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A behavioral screen for isolating zebrafish mutants with visual system defects

SUSAN E. BROCKERHOFF*, JAMES B. HURLEY†, ULRIKE JANSSEN-BIENHOLD‡, STEPHAN C. F. NEUHAUSS§, WOLFGANG DRIEVER§, AND JOHN E. DOWLING*

*Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138; †Howard Hughes Medical Institute, Box 357370, University of Washington, Seattle, WA 98195; ‡Department of Neurobiology, University of Oldenburg, Postfach 2503, 26111 Oldenburg, Germany; and §Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, 13th Street, Building 149, Charlestown, MA 02129

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ABSTRACT **Optokinetic and phototactic behaviors of zebrafish larvae were examined for their usefulness in screening for recessive defects in the visual system. The optokinetic response can be reliably and rapidly detected in 5-day larvae, whereas the phototactic response of larvae is variable and not robust enough to be useful for screening. We therefore measured optokinetic responses of mutagenized larvae as a genetic screen for visual system defects. Third-generation larvae, representing 266 mutagenized genomes, were examined for abnormal optokinetic responses. Eighteen optokinetic-defective mutants were identified and two mutants that did not show obvious morphological defects, *no optokinetic response a* (*noa*) and *partial optokinetic response a* (*poa*), were studied further. We recorded the electroretinogram (ERG) to determine whether these two mutations affect the retina. The b-wave of *noa* larvae was grossly abnormal, being delayed in onset and significantly reduced in amplitude. In contrast, the ERG waveform of *poa* larvae was normal, although the b-wave was reduced in amplitude in bright light. Histologically, the retinas of *noa* and *poa* larvae appeared normal. We conclude that *noa* larvae have a functional defect in the outer retina, whereas the outer retina of *poa* larvae is likely to be normal.**

Benzer (1) was the first to report that mutant *Drosophila* could be identified by their phototactic behavior. Subsequently, a number of nonphototactic mutants were found to have specific molecular defects in their photoreceptors (2). A phototaxis mutant that failed to respond to UV light, sevenless, lacks UV-sensitive photoreceptor cells (3); analysis of this mutant has defined the role of cell–cell interactions in ommatidial development (for review, see ref. 6).

Because there are significant differences between vertebrate and invertebrate eyes, genetic analysis of the *Drosophila* eye has provided only limited information about the vertebrate visual system. To apply a genetic analysis to the vertebrate visual system, we have turned to zebrafish (*Danio rerio*). Zebrafish are highly visual and exhibit vision-dependent behavior as early as 3 days postfertilization (pf) (4). They possess four types of cones and are tetrachromatic. Short single cones contain a UV-sensitive photopigment, whereas long single cones contain a blue-sensitive pigment; a green-sensitive pigment is in the short member of the double cones and a red-sensitive pigment is in the long member of the double cones (5). Rod photoreceptors are also present, so scotopic and photopic vision can be analyzed in this organism (7). Furthermore, early eye morphogenesis and organization of the zebrafish visual system are well characterized and similar to other vertebrates (8). Thus, information obtained from a genetic dissection of the zebrafish visual system should be applicable to other vertebrates.

Recently, two groups developed chemical mutagenesis procedures and methods for efficiently growing large numbers of zebrafish (9–12). These procedures have made it possible to conduct large-scale genetic screens in which zebrafish larvae from the third generation are analyzed for recessive mutations. Furthermore, a genetic linkage map in zebrafish is now available so mutant genes can be isolated by positional cloning (13).

We first characterized two visual behaviors—phototaxis and optokinetic responses—in wild-type zebrafish larvae (3–19 days pf). Preliminary experiments on wild-type larvae (4) suggested that both of these assays would be useful. We then analyzed the optokinetic responses of mutagenized larvae as a primary screen for detecting recessive defects in the visual system. As a secondary screen, we recorded the electroretinogram (ERG) from larvae 5–7 days pf to identify mutations that specifically affect the retina. We describe here the feasibility of this approach for identifying mutations affecting the visual system and describe two mutants isolated on the basis of their abnormal optokinetic response.

MATERIALS AND METHODS

Animals. AB strain zebrafish were obtained originally from Oregon (14) and propagated at Harvard University by inbreeding. The AB strain maintained at the Massachusetts General Hospital was also originally obtained in Oregon and was then selected over several generations to be free of lethal mutations (9). In this study, zebrafish between 3 and 19 days pf are referred to as larvae. The water used for fish was reverse-osmosis distilled and then reconstituted for fish compatibility by addition of salts (2 g of Instant Ocean per gal; 1 gal = 3.785 liters) and vitamins (Fritz, Dallas).

Mutagenesis. The procedures for mutagenesis and for conducting crosses to identify recessive mutations in the third generation of mutagenized fish have been described (9). Briefly, male AB fish were mutagenized with *N*-ethyl-*N*-nitrosourea (Sigma) and outcrossed with wild-type females. The resulting F_1 generation fish were crossed with each other or with wild-type fish to generate F_2 families. Pairs of F_2 siblings were then crossed to uncover recessive mutations in the F_3 generation. The total number of genomes screened was determined from the total number of F_2 families and the extent to which each F_2 family was examined. The probability of finding a mutation in a given F_2 family depends on the number of crosses performed from that family and the number of larvae examined from each cross. The number of mutagenized genomes screened per family = $(1 - 0.75^S) \times a$, where S is the sum of fractions of crosses screened per family = $x_1 + x_2 + \ldots$

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Abbreviations: pf, postfertilization; ERG, electroretinogram; OKN, optokinetic nystagmus.

$+ x_n$ and a is the number of mutagenized genomes crossed into a given F_2 family (value of 1 or 2); x_n is the fraction of cross n that was screened ($1 - 0.75^{L_n}$), where L_n is the number of larvae screened from cross n.

One *N*-ethyl-*N*-nitrosourea-induced allele of *noa*^{m631} and one allele of *poa*^{m724} were isolated. Thirty-eight of 122 larvae examined from crosses between *noa*-carrying G_0 fish (F_2 fish in original screen) showed no optokinetic response in white light. G_0 *noa*-carrying fish were outcrossed pairwise with AB fish and 13 *noa* carriers were identified in the F_1 generation. The optokinetic response of 442 larvae from pairwise crosses of *noa*-carrying F_1 fish were analyzed and 119 gave no optokinetic response. Of these 119 nonresponders, only 3 were noted as not having expanded melanophores (see *Results*).

One pair of *poa*-carrying G_0 fish was identified. The optokinetic responses of 57 larvae from crosses between these two fish were analyzed and 10 larvae had an abnormal partial optokinetic response. All 10 of these larvae had expanded melanophores. G_0 *poa*-carrying fish were outcrossed with AB fish and four F_1 carriers have been identified. The optokinetic responses of 26 larvae were analyzed from crosses between these F_1 fish; 6 larvae had a partial optokinetic response and all 6 were darker than wild-type larvae. Additional *poa* larvae were selected from additional crosses between the *poa*-carrying F_1 fish based on their unusual swimming behavior and darker pigmentation (see *Results*). Of ≈50 larvae selected in this way, all had a partial abnormal optokinetic response.

Finally, the optokinetic responses of 32 larvae from a cross between *noa*- and *poa*-carrying fish were analyzed and all showed a normal optokinetic response, suggesting that the *noa* and *poa* mutations are in different genes. Furthermore, the above data suggest that both mutations are recessive.

Behavioral Assays. A useful behavioral screen must be reliable and fast because recessive defects are detected in only 25% of a given population and thus large numbers of animals must be analyzed. Also, the assay should be conducted on young fish so that the cost and labor associated with raising many larvae is minimal.

Phototaxis. The phototactic response of AB larvae was measured using a 10.5 × 3 × 4 cm (length × width × height) acrylic box with a sliding partition separating two chambers referred to as A and B. To optimize fish health, larvae used in phototaxis assays were fed beginning 5 days pf, even though feeding larvae may not be practical in a mutant screen. Two types of experiments were done. In the first type of assay, 20–30 larvae were placed in the box in ambient light (30 lux) and allowed to distribute themselves between the two chambers. After 5 min, larvae on each side were counted. Chamber A was then illuminated with white light (100 lux) and chamber B was covered. Larvae in each chamber were counted again after 1–5 min. Larvae between 7 and 14 days pf were examined by this method. In the second type of assay, 10–30 larvae were placed in chamber B in darkness for 0–2 min with the partition closed. The partition was then removed and chamber A was exposed to ambient light, while chamber B was kept dark by covering it. After 1–3 min, larvae in each chamber were counted. In control experiments, the entire box was kept dark after raising the partition. Larvae between 7 and 19 days pf were examined by this method.

Optokinetic responses. The apparatus for measuring optokinetic responses is shown in Fig. 1. Ten to twenty 5- to 7-day mutagenized F_3 larvae were placed in a 35-mm Petri dish containing ≈4% methylcellulose (Sigma) to partially immobilize the fish. (Larvae kept in methylcellulose for >1 h continue to develop normally when returned to fish water.) Larvae were arranged for optimal viewing with a dissecting needle. The dish was placed in the center of a microscope stage to which a circular drum was mounted. The drum had 18° black and white vertical stripes on the inside and was turned at 6 rpm by a belt attached to an adjacent motor. For each larva, the drum was rotated in two directions and the eye movements were analyzed by watching the larva through the microscope. A response was considered positive if a single smooth pursuit and saccade eye movement in the proper direction was observed after starting drum rotation in each direction (see *Results*). A larva was considered abnormal if it showed no eye movements at all or if the eye movements were unusual, such as too fast, too small, or in the wrong direction. On average, 1 min was required to analyze each larva including time spent arranging it. Thus, we could screen ≈60 larvae per h or ≈500 larvae per day. Optimally, at least 10 larvae were examined from each cross and larvae from at least 6 crosses were examined from each line. Two hundred and forty-one genomes were screened with white light several log units above threshold. The stripes were illuminated from above and below with a fiber optic light source. The remaining 25 genomes were screened with 600-nm light <1 log unit above threshold. Colored illumination came from above using a fiber optic with a diffuser attached to a monochromator. In this method, the stage was illuminated from below with 750-nm light, which was detected by a near infrared video camera. Larvae between 3 and 10 days pf did not move their eyes in response to rotating stripes illuminated with 750-nm light.

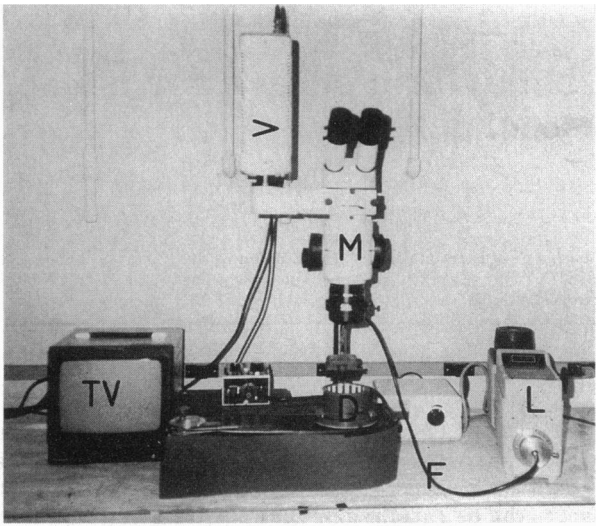

FIG. 1. Apparatus for measuring optokinetic responses. D, drum; F, fiber optic; L, monochromatic light source; M, microscope; V, videocamera.

Electroretinography. Dark-adapted (>2 h) zebrafish larvae (5–7 days pf) were anesthetized in 3-aminobenzoic acid methyl ester (100 μg/ml) (Sigma) in fish water for 2 min at room temperature. In dim red light, the anesthetized fish were covered with ≈3% methylcellulose in anesthetic and positioned on top of a sponge so that one eye pointed toward the light source (100-W halogen; maximum illuminance on head, 30,000 lux). For most recordings, a suction electrode (tip diameter, ≈10 μm) was applied to the cornea and a silver ground electrode was placed in the bath. Initially, a wire electrode was used to record ERGs as described (15). Since the larvae could not be submerged by this method, a tube was attached to the fish's mouth and anesthetic was flushed over the gills. Recordings obtained with a wire electrode were more variable and the experiments could not be sustained as long as when the suction electrode was used. Flashes (0.01–0.5 sec) produced with an electronically controlled shutter were attenuated by neutral density filters over a range of 6 log units. The responses were amplified, filtered so that a square test pulse decayed to $1/e$ in 0.14 sec, averaged, and recorded. Responses to dim flashes were averaged (n = 3–10). For bright flashes, only single responses were used unless it was clear that the first

flash did not attenuate subsequent responses. When recording an intensity-response series, dim flashes were presented first.

Histology and Immunocytochemistry. Larvae were fixed in 2.5% glutaraldehyde/1% paraformaldehyde, dehydrated through a series of ethanol washes, and embedded in Epon/Araldite (8). After resin polymerization, the specimens were sectioned at 1 μm, stained with an aqueous 1% methylene blue/1% azure II/1% borax solution, and examined under the light microscope. Whole-mount antibody staining was done as described (14) with the following modifications: 7-day larvae were treated with −20°C acetone for 17 min and then incubated in PBS containing proteinase K (2 μg/ml) (Sigma) for 90 min at room temperature. After this enzymatic digestion, larvae were fixed again for 30 min, incubated in blocking solution for 30 min, and continued through the remainder of the procedure. The antibody 7A11, kindly provided by Han Chang (Harvard University), was used at a 1:1 dilution.

RESULTS

Phototaxis. Initial screens for mutations that affect the visual system of *Drosophila* relied on phototactic behavior (1). To determine whether phototaxis could also be used to screen mutagenized zebrafish, we assayed zebrafish phototactic behavior by two simple protocols using a small box with two chambers separated by a removable partition. When both chambers were illuminated, 57% ± 11% of 7- to 14-day larvae were in one chamber; in contrast, if larvae were counted again after covering one chamber and illuminating the other, 85% ± 10% of larvae were in the illuminated chamber ($n = 22$). This demonstrates significant phototactic behavior in larval zebrafish ($P < 0.002$). In the second method, when both chambers were kept dark, 36% ± 15% of larvae migrated from the starting chamber to the other chamber ($n = 12$). When one chamber was illuminated and the starting chamber was kept dark, 49% ± 23% of larvae migrated to the illuminated chamber ($n = 36$). Although scores using the second method were not as striking, this method also demonstrated significant phototactic behavior in larval zebrafish ($P = 0.025$). In both types of assays, there appeared to be little difference in the responses of larvae between 7 and 14 days pf. Although these studies demonstrated significant phototactic responses in larval zebrafish, the responses were not as strong or as reliable as the optokinetic response (see below); thus, we did not use phototaxis to screen mutagenized zebrafish larvae.

Optokinetic Responses. The optokinetic response was consistent and robust. We observed optokinetic responses as early as 3 days pf, and by 5 days pf ≈98% of healthy AB zebrafish larvae ($n > 100$) showed a definitive optokinetic response consisting of smooth pursuit eye movements followed by rapid saccades in the opposite direction. Thus, rotating stripes elicited an optokinetic nystagmus (OKN) type response in larval zebrafish. Furthermore, the eye movements were instantly reversed when the direction of the rotating stripes was changed. Because optokinetic responses could be detected reliably by 5 days pf, and because of the efficiency of obtaining and observing these responses, we measured optokinetic responses as our primary assay of visual abilities in mutagenized larvae.

Larvae representing 266 mutagenized genomes (≈13,000 F_3 fish) were tested for defects in their optokinetic responses. Eighteen mutations causing optokinetic defects were identified. Sixteen of these mutants showed morphological defects in the eye and/or elsewhere and 15 of these 16 had a smaller-than-normal eye by day 5. These morphological mutants have not been examined in more detail in this study.

Two optokinetic-defective mutants did not show obvious morphological defects, although both were somewhat darker than their optokinetic-positive siblings (see below). We have analyzed these two mutants further. One showed no optokinetic response in either dim or bright white light and was therefore designated *noa* (*no optokinetic response a*). *noa* larvae can, however, move their eyes. They showed occasional random eye movements that were not consistent with the direction of drum rotation. The other mutant was called *poa* (*partial optokinetic response a*) because it displayed abnormal eye movements (see below) in response to the rotating stripes. Both mutants moved vigorously when touched with a fine probe. However, the swimming behavior of all *poa* larvae was abnormal; they typically swam on their sides. On the other hand, *noa* larvae usually swam normally but occasionally floated on their backs.

As noted above, both *noa* and *poa* larvae appeared darker than wild-type larvae by day 5; their melanophores were chronically expanded. Normally, in the light, melanophores are small because of the aggregation of melanin-containing organelles. In the dark, melanophores expand due to melanin dispersion. In *noa* and *poa*, the melanophores did not contract even after 10 min in bright light.

To characterize further the optokinetic responses of *poa* fish, we examined the responses of 10 mutant larvae with monochromatic light at 600, 500, and 400 nm at intensities less than a log unit above wild-type threshold levels. All 10 fish showed abnormal optokinetic responses at all three wavelengths. Two types of abnormal eye movements were observed in all 10 larvae. Sporadically, *poa* fish displayed very rapid eye movements back and forth in response to the rotating illuminated stripes. These rapid eye movements seemed consistent with the direction of drum rotation but were significantly smaller than the movements observed in wild-type larvae. Furthermore, rapid eye movements were often accompanied by tail twitching and head movements. On occasion, the eyes of *poa* larvae followed the stripes in a manner similar to those of wild-type larvae. However, when the eyes were maximally turned, they did not rapidly saccade back to their original position. Instead, they remained fixed in position in the direction of rotation.

Electroretinography. Fish with an abnormal optokinetic response could have defects at several possible loci. The vertebrate ERG originates in the outer retina (16) and is characterized by two prominent waves. An initial corneal negative a-wave originates from the photoreceptors, whereas a larger corneal positive b-wave reflects postsynaptic activity. Electroretinography thus provides a method for localizing defects to retinal loci.

Fig. 2*A* shows a series of ERG responses recorded from a *noa* larva (*Right*) and its OKN^+ sibling (*Left*) to 10-msec flashes of various intensities. The ERG of the normal larvae displayed a corneal negative a-wave followed by a larger corneal positive b-wave to the maximum intensity flash ($\log I = 0$). Both waves decreased in amplitude as the intensity of the flash decreased. In contrast, the ERG b-wave of *noa* larvae was highly abnormal; it was significantly delayed and very much reduced in amplitude. The a-wave of *noa* larvae, on the other hand, appeared quite normal. At maximum light intensity, abnormal b-wave responses were observed in 21 of 21 *noa* mutants. Normal responses were observed in 25 of 27 OKN^+ siblings examined. We were unable to record any ERG response from two OKN^+ larvae. Also, it should be noted that the maximum amplitude of the b-wave was quite variable (88 ± 80 for the 23 OKN^+ larvae stimulated at maximum intensity); however, it was consistently larger than the b-wave amplitude of *noa* larvae (18 ± 13; $P < 0.001$). Both suction and wire electrodes were used to obtain these data. Fig. 2*B* shows superimposed ERG responses of another pair of *noa* and OKN^+ siblings stimulated at the brightest light intensity for 0.5 sec. The delayed b-wave and reduced b-wave amplitude of the mutant are very obvious in this figure.

On the other hand, the ERGs recorded from *poa* mutant larvae were qualitatively normal compared to their normal

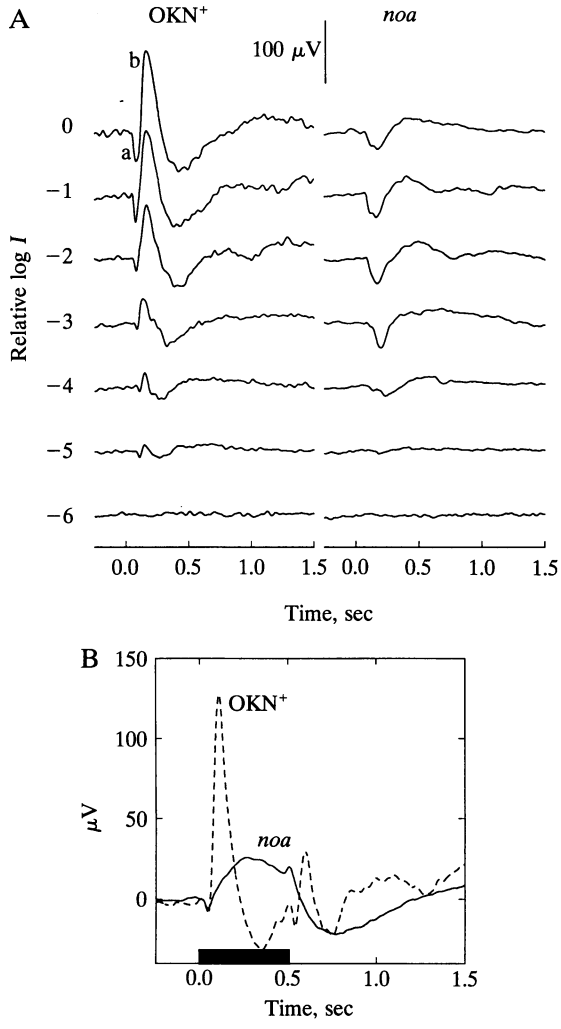

FIG. 2. ERGs from *noa* larvae. (*A*) ERG responses of 7-day *noa* (*Right*) and OKN$^+$ (*Left*) larvae to short, 0.01-sec flashes of various light intensities presented at time 0. Light intensities (log *I*) decrease by factors of 10 from top to bottom. The a- and b-waves are indicated in the top recording (*Left*). (*B*) Superimposed ERG responses from 6-day OKN$^+$ (dashed line) and *noa* (solid line) larvae obtained using the brightest intensity (log $I = 0$) 0.5-sec flash. Bar represents flash of light. At cessation of the prolonged stimulus, a complex off-response is evident in the recording from the OKN$^+$ larva. For more details on the vertebrate ERG, see ref. 16.

siblings. The waveforms of the a-wave and b-wave were indistinguishable from the normal response (Fig. 3*A*). Average b-wave amplitudes for *poa* larvae were, however, somewhat smaller than for normal larvae at the highest light intensities (Fig. 3*B*). Maximal b-wave amplitude was 109 ± 77 μV for OKN$^+$ larvae ($n = 14$) and 60 ± 29 μV for *poa* larvae ($n = 9$) ($P = 0.03$). Preliminary analyses of light and dark adaptation as well as recovery kinetics did not reveal any striking differences between the responses of *poa* and normal larvae (data not shown).

Morphological Analysis of *noa* and *poa*. Histological analysis of *noa* and *poa* eyes did not reveal any detectable morphological alterations of the retinas. Transverse retinal sections along the dorsal–ventral axis of the head from 5-day *noa* and *poa* larvae were compared with 5-day OKN$^+$ larvae (Fig. 4 *Left*). At day 5 pf in both mutant and OKN$^+$ larvae, the retina was fully laminated and cells in the inner and outer retina had differentiated. Photoreceptors at this stage are mostly immature and do not have substantial outer segments. Some rods can be identified in the ventral region of the retina and some short single cones can be identified scattered throughout the photoreceptor layer. Sections through the optic nerve of 5-day *noa* and *poa* larvae indicated that the nerve exited the eye at the appropriate position and had a normal morphology (Fig. 4 *Right*).

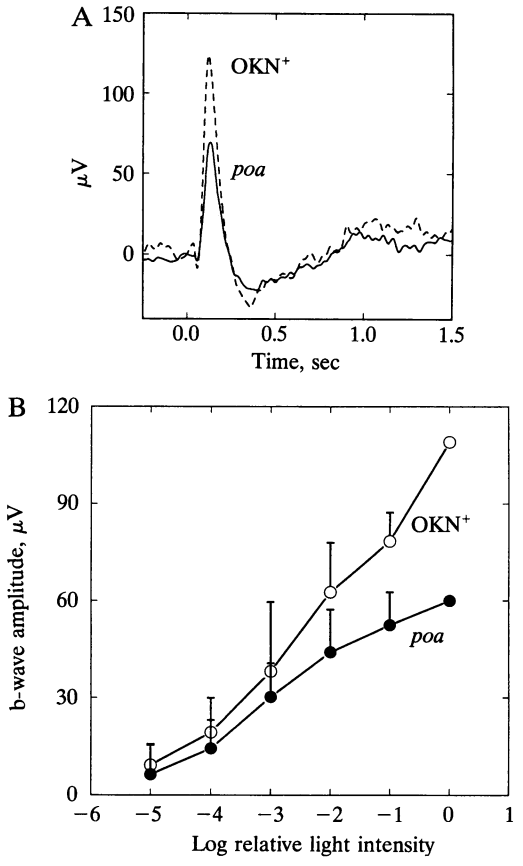

FIG. 3. ERGs from *poa* larvae. (*A*) Typical ERGs, in response to maximal intensity 10-msec flash, from a 6-day *poa* larva (solid line) and an OKN$^+$ sibling (dashed line). (*B*) b-wave amplitude plotted as a function of light intensity for 7-day OKN$^+$ (○) and *poa* (●) larvae. Responses were calculated relative to the maximum response for each larva. Relative values at each intensity were averaged and then multiplied by the average maximal response for OKN$^+$ or *poa* larvae.

We also examined ganglion cell projections in *noa* larvae with a monoclonal antibody that recognizes the optic nerve in zebrafish. The optic nerve appeared normal in all 47 7-day larvae produced from a cross of adult fish heterozygous for the *noa* mutation (data not shown). Since ≈25% of the population are expected to be homozygous for the *noa* mutation, 9–14 larvae presumably had the *noa* defect.

DISCUSSION

In this study, we showed that measuring optokinetic responses is a quick and reliable test of visual ability in zebrafish larvae and can be used to identify mutations that cause defects in the visual system. Although most fish lacking an optokinetic response had obvious morphological abnormalities and would have been detected in screens for morphological mutants (9, 10), two optokinetic-defective mutants, *noa* and *poa*, had no visible structural defects in the eye. Furthermore, the morphology of the brain and other organs as viewed under the dissecting microscope was normal in these two mutants. ERG analysis localized the defect in *noa* larvae to the outer retina and showed that the primary defect in *poa* larvae is not likely to be in the outer retina. The results demonstrate that a behavioral assay can be used to identify subtle mutations that alter visual function in zebrafish and that the ERG can then be used to define the nature and location of these mutations.

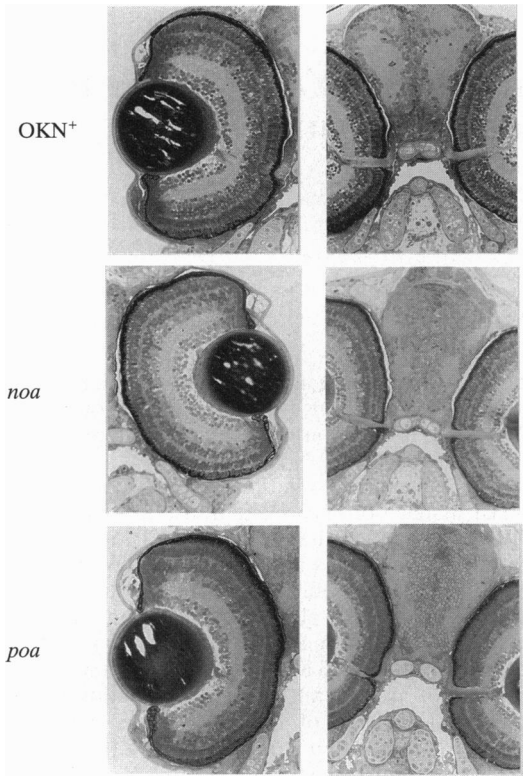

FIG. 4. Histological sections of an eye (*Left*) and optic nerve (*Right*) of day-5 OKN$^+$, *noa*, and *poa* larvae. Eyes and optic nerve are indistinguishable between the three animals.

The major ERG abnormality of *noa* larvae involves the b-wave, which is severely reduced in amplitude and delayed in onset. The b-wave currents are most likely generated in the Müller (glial) cells, but they reflect mainly on-bipolar cell activity (16). A defect in Müller cells, a defect in bipolar cells, or a defect in transmission between photoreceptor and bipolar cells could result in a reduced and delayed b-wave. We surmise, however, that a defect localized to the Müller cells would not abolish the flow of information through the retina and would not eliminate all eye movements. We suggest, therefore, that *noa* larvae have a defect in synaptic transmission between photoreceptors and bipolar cells or a defect in the bipolar cells themselves. That histological examination of *noa* retinas revealed no obvious structural changes in the retina suggests that the defect is likely to be at the molecular level.

In contrast, the optokinetic defect in *poa* larvae cannot be easily explained by a retinal defect. *poa* larvae display small and erratic eye movements in response to rotating stripes. Thus, visual information is being transmitted through the retina. ERG analysis of *poa* larvae confirmed that these fish do not have a dramatic defect in the outer retina. Furthermore, preliminary analyses indicated that they adapt to background light and recover from short flashes of light as well as wild-type larvae. Although, the average b-wave amplitude is smaller in *poa* larvae than in wild-type larvae at the brightest light intensities, the abnormal eye movements detected in *poa* larvae were observed in response to all light intensities.

Both *noa* and *poa* larvae have expanded melanophores by day 5. Melanophores are melanin-containing organelles responsible for color changes in many cold-blooded vertebrates (17). In the light, melanin aggregates and the melanophores appear smaller in size. In contrast to this normal response, the melanin granules remain dispersed in the melanophores of *noa* and *poa* larvae even after prolonged exposure to bright light. This phenotype in *noa* larvae may be due to the outer retinal defect. For *poa* larvae the cause of this phenotype is unclear.

Assuming that all loci affecting optokinetic responses in the genome mutate at a rate comparable to the ones used as tester loci (≈1:1000) (9), and assuming a Poisson distribution of mutations per genome, we would need to screen 5000 genomes to identify 99.3% or 2000 genomes to identify 87% of all loci causing an optokinetic defect. Thus, in this study, analyzing 266 mutagenized genomes, we have conducted a small screen and should have recovered 5–10% of all genes that can mutate to optokinetic-defective phenotypes. This figure is a very coarse estimate, since good data on the relative mutability of a larger group of genes are not available for zebrafish so far. Thus, this study is a preliminary presentation of the types of mutants that should be identified through screening optokinetic responses.

The bulk of the screening presented in this study was done with white light well above threshold levels, which biased our screen toward identifying mutations that affected all classes of photoreceptors. We also can screen for mutants by using individual wavelengths at light intensities near threshold. This type of screening strategy may identify mutants with defects in specific photoreceptor types. Recently, we have begun using dim red light to screen for mutants with defects that affect only red photoreceptor pathways. Finally, by screening with light intensities near absolute threshold levels after exposure of the larvae to darkness or various intensities of background illumination, we may identify mutants that are less sensitive to light than wild-type fish or that have a defect in light or dark adaptation.

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