double eye, no. (% of live)	Dead,* no.
0	0	0 (0)	100 (100)	0 (0)	0
	3	0 (0)	99 (100)†	0 (0)	1
250	0	65 (98)	1 (2)	0 (0)	34
	1	29 (39)	39 (53)	6 (8)	26
	3	17 (21)	31 (38)	34 (41)	18

*Or monsters (i.e., too deformed to analyze).
†These embryos showed an abnormally thick optic stalk, as illustrated in Fig. 4*C*.

50% of the citral-treated embryos, differentiation proceeds normally dorsal to the optic disk: all retinal cell types seem to be present, arranged in their proper laminae, and there is a normal ciliary margin (Fig. 5*B*). Ventral to the optic disk, however, only a few cells appear, which resemble undifferentiated neuroepithelial cells. In the remaining 50% of embryos, the ventral retina also fails to develop, but the dorsal retina develops complex lamination patterns, and often more than one inner plexiform layer forms (large arrow in Fig. 5*C*). Because of the abnormal lamination, some cells can be found in unusual environments. For example, in many of these retinas, photoreceptors and their outer segments are surrounded by inner nuclear-layer cells and not by pigment epithelium, and consequently their outer segments atrophy, and the photoreceptor cells die (small arrow in Fig. 5*C*). With the location of the optic disk as a positional marker, these histological sections demonstrate that the inhibition of retinoic acid synthesis in the developing eye anlage results in failure of ventral retina formation.

DISCUSSION

The asymmetry of the retina along the dorsoventral axis is most evident morphologically by the presence of a ventral choroid fissure in the developing eye. Dorsoventral axial information in the retina is established very early in eye development, as shown by eye rotation and transplantation studies (21). Several markers have been identified that distinguish between dorsal and ventral retina (22–24), but what role these molecules play in dorsoventral patterning remains to be established.

In zebrafish (data not shown), mice (3), *Xenopus* (N.M.-A., P.M., and U.C.D., unpublished observations), and chicken (P.M. and U.C.D., unpublished observations), retinoic acid levels are higher in the ventral half of the embryonic retina. In all these species, the retinoic acid synthetic enzymes are also distributed asymmetrically along the dorsoventral axis, suggesting that the asymmetry of retinoic acid is established at the level of synthesis. Such interspecies conservation, coupled with the role that retinoids play in other developing systems, suggests that retinoic acid may function in the establishment of the dorsoventral axis in the vertebrate retina.

In the adult mouse, the V-enzyme completely disappears in the retina and the D-enzyme remains with greatly reduced activity (14). In the adult zebrafish, both enzymes persist and retain their asymmetrical distribution. This difference probably reflects the fact that in zebrafish, as in other cold-blooded vertebrates (25), the retina continues to grow throughout life, ever expanding in its periphery. Any mechanism important in setting up dorsoventral differences in an embryonic retina is thus likely to persist in the periphery of the retina of adult zebrafish. Retinoic acid and retinoic acid synthetic machinery fulfill this requirement.

Experiments providing too much or too little retinoic acid to developing eyes provide direct evidence that retinoic acid is important in the development of the retinal dorsoventral axis. Exogenous retinoic acid exposure of neurulating zebrafish embryos, during the formation of the optic primordia, leads to the development of excessive ventral retinal tissue, which forms a second retina (15). Conversely, inhibition of the retinoic acid synthetic machinery in developing fish produces eyes lacking ventral retinas—an effect that can be reversed, at least partially, by adding exogenous retinoic acid. An interesting question is why the retina ceases to be susceptible to retinoic acid-induced morphological alterations by about 24 hr of development. This desensitization may be attributable to increasing levels of endogenous retinoic acid, since inhibition of synthesis of endogenous retinoic acid, by means of treatment with citral, lengthens the period

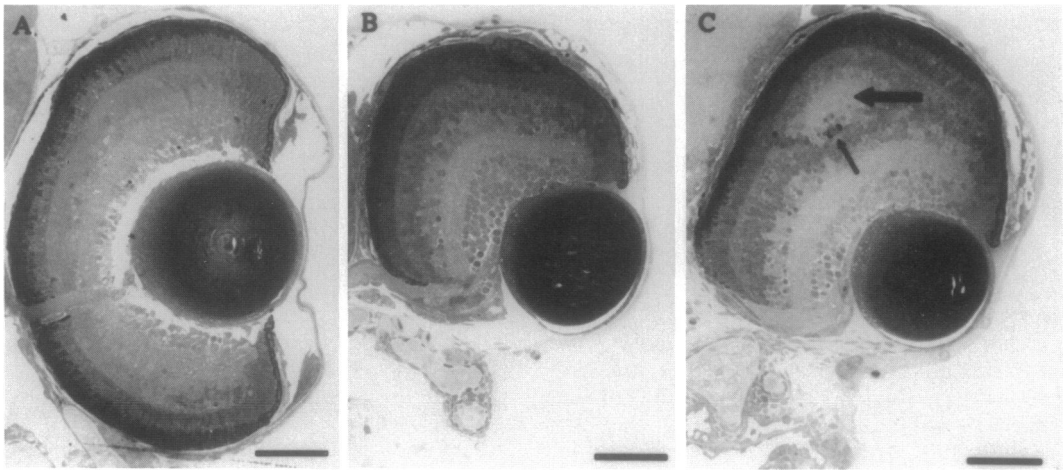

FIG. 5. Absence of a ventral retina as shown by transverse sections through the eyes of 5-day control embryo (*A*) and embryos which had been treated with 250 μM citral for 2 hr starting at the 2- to 4-somite stage (*B* and *C*). Large arrow points to secondary inner plexiform layer. Small arrow points to degenerating photoreceptor cells. (Bars = 50 μm.)

during which the eyes are susceptible to retinoic acid-induced retinal duplications (Table 2 and Fig. 5*D*).

We propose that dorsal retinal identity represents either a default state or one requiring low levels of retinoic acid and that the determination of the ventral retina is dependent on higher levels of retinoic acid. It is intriguing that a malformation of the ventral eye has been observed in *Drosophila* carrying a dominant negative mutation of the retinoic acid X receptor homolog ultraspiracle (26). This suggests that retinoic acid, or a derivative, may share a common role in eye development of both vertebrates and invertebrates. While very little is known about retinoids in *Drosophila*, in vertebrates the homeobox genes appear to mediate at least some of the effects of retinoids in development. This may be true for the retina as well, since a malformation of the ventral eye in *Drosophila* has also been observed in mutants at deformed, a locus that codes for a homeobox-containing protein (27).

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