Histology-Based Screen for Zebrafish Mutants with Abnormal Cell Differentiation

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The power of histology to define states of cell differentiation was used as the basis of a mutagenesis screen in zebrafish. In this screen, 7-day-old parthenogenetic half-tetrad larvae from potential carrier females were screened for mutations affecting cell differentiation in hematoxylin and eosin-stained tissue sections. Seven, noncomplementing, recessive mutations were found. Two mutations affect only the retina: segmented photoreceptors (spr) show a discontinuous photoreceptor cell layer; vestigial outer segments (vos) has fewer photoreceptor cells and degenerated outer segments within this cell layer. Three mutants have gut-specific defects: the epithelial cells of kirby (kby) are replaced by ballooned cells; the intestines of stuffy (sfy) and stuffed (sfd) contain increased luminal mucus. Two mutations affect multiple organs: disordered neural retina (dnr) has disrupted retinal layering and mild nuclear abnormalities in the gut and liver; and in huli hutu (hht), the retinal cell layers are disorganized and multiple organs have mild to severe nuclear abnormalities that are reminiscent of the atypia of human neoplasia. Each mutation appears to be homozygous lethal. This screen is proof of principle for the feasibility of histologic screens to yield novel mutations, including potential models of human disease. The throughput for this type of screen may be enhanced by automation. Developmental Dynamics 228:414–423, 2003.

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Key words: zebrafish mutant; larval arrays; histology; cell differentiation; retina; cancer

Received 14 May 2003; Accepted 4 August 2003

INTRODUCTION

Infinite combinations of architectural and cytologic features of tissue cells are detectable by the light microscopic study of stained tissue sections. Histologic studies distinguish a multitude of cell types, cell processes, and abnormalities. This power is routinely exploited in the field of pathology, where histologic features are used to assess biologic behavior, identify abnormalities in cell differentiation, and evaluate cell injury in human diseases, including cancer (Demay, 1996; Cotran et al., 1999). We reasoned that the small size of zebrafish larvae containing primordia of most adult tissues, combined with the high resolution and sensitivity associated with light microscopy of tissue sections, could be harnessed to detect subtle changes in cellular structure. Cloning of such mutations would allow the dissection of their genetic basis. The subtlety of many lesions routinely assessed in pathology indicate that many abnormalities in organogenesis and/or cytology would be difficult or impossible to detect by microscopy of live larvae, even when transparent. Pigmentation of the larvae further impairs detection of cel-
cular changes. We expected a histology-based screen to find previously undiscovered mutants for two reasons. First, stained tissue sections provide a higher resolution than unsectioned, unstained, whole embryos, as used in two large published screens (Haffter et al., 1996; Dreier et al., 1996). Second, organogenesis is more advanced in 7-day larvae than in embryos or younger larvae, which permits identification of mutations affecting later stages of cell differentiation. The ability to embed zebrafish larval arrays in agarose consisting of 64 larvae per block, developed in our laboratory (Tsao-Wu et al., 1998) made the screen possible. The first histology-based genetic screen, performed in Drosophila, was reported by Heisenberg and Bohl (1978). Here, we report the first histology-based genetic screen in a vertebrate.

Heterozygous mutations in carrier females were rendered homozygous in their progeny by gynogenesis (Streisinger et al., 1981; Cheng and Moore, 1997). Seven recessive mutations with novel phenotypes were obtained from this initial screen, demonstrating that histology-based screens can detect interesting mutants that do not otherwise exhibit any easily identifiable nonhistologic phenotypes. The mutants reported here include two with defective retinal photoreceptor differentiation, three with abnormal differentiation of the intestinal epithelium, and two with multiorgan defects. The latter two show disruption of retinal layers in addition to cytologic abnormalities in multiple organs. The mutants found include models of retinal dysplasia and of cytologic dysplasia seen in human cancer. The mutations identified in this screen are different from those reported in earlier zebrafish screens, further validating the utility of histology-based screens.

RESULTS AND DISCUSSION

Half-tetrad progeny were produced from F1 females carrying N-ethyl-N-nitrosourea (ethylnitrosourea, ENU)-induced mutations and were screened for histologic defects at 7 days of development (Fig. 1). The small size of zebrafish larvae (~5-mm-long at 7 days postfertilization) made it possible to fit a section from an array of 64 larvae on one microscope slide (Tsao-Wu et al., 1998). The screen was performed at 7 days, because most organs have distinct cell patterns and appearances by that age (Kimmel et al., 1995). Larvae with gross abnormalities noted under a dissection microscope were discarded, based on the assumption that mutations causing such defects would have been found in past genetic screens. We chose not to use older larvae because gut mutations affecting absorption would be expected to be lost beginning on day 8, when yolk is normally depleted. A total of 3,281 larvae from 72 histology arrays of up to 7-day-old parthenogenetic half-tetrad larvae per F1 female were initially screened at ×25–×400 magnification, and as necessary, examined at ×1,000 magnification for additional cytologic detail. Among the embedded larvae, 2,038 (62%) larvae were screened for eye morphology and 1,670 (51%) were screened for intestinal morphology in at least one of three stained sections. Seven putative mutants were obtained, all of which showed transmission to at least one succeeding generation and exhibited Mendelian inheritance. Five organ-specific and two pleiotropic mutations were found (Table 1). Of these, vos and hht are described in greater detail.

Eye-Specific Mutants

The two eye mutants vestigial outer segments (vos) and segmented photoreceptors (spr) appear normal under the dissecting microscope. Histologic examination of these two mutants revealed defects specific to the photoreceptor cell layer (PCL) of the retina. The photoreceptor cells of the wild-type 7-day zebrafish eye are elongated and are oriented perpendicular to the outer circumference of the eye (Fig. 2A,B). The PCL consists of the outer nuclear layer, and inner and outer segments. The outer nuclear layer stains blue with

Fig. 1. Genetic screen for histologic mutants in zebrafish. N-ethyl-N-nitrosourea (ethylnitrosourea, ENU) was used to mutagenize adult males, which were outcrossed with homozygous golden (gol) females to generate F1 progeny for screening. Early pressure partheno-
genesis (EP) was used to generate half-tetrad from F1 females. At 7 days of age, half-tetrad larvae were fixed in 10% neutral buffered formalin, and up to 64 sibs from individual EP families were arrayed in individual agarose blocks. After embedding in paraffin, 6-μm-thick sections were cut and stained with hematoxylin and eosin for histologic screening. F1 carriers that generated half-tetrad progeny with interesting phenotypes were then outcrossed for further study, including confirmation of Mendelian inheritance. m, mutant.

Fig. 2. vos specifically affects the photoreceptor cell layer of the developing zebrafish retina. Figure depicts coronal sections through the eye. A–D: Wild-type retina. E–H: vos mutant retina. A,B,E,F: hematoxylin and eosin-stained sections; C,D,G,H: are scanning electron photomicrographs. B,F: Enlarged views of the photoreceptor cell layer (indicated between arrowheads in A and E, respectively). C,D,G,H: Outer segments of the photoreceptor cells; D and H are the magnified images of the boxed area in C and G, respectively. npef/c, retinal pigment epithelium/cells; is/OS, inner segment/outer segment of the photoreceptor cells; onl, outer nuclear layer; inl, inner nuclear layer; ipil, inner plexiform layer; gcl, ganglion cell layer; opil, outer plexiform layer; the space between the white arrowheads indicates the thickness of the photoreceptor cell layer; asterisks, photoreceptor outer segment; red arrows, vacuolar spaces; black arrowheads, mitochondria. Scale bars = 10 μm in A,B,E,F, 1 μm in C,D,G,H.

Fig. 3. The loss of outer segments of the photoreceptor cells in vos mutants occurs at 5 days postfertilization (dpf). Embryos (from 3 to 6 dpf) from pairwise crosses of previously identified vos heterozygotes were fixed, embedded, sectioned, stained with hematoxylin and eosin, and were examined under the light microscope for any abnormalities in the developing retina. At 3 and 4 dpf, the developing retina looked normal in all embryos (data not shown). At 5 dpf, in approximately 25% of the embryos (vos), the outer segments are not apparent (A,B, wild-type; C,D, vos). In addition, the retinal pigment epithelium layer in vos appears to be thicker than the wild-type. At 6 dpf, approximately 25% of the embryos (vos) continue to exhibit the loss of outer segments (data not shown). A,C are low-power views showing all the cell layers of the developing retina; B,D, are enlarged views of the photoreceptor cell layer (between red arrowheads). Scale bars = 10 μm.
vacuolar spaces suggestive of disordered neural retina (dnr) are also scorable by light microscopy (Fig. 2E,F). The photoreceptor cell layer in spr mutants is formed in patches. At 7 days postfertilization, spr mutants do not have a continuous photoreceptor cell layer (PCL). The PCL is present as patches (indicated by white arrowheads). There is also no distinct outer plexiform layer in spr mutants (compare with wild-type in Fig. 2A). Scale bar = 10 \( \mu \text{m} \).

Intestinal epithelial cells are ballooned in kby (C) and lack the brush border (D); the lumen is indicated by asterisks. Mucus accumulates in sfy (E); abnormal clumps of mucous cells are occasionally found in sfy mutants (E, F, black arrowheads). wt, wild-type. Scale bar = 10 \( \mu \text{m} \) in A-F.

Mutants with multiorgan defects. The mutations dnr (A,B,D) and hht (E-H) show architectural and cytologic effects in multiple organs. At low power, disruption of the neural retina is evident in both dnr (A) and hht (E). The dnr mutation shows cellular atypia in the intestine (white arrowheads in B; compare normal size with normal intestine in Fig. 3A) and liver (white arrowhead in D; compare with normal liver shown in C). Atypical cells of hht (F,G) include giant cells in the intestine (white arrowheads in F; a single giant cell is shown in G), pancreas (P), central nervous system, and other endodermally derived epithelia. An atypical mitosis in hht pancreas is shown in F (downward arrow, lower left) and H (arrowhead). Cells with nuclear atypia were present in proximal intestine and pancreas (F, black arrows). Scale bars = 10 \( \mu \text{m} \) in A-H.

The distributions of nuclear areas in wild-type vs. hht intestine. The area of 100 hht and 100 wild-type intestinal nuclei were obtained as described in Experimental Procedures section and plotted by size, wild-type in black and hht in red.
was less than 10% of that seen in wild-type retina (not shown).

However, the electron photomicrographs show that vestigial outer segments are present in vos (Fig. 2G,H); in the outer segments in vos are oriented parallel, rather than perpendicular, to the circumference of the eye (Fig. 2H). To determine the time of onset of the vos phenotype, we examined larvae obtained from a pair of vos heterozygous parents from 2 to 7 days postfertilization (dpf). Histologically, photoreceptors can be distinguished at 2 dpf with the outer segments beginning to appear by 2.5 dpf (Branchek and Bremiller, 1984). The first defects were detected in the photoreceptors of vos larvae at day 5, when 25% of the larvae from vos heterozygous parents from 2 to 7 days postfertilization (dpf) had defects (Branchek and Bremiller, 1984). We do not know whether vos mutants first develop an intact PCL that is then lost in patches or whether the PCL forms only in patches to begin with. This mutant did not exhibit any other defects identifiable under the dissection microscope. This defect was fully penetrant and followed Mendelian inheritance. Unfortunately, the vos mutation was lost before we could perform further characterization.

### Gut-Specific Mutants

Three mutations, kirby (kby), stuffy (sfr), and stuffed (sf) affect only the intestine. kby is characterized by balloononed gut epithelial cells, which also display perinuclear clearing (Fig. 5C,D; compare with the corresponding wild-type sections in 5A,B). In addition, the epithelial cells are deficient in brush borders (Fig. 5D). The balloononed cells suggest degenerative changes, while brush border deficiency suggests a defect in cell differentiation. The nuclei in kby intestinal epithelium (Fig. 5D) are larger and more variable in size and shape than wild-type (Fig. 5B). Occasional nuclear fragments and pyknotic nuclei are also found in kby (not shown). Further study of this mutant may provide insights into mechanisms of cell differentiation, nuclear atypia, and cell degeneration in the intestine.

The proximal intestine of wild-type 7-day larvae contains little to no mucus (Fig. 5A,B). In the distal intestine, small amounts of mucus are associated with scattered, solitary mucus cells surrounded by absorptive epithelium. However, in the two intestinal mutations, sfr and sf, there is increased luminal mucus in the proximal intestine (Fig. 5E, only sfr is shown). In addition, in sfr, clusters of mucus cells appear in the proximal intestinal epithelium (Fig. 5E,F, indicated by arrowheads). The mucus present in these mutants stains with the same weak intensity as in wild-type with both mucicarmine and colloidal iron mucus stains (Luna, 1960; not shown). All three gut mutants are fully penetrant and show Mendelian inheritance in outcrosses. None of these phenotypes were described among the previously described zebrafish intestinal mutants (Pack et al., 1996; Chen et al., 1996).

### Pleiotropic Mutations

Two mutations showed multiorgan and cytologic defects (Fig. 6). The first, disordered neural retina (dnr), exhibits defects in the eye, intestine, and the liver (Fig. 6). The eyes of dnr mutants show severely disrupted architecture of the neural retina (Fig. 6A). Histologically, the lens and retinal layers of dnr are poorly formed. The different layers of the retina are not distinct and have a disordered appearance. The dnr retina contains scattered nuclear fragments and vacuolar spaces. This eye phenotype is detectable as early as day 3 (not shown). The dnr gut phenotype is characterized by a consistent nuclear pleomorphism of the intestine (Fig. 6B) and nuclear enlargement in the liver (Fig. 6D; compare with nuclei of normal liver in 6C). The nuclear atypia in the intestine and liver of dnr mutants cosegregated consistently with the eye phenotype, suggesting that a mutation in the dnr gene is responsible for these defects. The dnr mutants could be identified by their smaller eye phenotype with the help of a dissection microscope. However, their gut defects could not be detected without histologic examination. The mutant larvae do not survive to adulthood. Unfortunately, this mutant has also been lost before further characterization was possible.

The second pleiotropic mutation, hull futu (hhf), causes striking architectural and cytologic changes in several organs (Fig. 6E-H). The eyes of a variable fraction of mutants are reduced in size. The cell types of different retinal layers are mixed together to varying degrees. In the most extreme cases, no discernible retinal layering is evident, similar to what is seen in dnr. The eye phenotype is detectable at 2 dpf (not shown) and can be mild to severe at 7 dpf. The retinal disorganization is also associated with scattered nuclear fragments suggestive of apoptosis (Fig. 6E). Accordingly, the
number of nuclei per cross-section of the eye was 25% to 75% of normal. The decrease in cell number and severity of retinal disorganization were roughly proportional to the degree of disruption of layers and to eye size, which ranged from normal to approximately 75% of normal. The nuclear defect is not evident in muscle, blood, gills, or blood vessels.

The most interesting of the hht phenotypes is severe cytologic atypia in the intestine, pancreas, liver, swim bladder, and pneumatic duct. This atypia includes a great variability in nuclear size and shape, frequent multinucleation, and architectural disorganization (Fig. 6F,G). There were occasional giant cells in the intestine and pancreas (Fig. 6F,G). Despite architectural disorganization (Fig. 6F,G).

An intriguing but rare finding was tripled mitosis (Fig. 6H); the significance and potential relationship of this phenomenon to the other phenotypes of hht remains to be determined.

To quantify the degree of nuclear enlargement in hht intestine, cross-sectional nuclear areas were measured digitally for the intestinal epithelial nuclei of 100 cells of hht larvae and wild-type siblings. The area of hht nuclei vary in size and are significantly larger than wild-type (Fig. 7); mean nuclear areas were 34.8 \( \mu \text{m}^2 \) for hht and 12.1 \( \mu \text{m}^2 \) for wild-type. Because the density of stain is at least as dark as wild-type, we expect this difference to reflect differences in DNA content. The degree of nuclear irregularity, which correlates with malignant potential in human cancer (DeMay, 1996), was measured as the ratio of circumference to nuclear area (“circularity”): greater values correlate with increased irregularity. The mean circularity was 16.9 for hht and 14.3 for wild-type. Thus, the nuclei of intestinal epithelial cells in hht are larger, more variable in size, and are irregular in outline compared with wild-type.

### Complementation Analyses

To determine the number of genes corresponding to the seven recessive mutations found, we performed complementation crosses and half-tetrad mapping. Histologic analysis of reciprocal complementation crosses between known carriers of the four mutations with eye phenotypes (spr, vos, dnr, and hht) showed only wild-type progeny, consistent with these mutations occurring in four different genes. Half-tetrad genetic mapping (Streisinger et al., 1986; Mohideen et al., 2000) of the five mutants with intestinal phenotypes (kby, sfy, sfd, dnr, and hht) showed that they reside on different chromosomes. Each of the seven mutants were obtained from a different ENU-treated G0 male. Taken together, the data suggest that we have identified seven different single locus mutations.

### Genetic Map Positions

Five mutations (vos, kby, sfy, sfd, and hht) were mapped to specific chromosomes arms on the SSR genetic map by centromere-linkage mapping (Table 1); fine mapping for vos and hht are in progress. Mapping of spr and dnr is incomplete due to loss of these mutant lines.

### Viability

The mutations identified in this screen are lethal after 8 dpf. Up to this time, the eye-specific mutants spr and vos, as well as the gut mutants kby, sfy, and sfd and the multi-organ mutant dnr look normal, with some hht mutants exhibiting small eyes. However, they all die soon after 8 dpf. We speculate that the histologic defects in spr and vos might render them blind, which inhibits their feeding ability. After 8 dpf, the yolk becomes depleted and the larvae depend on food from their environment for survival. We also believe that, in kby, sfy, and sfd, their histologic gut defects may cause them to die because of insufficient food absorption. The combined defects in both the eye and gut in dnr and hht confer both disadvantages. Progeny from crosses between heterozygotes of these mutations have been grown to adulthood. Homozygotes have not been recovered. In the case of vos and hht, we have genotyped adults with tightly linked SSR markers and have not detected any homozygotes among progeny derived from known heterozygotes.

### Frequency of Mutants

In this screen, half-tetrad progeny from heterozygous mothers were screened for homozygous mutations affecting their histologic appearance. Because centromeric mutations are more frequently homozygous than are telomeric mutations in half-tetradss (Streisinger et al., 1986), half-tetrad screens have a bias toward detecting mutations that tend to be close to centromeres (Grunwald and Streisinger, 1992; Henion et al., 1996). Thus, the method used in this report will underestimate the total number of mutations that were induced and that could be detected in the histologic screen. Three levels of histologic sections were examined per larva in this screen. Examining additional levels per block, while increasing the amount of work per block, might be expected to yield additional mutants. Under our conditions, approximately 1 in 10 mutagenized F1 individuals had a histologically detectable mutation, whereas 1 in 1,000 F1s had a mutation at a single predefined locus, golden. We estimate that approximately 100 genes exist that, when mutated, will yield defects not seen in the dissecting scope but seen with hematoxylin and eosin.

### Relevance to Human Disease

The mechanisms responsible for defective photoreceptor layers in spr and vos may relate to one or more human genetic retinal diseases (He Witt and Adler, 1994). In particular, the histologic phenotypes of photoreceptor cell layer and pigment cells of vos are similar to human retinal degenerative diseases such as retinitis pigmentosa. For example, a similar deficiency of outer segments is seen in mice with mutations in the otx-related homeobox gene cux (Furukawa et al., 1997, 1999). Mutant alleles of cux in man are associated with retinal degeneration syndromes, including cone–rod dystrophy, Leber congeni-
tal amaurosis, and retinitis pigmentosa (Furukawa et al., 1997, 1999; Freund et al., 1997; Swain et al., 1997; Sohocki et al., 1998; Swaroop et al., 1999). The zebrafish cdx homolog has been cloned and subsequently mapped to LG05 on the zebrafish genetic linkage map (Liu et al., 2001). However, we have confirmed that vos and spr are not alleles of cdx; because the vos locus is on LG23 and spr is not linked to LG05.

Histologic sections allow us to detect changes otherwise hidden beneath normal, opaque choroidal pigments that appear after day 3 in zebrafish. Both mutant phenotypes are clearly different from those with photoreceptor defects found in earlier screens (Malicki et al., 1996; Fadool et al., 1997; Neuhauss et al., 1999). The photoreceptor mutants identified in previous screens have small eyes and are usually associated with additional phenotypes such as poor touch response, brain defects, and abnormal pigmentation. The vos and spr mutants have normal-sized eyes and do not have any other scoreable defects. Only upon histologic examination are their photoreceptor cell defects apparent. It is unclear whether the primary defect in either mutant lies in the photoreceptor cells themselves or surrounding cells, such as the pigmented epithelium, upon which differentiation or maintenance of the photoreceptor outer segment depends (Hewitt and Adler, 1994).

The common histologic phenotypes of human cancer include disorganization of tissue architecture, invasion, and a variety of cytologic features, including nuclear hyperchromasia, nuclear pleomorphism, irregular nuclear outlines and chromatin patterns, multinucleation, and multipolar mitoses (Frost, 1986; Dernay, 1996; Cotran et al., 1999). Despite the critical importance of these histopathologic phenotypes in clinical medicine, their genetic origins remains largely unknown. To our knowledge, the hht mutation is the first vertebrate mutant to be identified by its atypical histologic phenotype. Because the hht phenotype includes several of the cytologic features of high grade dysplasia, which frequently progresses to cancer, further studies may contribute to our understanding of cytologic cancer phenotypes. It will be interesting to determine whether hht heterozygotes are susceptible to cancer.

Rudolf Virchow not only played a key role in defining the cellular basis of disease, largely as revealed by histologic analysis, but also pointed out the importance of genetics in gaining understanding of normal and abnormal cell physiology (Virchow, 1855). This screen reflects the first application of a classic genetic approach to these fundamental ideas.

Potential Throughput of Histologic Screens

A histology screen such as the one reported here involves several steps, some of which can be automated to improve the throughput of the screen. First, the embedding step involves careful positioning of individual larvae into each agarose well. The maximum speed we have achieved for embedding is approximately 10 min per block of 64 larvae, or approximately 10 sec per larva. We imagine that a mold that closely matches the shape of the larva may allow more rapid positioning than presently possible. There is potential for robotic positioning of the embryos (Schneider, 2003) and automated removal of excess water from the well that otherwise causes poor sections. The next step that could be automated is that of scoring sectioned and stained embryos. While screening of slides of larval arrays is most rapidly done by an experienced human operator, written and image documentation of mutant phenotypes can be greatly facilitated by combining database software with virtual slide scanning software such as that offered by MicroBrightField (Williston, VT) or Aperio Technologies (Vista, CA). The virtual images may be digitized, individual embryos recognized and labeled, and linkage to appropriate database fields be automated. Our laboratory is currently developing such automations to facilitate histologic screening of zebrafish larvae. Increases in the throughput of histologic screens can be applied not only for genetic screens but also for toxicologic, forward genetic (Nasevicius and Ekker, 2000), and chemical screens (Peterson et al., 2000).

EXPERIMENTAL PROCEDURES

Zebrafish Strains and Fish Care

Wild-type male zebrafish used for mutagenesis were obtained from Ekkwill (Gibsonton, FL) and Liles (Ruskin, FL); microsatellite alleles in the two strains were similar at several loci (unpublished data). Homozygous goldenP homozygous females used for crosses with mutagenized males were descendants of goldenP fish obtained from the Zebrafish Resource Center, University of Oregon. A strain obtained from India was initially used to introduce polymorphisms by crosses to identified female carriers; these were discarded after poor breeding of the progeny. The fish were raised in a recirculating system as described (Beckwith et al., 2000). Fish were maintained and bred for generating larvae as previously described (Westerfield, 1996). Before fixation for histology, larvae were anesthetized using tricaine methane sulphonate (Westerfield, 1996).

Mutagenesis

Males, 7- to 9-month-old, were used for mutagenesis. The males were selected for high fertility by previous pair-wise matings. Germline mutations were induced in adult male zebrafish with 2.5 mM ENU (Sigma), as described (Mullins et al., 1994; Beckwith et al., 2000). Mutagenized males were first crossed 1 week after mutagenesis to enable expulsion of sperm with single-strand DNA damage. Beginning 2 weeks postmutagenesis, males were outcrossed to homozygous goldenP females to determine germline mutation rates and to generate F1 for screening.

Early Pressure Parthenogenesis

Mutations induced by ENU in G0 males and carried in ~6-month-old F1 females were made homozygous by “early pressure” gynogenesis (Fig. 1; Streisinger et al., 1981) using conditions described previously (Gestl et al., 2001). However, we have confirmed that vos and spr are not alleles of cdx; because the vos locus is on LG23 and spr is not linked to LG05.

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for further study. Outcrossed to the India or AB strains interesting histologic phenotypes were for further study or for extracting DNA remaining sections were left unstained protocols (Luna, 1960), and the re- toxin and eosin by using standard slide per set was stained with hema- matoxylin and eosin by using standard protocols (Luna, 1960), and the remaining sections were left unstained for further study or for extracting DNA for genetic mapping. F1 females that produced half-tetrad progeny with interesting histologic phenotypes were outcrossed to the India or AB strains for further study.

Histology

Seven-day-old larvae without any visible defects were fixed in 10% neutral buffered formalin for 16 to 64 hr at 4°C. Arrays of 32 to 64 larvae per clutch were embedded in individual agarose blocks, processed into paraffin, and cut into 6-μm-thick sections, three sets at a time as described previously (Tsao-Wu et al., 1998). One slide per set was stained with hema- toxylin and eosin by using standard methods. The 35-mm slides were scanned using a Polaroid SprintScan 35 Plus scanner (Cambridge, MA), and labelling and adjustments were done with Adobe Photoshop.

Genetic Mapping

Mutations were first mapped to a chromosome arm by centromere linkage analysis as used by Johnson et al. (1995), using markers described previously (Mohideen et al., 2000). For genetic mapping, DNA from mutant half-tetrad larvae as well as from wild-type sibs were extracted from corresponding unstained, deparaaffinized sections as described previously (Tsao-Wu et al., 1998). Centromere-linkage analysis was performed using SSR markers that are linked to the centromeres of the 25 linkage groups (Mohideen et al., 2000). Upon establishing linkage to a specific linkage group, markers flanking the centromere were used to determine the specific arm on which the mutation is located.

Electron Microscopy

Larvae were fixed for electron microscopy in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M sodium cacodylate buffer (Sigma) at 4°C for 2 hr, or in 1% glutaraldehyde 4% Ultra- pure formalin (EM Sciences) in 0.1 M cacodylate buffer at 4°C for 2 hr. Fixed larvae were washed in 0.1 M cacodylate buffer on ice, post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr, dehydrated in prechilled graded alcohol solutions, and embedded in EMBed 812 (EM Sciences). Light microscopy of methylene blue–stained 1-μm transverse sections were used to screen sections for electron micro- copy. Ultra-thin, silver–grey sections (60 nm) were cut from preselected blocks, stained with uranyl acetate and lead citrate, and examined on a Philips 400 transmission electron micro- scope.

Photography

Sections were photographed with Kodak Ektachrome T160 slide film on a Zeiss Axioshot with a Zeiss MC spot camera, using a variety of objectives. The 35-mm slides were scanned using a Polaroid SprintScan 35 Plus scanner (Cambridge, MA), and labelling and adjustments were done with Adobe Photoshop.

Morphometry

For cell counts in hht, nuclei were counted manually on 8-× 10-inch enlargements obtained by using a color photocopier of full coronal sections of normal and hht eyes. Nuclear size and irregularity were determined by digital photography of images from 6-μm thick, hematoxylin- and eosin-stained sections of 7-day-old wild-type and hht siblings derived from early pressure parthe- nogenesis or crosses. Digitized images were acquired from the slides by using an Olympus BH-2 microscope with a Zeiss ×40 dry objective, a Sony DKC-5000 digital camera, and Adobe Photoshop. Outlines of digital images of 100 nuclei, each from several wild-type and mutant larvae, were traced by using Opti- mas 4.2 software (Silver Spring, MD) to calculate nuclear areas and circumference/area ratios.

Acknowledgments

We thank Lynn Budgen and Xiao- Hong Wang for histologic work, Dr. Ian Zagon for help with determining nuclear areas, Roland Meyers for electron microscopy, Michele Aros for technical assistance, and Peggy Hublel for fish facility management. This work was supported by the Jake Gittlen Memorial Golf Tournament and by grants from the NIH (to K.C.C. and J.L.M.) and the NSF (to K.C.C.).

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