

## Genetic dissection of the retinotectal projection

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### SUMMARY

A systematic search for mutations affecting the retinotectal projection in zebrafish larvae was performed, as part of the large-scale Tübingen screen for homozygous diploid mutants in embryonic development. 2,746 inbred lines (F<sub>2</sub> families) from males mutagenized with ethylnitroso urea were screened. In wild-type larvae, developing retinal axons travel along a stereotyped route to the contralateral optic tectum. Here, their terminals form a highly ordered retinotopic map. To detect deviations from this pattern, an axon tracing assay was developed that permits screening of large numbers of mutagenized fish. Two fluorescent tracer dyes (DiI and DiO) were injected at opposite poles of the eyes of day-5 aldehyde-fixed larvae. 12 hours later, retinal axons were labelled over their entire length, and could be observed through the intact skin. The assay procedure (aldehyde fixation, mounting, injection of dyes, microscopic analysis) took about 1 minute per fish. In total, 125,000 individual fish larvae were processed.

During the screen, 114 mutations in approx. 35 genes were discovered. For the mutants subjected to complementation testing, the number of alleles per locus ranges from 1 to 15. The mutations affect distinct steps in the retinotectal pathway, from pathfinding between eye and tectum to map formation along the dorsal-ventral and the anterior-posterior axis of the tectum. Mutations that disturb axon pathfinding to the tectum for the most part do not disrupt retinotopic mapping, and vice versa. The majority of the mutants display associated defects in other tissues and die before day 10. These mutants provide new tools for studying the formation of neuronal maps. The results of this screen show that a large-scale genetic approach can be applied to relatively late and circumscribed developmental processes in the vertebrate brain.

Key words: zebrafish, *Danio rerio*, mutant screen, visual system, brain map, neuronal specificity

### INTRODUCTION

During development of the nervous system, neurons become connected to each other in a highly specific and spatially ordered fashion (see Goodman and Shatz, 1993, for a review). A classical system for the study of neuronal specificity is the retinotectal projection of vertebrates (Sperry, 1963). This projection is set up in two steps. First, after leaving the eye, the growth cones of retinal ganglion cells navigate through the brain to find the contralateral tectal lobe, their principal target (see Chien and Harris, 1994, for a review). Second, once they have entered the tectum, retinal axons travel further to their individual target sites within the tectal field. Here, the projection is topographically organized in that neighbouring retinal cells connect to neighboring places in the tectum (see Sanes, 1993, for a review). Thus, the retinal image is topologically mapped onto an area of the brain that is concerned with processing of visual information.

Little is known about how retinotectal specificity is achieved. Map formation, for the most part, does not depend on visual input or other forms of electrical activity and is either precise from the outset (in fish and amphibians) or follows a

fixed sequence of axon navigation and branching (in chick and rodents; for reviews see O'Leary and Simon, 1992; Holt and Harris, 1993). These observations, among others, suggest a high degree of molecular specificity (or 'chemoaffinity'; Sperry, 1963) between ingrowing fibers and their targets. According to one model, the directional information to the ingrowing neurites could be provided by just a few guidance molecules distributed as gradients in the tectum (Gierer, 1983). The growth cones sample environmental information by means of surface receptors and associated signal transduction cascades that redirect the cytoskeletal growth machinery. The expression of both the tectal guidance cues and the retinal receptors must be tightly regulated in time and space and may link the retinotectal projection to earlier developmental processes.

Two strategies for the most part have been employed to identify molecules that guide retinal axons to their targets and provide for retinotectal specificity. In one approach, candidate molecules were isolated by screens for monoclonal antibodies that recognize antigens with striking expression patterns in the tissue. Several molecules that are expressed as gradients in the retina or the tectum were discovered (e.g., Trisler et al., 1981;

Trisler and Collins, 1987; Constantine-Paton et al., 1986; McLoon, 1991). However, the demonstration of a specific function for these molecules generally proved difficult. A different means of analysis was the biochemical approach that used functional assays to reproduce the process of interest (or part of it) in vitro. One of the first such in vitro assays showed that retinal axons from the temporal half of the retina prefer to grow on anterior tectal cells over posterior cells, resembling their in vivo behavior (Bonhoeffer and Huf, 1982). Using a modification of this assay, the stripe choice assay (Walter et al., 1987), it was possible to isolate and microsequence one of the tectal proteins involved, and clone the corresponding gene (RAGS: Drescher et al., 1995). The strength of the functional biochemical approach is further exemplified by the discovery of axon guidance molecules in other systems (collapsin I: Raper and Kapfhammer, 1990; Luo et al., 1993; netrins: Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994). However, this approach is limited to systems where adequate in vitro assays are available.

Here, we set out to explore the retinotectal projection in a third, more systematic approach by taking advantage of techniques recently developed to generate and isolate mutants in zebrafish (Grunwald and Streisinger, 1992; Mullins et al., 1994; Driever et al., 1994). Mutational disturbances offer the possibility to obtain a more complete and unbiased picture of processes that lead to an ordered projection, even before knowing the mutated genes. Inherently, a mutant screen cannot be targeted to gene products directly involved in axon guidance (like cell surface molecules), since projection defects may also arise from mutations in upstream genetic pathways that control the development of key tissues. This can be considered as an advantage since it helps to put axon guidance in a more general developmental context. A combination of functional approaches, biochemical as well as genetic, is likely to identify the key molecular players much more efficiently than any single approach. So far, large-scale genetic screens in animals have only been possible for *Drosophila melanogaster* and *Caenorhabditis elegans* (e.g., Nüsslein-Volhard and Wieschaus, 1980). In these organisms, mutant screens have recently uncovered genes that are important for axon guidance and neuronal target recognition (Hedgecock et al., 1985; Kunes et al., 1993; Seeger et al., 1993; van Vactor et al., 1993; Martin et al., 1995). Before the advent of zebrafish genetics, screens of a similar dimension could not be carried out on a vertebrate.

The success of a mutant screen in general depends on whether the process of interest can be disturbed by mutation of a single gene. Vertebrates, as a rule, possess families of genes with overlapping expression patterns and seemingly overlapping functions (e.g., Joyner et al., 1991). Based on this observation, it is currently debated to what extent single genes determine phenotypic features in vertebrates. Redundant or regulatory mechanisms may compensate for loss of a single gene product, thus making it difficult to identify genes by loss-of-function phenotypes. By screening for mutations in a circumscribed developmental process, like in the retinotectal projection, we could test whether 'forward genetics' is useful at all to discover interesting new genes in the vertebrate genome.

Zebrafish are well suited for studying the retinotectal projection by a genetic screen. The development of the projection has been described in detail (Stuermer, 1988; Stuermer et al., 1990; Kaethner and Stuermer, 1992, 1994; Burrill and Easter,

1994, 1995) providing indispensable background information for the analysis of mutants. Retinal axons leave the eye during the second day postfertilization (dpf) and occupy their appropriate tectal zone by the end of the third day. Thus, a precise retinotopic map is formed very early, when the tectal neuropil is only 200  $\mu\text{m}$  in diameter. The distance from the eye to the tectum is less than 500  $\mu\text{m}$  allowing lipophilic axon tracer studies in fixed tissue (Godement et al., 1987). While tecta and retinae grow by addition of new cells, the map adapts accordingly thus ensuring a stable representation of the retinal image for the rest of the animal's life. Fig. 1 gives a semi-schematic overview of the retinotectal projection in zebrafish larvae at the stage screened (5 dpf).

For any systematic mutant screen, two technical requirements have to be met. First, methods have to be established to produce and keep large numbers of inbred lines. This was done for the Tübingen screen as described by Mullins et al. (1994), Brand et al. (1995), and Haffter et al. (1996). The retinotectal screen was conducted after the screen for early embryonic mutations performed in our institute (Haffter et al., 1996). Second, a quick and reliable screening assay needs to be developed to handle large numbers of mutagenized individuals. This paper describes the design of the retinotectal screen, the screening method, and the general outcome of the screen. A description of the mutant phenotypes is given in two accompanying articles (Trowe et al., 1996; Karlstrom et al., 1996).

## MATERIALS AND METHODS

### Mutagenesis and general conditions of the screen

Mutagenesis of the founder population (males of  $F_0$ ) and production of homozygous diploid larvae by inbreeding over two generations ( $F_1$  and  $F_2$ ) were performed in the Department of Genetics of our institute (Mullins et al., 1994, and Haffter et al., 1996).  $F_2$  lines ('families') were screened by random crosses between siblings. The resulting  $F_3$  progeny was analyzed. If a mutation was present in a family, then 25% of the crosses yielded homozygous mutant embryos. Generally, fish were raised and bred as described by Mullins et al. (1994) and Brand et al. (1995). For the retinotectal screen, larvae were screened at 6 dpf, at a stage in wild type when an orderly retinotopic map has already formed (Stuermer, 1988). The same fish were previously screened morphologically for early embryonic mutations. No care was taken to control the duration or degree of visual stimulation of the larvae.

On average, slightly more than one mutation affecting early embryonic development could be identified in any  $F_2$  family screened (Haffter et al., 1996). These mutant embryos were sorted out into separate Petri dishes prior to the retinotectal screen. We screened both unsorted and presorted batches. Most of the retinotectal mutants identified display other abnormalities (see Results) and were therefore detected in these presorted batches.

We screened the same  $F_2$  families (2,746) as Haffter et al. (1996). However, since the time spent for processing an individual fish was longer for the retinotectal screen, we analyzed only 8 individual larvae for each cross (as compared to 12-30 in the preceding screen). This means that for most mutants only one or two individuals were seen in the primary screen. We calculated that this reduction should not change the screening output to any significant extent. The basis for this calculation is given in the Appendix to this article.

### Fixation and mounting of larvae

The medium (E3; Brand et al., 1995) was drained from the larvae and they were floated into multi-well plates (24 wells; Nunc) with a few

drops of fresh medium. The larvae were then exposed to bright light under a halogen lamp for at least 1 minute. This treatment induces their melanophores to aggregate pigment granules and become translucent. Otherwise melanophores with dispersed granules sitting in the dorsal head epidermis may obscure the view of the optic tectum and thereby later impair the analysis of retinotectal phenotypes. For fixation, approx. 3 ml of ice-cold fixation buffer (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) were added to each well. The minimum fixation time was 12 hours at room temperature. During the screen, fish were processed further within a week after fixation. If necessary fish could be stored in fixation buffer for long periods before injection.

Fixed fish larvae were mounted at defined positions and orientations in agarose gels. This was done in order to achieve quick and reproducible dye injections and fast microscopic analysis. This also made it possible to keep track of individual fish by their spatial coordinates in the gel. During the primary screen, 8 fish from each clutch were mounted and their identities noted on a protocol sheet. The mounting apparatus is shown in Fig. 2. The bottom consists of a rectangular piece of aluminium containing a perpendicular array of 8 vertical and 12 horizontal grooves (shown in detail in Fig. 2D). The apparatus was built by the institute's machine shop. The pieces making up the agarose container (the aluminium bottom and the side walls) are sealed with silicone grease (Baysilone, highly viscous; Bayer). The grooves serve as molds for the fish providing for 96 individual mounting positions (the crossing-points of the grooves; Fig. 2D). The aluminium piece can be removed from the apparatus and stored in acetone for cleaning. Before use, it is washed in ethanol, dried, and briefly immersed in a silicone solution (Wacker, SF18). The silicone provides for an anti-adhesive coating so that, after mounting, the agarose gel containing the fish can easily be removed. The silicone coating has to be renewed once in about every ten uses.

The most appropriate mounting medium was found to be low-melting temperature type agarose (Sigma) at a concentration of 1.2% in low-strength phosphate-buffered saline (PBS). (To obtain low-strength PBS, regular PBS is diluted 1:3 in deionized water.) The agarose is heated to 100°C and poured into the mounting apparatus. Here, it is allowed to cool down to 45°C, and the mounting apparatus is held at this temperature to keep the agarose liquid. The temperature is controlled by pumping heated water through copper tubes at the bottom of the apparatus (not shown in Fig. 2).

Prior to mounting, the larvae from one cross are transferred from fixation buffer into one of twelve small chambers filled with low-strength PBS (Fig. 2C). The chambers were milled as rows of six into two blocks of plastic, which can be inserted into the side walls of the mounting apparatus (Fig. 2A,B). Larvae are placed with watchmaker's forceps into their positions within the molds. To carry the fish without breaking them small notches were ground into the tips of the forceps. The fish are mounted dorsal side down into the grooves as close as possible to the bottom. They are exactly positioned under a dissecting microscope with a steel needle. The dissecting microscope is mounted on two rails so that it can be moved in both horizontal directions. By this means, the field of view can be changed while the mounting apparatus itself is held in place. Later, the agarose block is removed from the apparatus and flipped so that the dorsal aspect of the fish is exposed to the surface of the agarose gel, accessible for injection and for microscopic analysis of the tectum.

After mounting, the agarose is cooled by pumping ice-cold water through the apparatus. The block of gel containing the fish is removed with a broad spatula and placed into a Petri dish for further processing. Fig. 3 shows a photograph of an array of 96 fish larvae mounted in agarose and a close-up view of an individual fish from this array after injection.

### Injection of dyes

Ready-to-use solutions of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate; Molecular Probes, cat.-no. D-282) and

DiO (3,3'-dioctadecyl oxacarbocyanine perchlorate; Molecular Probes, cat. no. D-275) were prepared in quantities of 2 ml. Aliquots of these solutions (100 µl) were stored at -80°C and thawed before use. DiI was dissolved in 92% dimethylformamide/8% H<sub>2</sub>O at 37.5 mg/ml. The solution was briefly heated to 50°C to completely dissolve DiI. DiO (50 mg/ml) was dissolved in chloroform at 50°C together with an equal amount of 1-amino-octadecane (Riedel-de Haen). (1-amino-octadecane was found to improve the solubility of DiO.) DiO and 1-amino-octadecane were precipitated with two volumes of methanol and spun down in a bench centrifuge (Microcentaur, MSE; 13,000 rpm). The supernatant was discarded and the pellet was completely dried. DiO/1-amino-octadecane crystals were redissolved in dimethylformamide at 47.5 mg/ml. The solution was bath-sonicated for 30 seconds, heated to 50°C for at least 5 minutes, and centrifuged again. The supernatant was used for dye injections.

The injection apparatus is depicted in Fig. 4. It was built in the institute's machine shop. Its centrepiece consists of an electrolytically sharpened tungsten needle (Clark Elektromedica Instruments) that is connected to a low-power loudspeaker by an aluminium rod. The tungsten needle is inserted into a syringe cannula (0.4 mm outer diameter), which is fixed to a micromanipulator. A narrow pipette tip (GELoader, Eppendorf) helps to guide the needle into the cannula and serves as a dye reservoir (Fig. 4). The loudspeaker can be induced to vibrate at 50 Hz with a foot pedal. The amplitude of vibration is regulated by a transformer. An amplitude that produces a vibration distance of 0.5 mm at the tip of the needle was found to be useful for injection.

Before injection, excess water is dried from the agarose block with a paper towel. Subsequently, the gel is mounted onto a plexiglass holder. The holder can be inserted into a plexiglass frame fixed to a modified microscope stage. The block is held in a tilted position below the injection needle so that the position of the exposed eye of a fish can be controlled visually (Fig. 4). The modified microscope stage can be moved in X-Y-direction and elevated by its focus control knobs. Dye solutions (5 µl) are put into the pipette tips ensheathing the injection needle (Fig. 4). Routinely, DiI was injected into a subregion of the temporal-ventral quadrant of the eye (as shown in red in Fig. 1). DiO injection was restricted to the nasal-dorsal part (green in Fig. 1). As soon as the dye solution comes in contact with saline medium (such as in agarose gel or fish tissue) it precipitates. By this physical process, the dye can be deposited precisely at the site of injection. DiI and DiO were injected separately at two injection stations.

Injections are done by elevating the eyes of a properly positioned fish into the vibrating needle. The vibration serves two purposes. First, it helps to penetrate the retinal epithelium (which is so compact at this stage that a fine glass needle pressed against it would break). Second, the vibration continuously transports dye to the tip of the needle. An optimum transport of dye is achieved when the tip of the needle at its extreme just disappears in the cannula before pushing the foot pedal. A good injection is achieved when the needle visibly penetrates the outer surface of the eye. The amount of dye is controlled by the duration of vibrations. One injection usually takes a second or less. The 96 fish in a block are injected row after row. After having injected one row, the holder of the agarose block can be moved down manually so that it clicks into place one row further up (Fig. 5B,C). After the two injection rounds, the agarose block is rinsed briefly in low-strength PBS to remove free-floating dye crystals and stored at room temperature overnight. DiI and DiO diffuse within membranes and, by this passive process, stain the axons of retinal ganglion cells (Godement et al., 1987). A few hours after injection, the dye label can be seen within the axons, under the fluorescence microscope.

Occasionally, the cannula becomes clogged with dye crystals. These precipitates are removed completely by flushing the cannula with dimethylformamide (in the case of DiI) or chloroform (DiO). After refilling the system with dye solutions, test injections are made

into the agarose. The injection needle has to be sharpened electrolytically at regular intervals.

### Analysis of the retinotectal projection and complementation tests

The agarose blocks were placed into a plastic dish, which was put onto the stage of a Zeiss Axioskop microscope. Fish were analyzed at 100× magnification (10× objective, Zeiss Neofluar). We used three fluorescence filter sets, one to visualize DiI (Zeiss No. 17; 546 nm excitation, 580 nm dichroic mirror, >590 nm emission), one for DiO (Zeiss No. 15; 485 nm excitation, 510 nm dichroic mirror, 515–565 nm emission), and one combined filter (Zeiss No. 23) to visualize DiI and DiO, simultaneously. Usually, for analysis of a single fish, the filter sets were switched between the combined filter and the DiO filter, because DiO labellings were less intense than those of DiI and therefore harder to detect in combination with DiI.

During the screen, every single fish was inspected twice by independent observers to reduce the chance that a mutant would be overlooked. Graded scores were noted on a protocol sheet, depending on how obvious the phenotype was. The phenotype was scored as either 'clear wild type', or 'uncertain wild type', or 'uncertain mutant', or 'clear mutant'. In case of a putative mutant, a short description was included. Then, 16 sibling fish (from the same clutch) were analyzed the following days in a secondary screen. If the presence of a mutation was confirmed the parents were mated again and the offspring analyzed in a final 'rescreen'. Criteria for final selection of a mutant were the consistency of its phenotype and its phenotypic expression in 25% of the siblings in a clutch, as expected from Mendelian inheritance of a recessive single-gene mutation. Mutants with similar phenotypes were tested for complementation.

## RESULTS

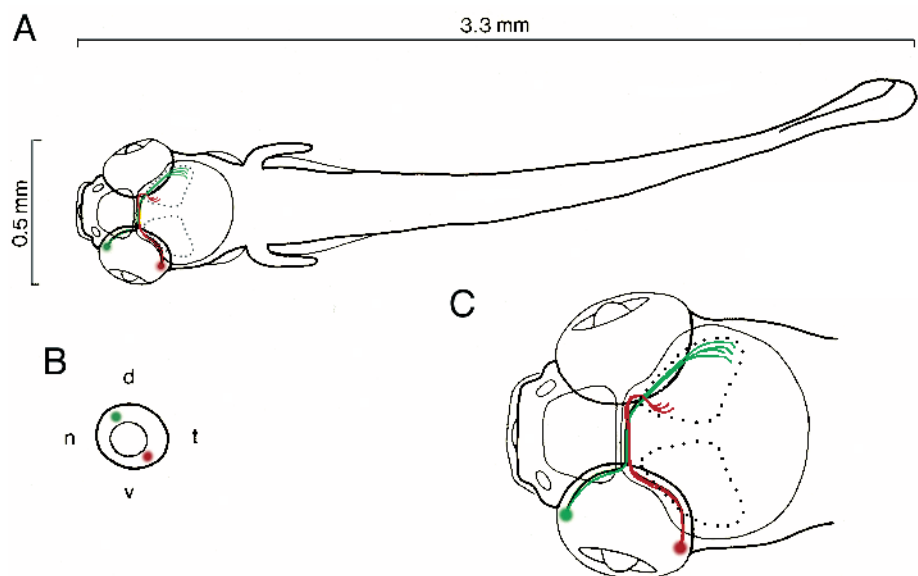
### Principle of the screening method

In wild-type zebrafish, retinal axons leave the eye and grow within the optic nerve to the contralateral side of the brain. After crossing the midline, they turn towards the tectum and segregate into one of two branches depending on their dorsal or ventral origin in the retina. Dorsal retinal cells mainly project via the ventral branch to the ventral part of the tectal neuropil; ventral cells mainly take the dorsal route to the dorsal tectum. Axons of nasal retinal origin grow to the posterior region of the tectum, temporal axons terminate in the anterior region. Stuermer (1988) and Kaethner and Stuermer (1992) have previously described the time course of the projection and the high accuracy of initial mapping.

Based on these findings, we established a method for observing retinal fiber growth and retinotectal topography that could be used for a mutant screen. This method employed the injection of two fluorescent tracers, the lipophilic carbocyanine dyes DiI and

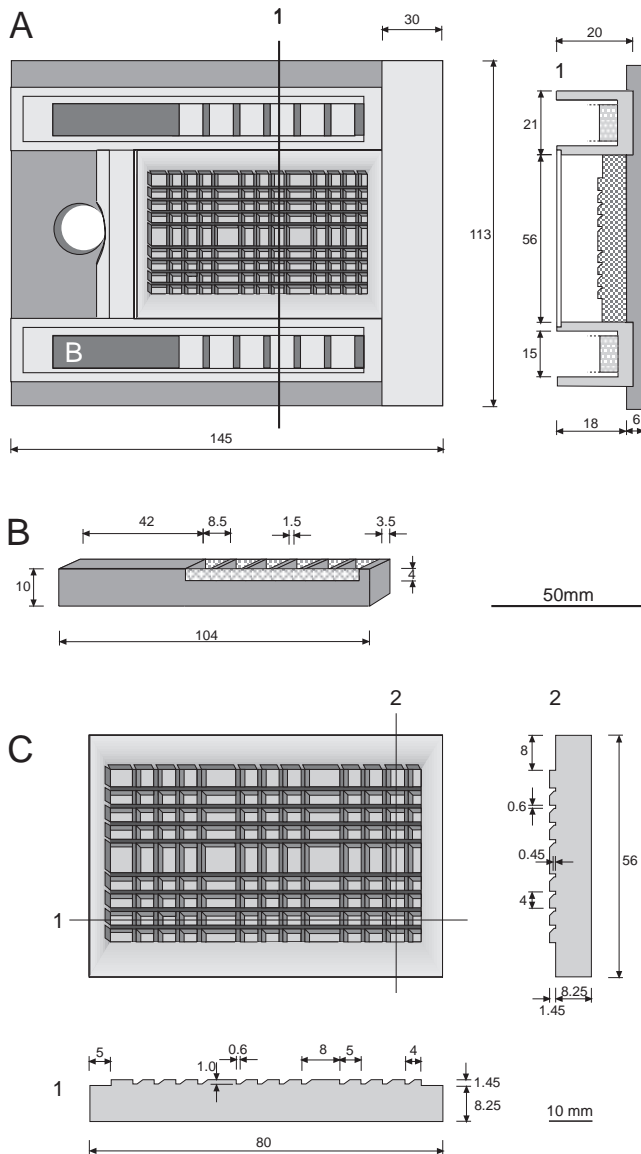
DiO, at two distinct positions into the eye of fixed zebrafish larvae. The injected dyes labelled two separate populations of retinal ganglion cell axons, by diffusion within axonal cell membranes (Godement et al., 1987). Routinely, DiI was injected into the temporal-ventral region of the retina, and DiO into the nasal-dorsal region (as depicted in Fig. 1). Therefore, in wildtype, DiI-labelled retinal fibers terminated in the anterior-dorsal quadrant of the tectum, whereas DiO-labelled fibers projected to the posterior-ventral region. By choosing this injection protocol, we could analyze both axes of the tectum simultaneously.

By microscopic inspection through the dorsal skin of intact fish, we could observe the pathway taken by retinal axons. The fiber patterns were scored as mutant if a deviation from wildtype was recognized. We saved mutants that were defective in pathfinding to the tectum, as well as those that displayed aberrant topography. Mutants with disturbed guidance or outgrowth within the retina could not be recognized without ambiguity because they could not be distinguished from wildtype fish with incomplete labelling. Fig. 5 shows three examples of wildtype fish and one arbitrarily chosen mutant. It is seen that the projection, as revealed by DiI and DiO axon tracing, is similar in every wildtype animal. This indicates that non-genetic contributions to variability are small and that the anatomical method yields consistent labelling patterns. In the representative case shown here (Fig. 5), the mutant can clearly be distinguished from wildtype because of its aberrant projection along the dorsal edge of the tectum.



**Fig. 1.** Schematic representation of the retinotectal projection and principle of the screening assay. A illustrates the size of a zebrafish larva at 5 days of development together with parts of the retinotectal map. B is a lateral view of the eye showing the sites of dye injections. C is a dorsal view of the head of a larva. Axons originating from the nasal-dorsal region of the retina (in green) project to the posterior-ventral region of the contralateral tectum. Axons originating from the temporal-ventral region of the retina (in red) project to the anterior-dorsal region of the tectum. The map is continuous in that the nasal-ventral retina projects to the posterior-dorsal tectum and the temporal-dorsal retina to the anterior-ventral tectum (not shown). For the screen, green axons, in this diagram, were labelled with DiO and red axons with DiI. d, dorsal; n, nasal; t, temporal; v, ventral.

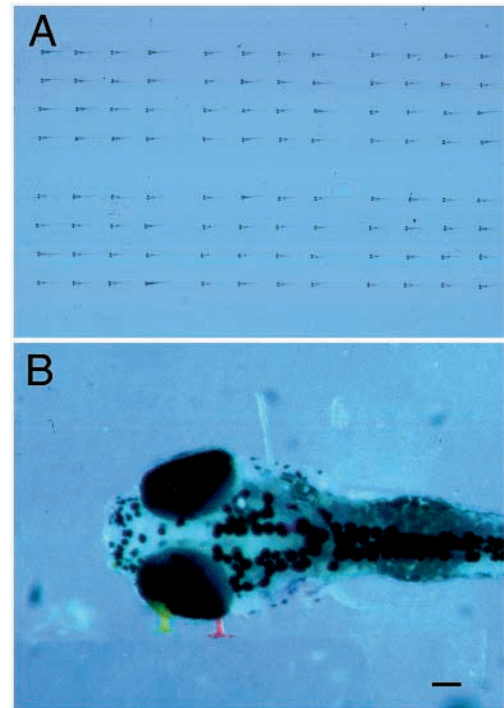




**Fig. 2.** Mounting apparatus. For mounting, warm agarose was poured into the apparatus. Dimensions are in mm. (A) Top-view (left) and cross section (right) of the assembly. The line at 1 indicates the position of the cross section. The letter B indicates one of the two chambered blocks that contain the fish larvae before mounting. (B) Dimensions of the chambered plastic block. The side-walls of the 6 chambers consist of wire mesh so that, during transfer of larvae, superfluous medium can escape, while the larvae are kept inside the chamber. (C) Details of the aluminium block. Top-view and two cross sections at the positions indicated. Larvae were mounted upside-down and anterior to the left. The horizontal grooves were designed so that a larva could be placed inside and stabilized. The vertical grooves provided room for the pectoral fins of the fish. The geometry of the grooves was optimized (shown in 1 and 2) for stability of the fish in the agarose gel as well as accessibility of its left eye for injection.

### Assessment of the assay for screening

The anatomical assay developed for our screen proved to be sufficiently quick, reliable and sensitive for mass screening. The screening assay was designed to handle large numbers of



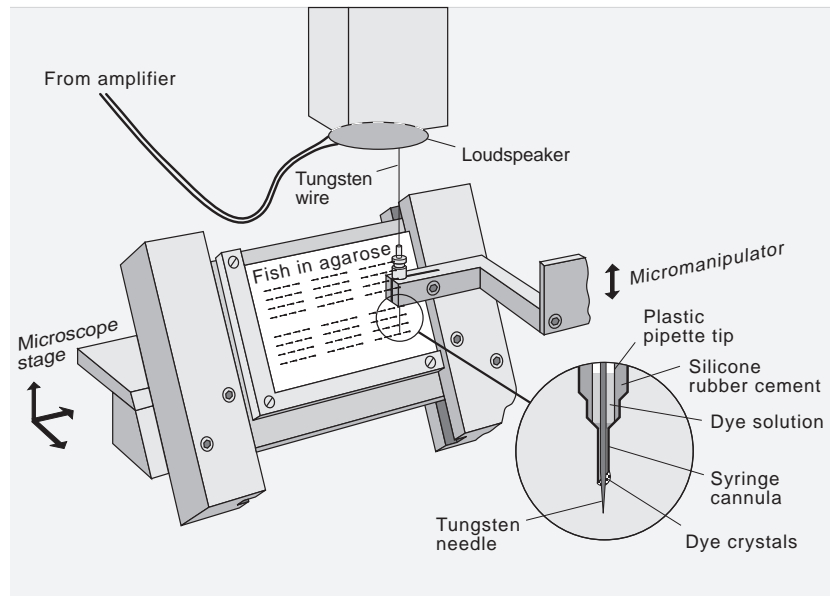
**Fig. 3.** (A) Photograph of an agarose gel containing 96 fish larvae after mounting, ready for injection of DiI and DiO. (B) Close-up view of an individual larva in the agarose gel after it had received injections of DiI (red dye deposit) and DiO (green dye deposit) into the left eye (bottom). Scale bar: 100  $\mu$ m.

fish. We could process more than 2,000 larvae per day if necessary. The whole procedure as described in Materials and Methods (transfer, fixation, and mounting of larvae, two rounds of dye injections, and microscopic analysis) required about one minute of hands-on work per individual fish. The 96 fish in one block of agarose were mounted in 45 minutes, injected in 30 minutes, and analyzed in 15 minutes.

Also, recognition of mutants was reproducible. In a blind test with fish having known genotypes, four experimenters involved in the routine evaluation independently scored wildtype and mutant fish with an accuracy of nearly 100%. The reliability and speed by which fish were screened increased during the screen with the growing confidence and experience of the individual researchers. This is illustrated by the fact that at the very beginning of the screen about 80% of putative mutants could not be confirmed in the rescreen. After a few weeks, the fraction of these 'false positives' decreased to 20% (and remained at this level until the end of the screen). Several error sources were responsible for this background of false positives: variability in raising conditions (e.g. in the water composition), overcautious or biased evaluation, and, most prominently, the varying quality of dye injections. For DiI, the labelling efficiency was more than 90%, for DiO it was less than 80%. Accordingly, double-labellings were seen in about 72% of fish injected. Candidate mutants were subjected to a three-step screening procedure, as described in Materials and Methods, largely eliminating the background caused by non-genetic variation.

### General results of the screen

The mutagenized inbred lines of fish (families of the F<sub>2</sub> gen-



**Fig. 4.** Injection apparatus. The central part of the assembly and a schematic close-up view of the injection device are shown. A loudspeaker induces the tungsten needle to vibrate. DiI or DiO is transported to the tip of the needle and deposited in the tissue. For details see Materials and Methods.

eration) and the crosses among F<sub>2</sub> siblings were identical to those in the preceding screens for earlier embryonic mutations (Haffter et al., 1996). 2,746 families were screened, with an average of 5.2 crosses within a family. For the analysis of the retinotectal projection (and only for this part of the Tübingen screen), eight randomly chosen F<sub>3</sub> fish were analyzed for each cross. In total, 125,000 individual fish were analyzed in the retinotectal screen.

During the screen, we originally discovered 154 families displaying mutant phenotypes. 40 mutants showed very general defects in brain development, like necroses, and were discarded after re-examination. 114 mutants were considered defective in axon targeting and were kept. Extensive complementation tests were performed for 105 mutants. These mutations fall into 28 complementation groups, giving on average 3.7 alleles per locus (ranging from 1 to 15). For the nine remaining mutants, complementation analysis is still being done. A preliminary evaluation of their phenotypes suggests that they mostly represent single alleles. We therefore estimate that we have identified slightly more than 35 individual loci. Table 1 gives a list of the genes identified, together with the number of alleles and cross-references to descriptions of their phenotypes (Karlstrom et al., 1996 and Trowe et al., 1996).

Retinotectal phenotypes were often discovered in mutant larvae that had been presorted for an earlier defect. After the primary screen, complementation tests then uncovered further alleles. In addition, we later tested mutants that, based on their visible phenotype, were candidates for a defective retinotectal phenotype as well. This was possible because defects in the retinotectal projection are often part of a phenotypic syndrome: defects in pathfinding to the tectum appear to be always associated with either notochord mutations or with other midline abnormalities; mutants with disturbed fiber sorting in the optic tract always have jaw defects; mutants with defects in mapping mostly have motility defects; and, lastly, some mutants display a combined pathfinding and pigmentation syndrome. Mutants

**Table 1. Mutants of the retinotectal projection**

Retinotectal process affected	Name of gene	No. alleles	Description of retinotectal phenotype	Major description in this issue
Pathfinding from eye to tectum	<i>astray</i>	4	a	a
	<i>bashful</i>	15	a	k
	<i>belladonna</i>	1	a	a
	<i>blowout</i>	1	a	a
	<i>chameleon</i>	5	a	d
	<i>cyclops</i>	2	a	d
	<i>esrom</i>	14	a	c
	<i>detour</i>	3	a	d
	<i>grumpy</i>	7	a	k
	<i>iguana</i>	2	a	d
	<i>sleepy</i>	9	a	k
	<i>tilsit</i>	1	a	c
	<i>tofu</i>	1	a	c
	<i>umleitung</i>	1	a	a
<i>you-too</i>	2	a	d	
Fiber sorting in the optic tract	<i>boxer</i>	8	a,b	b
	<i>dackel</i>	3	a,b	e
	<i>pinscher</i>	1	a,b	b
	<i>nevermind</i>	2	b	b
Anterior-posterior map	<i>macho</i>	1	b	f
	<i>gnarled</i>	1	b	b
Dorsal-ventral map	<i>who-cares</i>	1	b	b
	<i>nevermind</i>	2	b	b
Arbor formation on the tectum	<i>blumenkohl</i>	1	b	b
	<i>braindead</i>	10	b	b
	<i>esrom</i>	14	b	c
	<i>delayed fade</i>	1	b	g
	<i>tilsit</i>	1	b	c
Small tectum	<i>tofu</i>	1	b	c
	<i>no isthmus</i>	6	b	i
	<i>eisspalte</i>	1	b	h
	<i>tiny neuropil</i>	1	b	b
	[uncomplemented]	9	b	b

References: a, Karlstrom et al. (1996); b, Trowe et al. (1996); c, Odenthal et al. (1996b); d, Brand et al. (1996b); e, van Eeden et al. (1996); f, Granato et al. (1996); g, Kelsh et al. (1996); h, Jiang et al. (1996); i, Brand et al. (1996a); k, Odenthal et al. (1996a).

displaying visible features related to these syndromes sometimes turned out to be retinotectal mutants after reexamination. In total, 18 new alleles of retinotectal genes and even four new genes were identified during closer analysis of the mutants found in the general screen. In turn, in the retinotectal screen, seven new alleles of genes involved in earlier processes were discovered. These numbers demonstrate how the individual parts of the screen helped each other in the identification of mutants.

Most of the mutants display phenotypic aberrations in other tissues as well as in the retinotectal pathway, mainly in the nervous system. Only nine genetic loci (of 28) were isolated exclusively for their retinotectal phenotype. Two of them (*belladonna* and *astray*) are the only larval viable mutants identified so far in our screen. This suggests that most of the genes that are involved in retinotectal targeting are essential components of other developmental processes as well.

The frequency distribution of alleles isolated in the retinotectal screen shows that saturation of the genome has not been reached. In particular, the high incidence of single-allele loci (about half of our mutants) suggests that we have missed several relevant genes. The same applies to the Tübingen screen in general (Haffter et al., 1996). Based on a realistic mutation rate and a simple model, we calculated beforehand that a standard locus should have a chance of 0.84-0.97 to be identified by mutation (see Appendix). The average number of mutant alleles per locus is consistent with this model (2-4 in the model vs. 3.7 in the screen). However, it appears likely that the mutation rate varies considerably between different loci. Less mutable genes may have escaped discovery. An extension of the screen to reach saturation would be desirable, but difficult. However, the retinotectal phenotypes already found comprise a comprehensive collection of what can be found at all in a mutant screen.

### Retinotectal mutants

The retinotectal mutants can be tentatively classified according to whether they affect (i) the pathway from the retina to the tectum (pathfinding mutants; Karlstrom et al., 1996), (ii) dorsal-ventral fiber-sorting in the optic tract (fiber sorting mutants; Karlstrom et al., 1996; Trowe et al., 1996), (iii) the retinotectal topography along the dorsal-ventral axis or (iv) the topography along the anterior-posterior axis (mapping mutants; Trowe et al., 1996), (v) the formation of axonal arbors in the tectum (arborization mutants; Trowe et al., 1996), or (vi) the general development of the tectum (tectum size mutants; Trowe et al., 1996). Some overlap is observed between the pathfinding and the arborization mutants, as well as between the fiber sorting and the dorsal-ventral mapping mutants. For the pathfinding and fiber sorting mutants, the average number of alleles per locus is 4.1. In contrast, the screen for mapping mutations has uncovered only 5 mutations in 4 genes (i.e., on average 1.25; Table 1).

The pathfinding mutants (15 genes; Karlstrom et al., 1996), show a variety of phenotypes, with axons making pathfinding errors at several different points between the eye and the contralateral tectum (*astray*, *bashful*, *belladonna*, *blowout*, *chameleon*, *cyclops*, *esrom*, *detour*, *grumpy*, *iguana*, *sleepy*, *tilsit*, *tofu*, *umleitung*, *you-too*). For example, in some mutants axons may not cross the midline and instead project to the ipsilateral tectum, whereas in other mutants, axons may project

into forebrain areas in addition to the tectum. Remarkably, in all of the pathfinding mutants, those axons that invade the tectum form a proper retinotopic map.

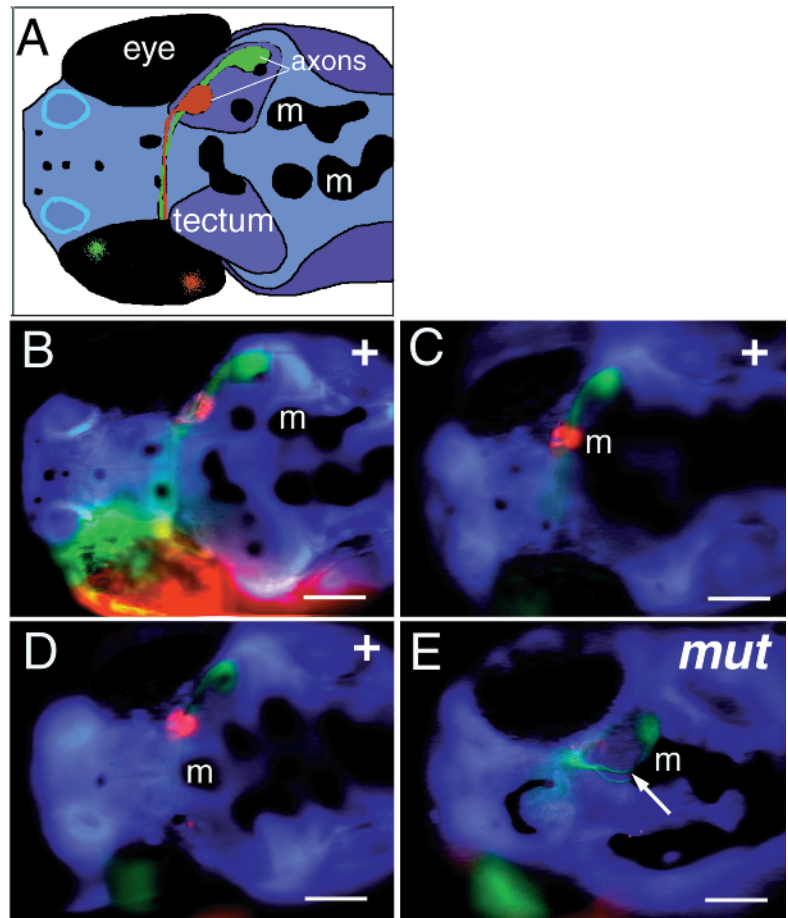
In the fiber-sorting mutants (4 genes; Karlstrom et al., 1996; Trowe et al., 1996), position-specific properties of retinal axons along the dorsal-ventral axis are affected: fibers do not sort out into a dorsal and a ventral branch before entering the tectum (*boxer*, *dackel*, *pinscher*, *nevermind*). Axons in *boxer*, *dackel* and *pinscher* correct their course in the tectum and map normally, while axons in *nevermind* do not project properly to their target area. The latter, therefore, together with *who-cares* form the small group of dorsal-ventral mapping mutants (2 genes; Trowe et al., 1996).

Mutations in 2 other genes (*gnarled*, *macho*) affect anterior-posterior mapping (Trowe et al., 1996). Formation of retinal arbors is disturbed in *esrom*, *tofu* and *tilsit* (in addition to their pathfinding defect), as well as in *blumenkohl*, *braindead* and *delayed fade* (Trowe et al., 1996). Mutants in 3 other genes (*eisspalte*, *tiny neuropil*, *no isthmus*) show a small-sized tectum (Trowe et al., 1996). Of the nine mutations that still await complementation testing, most have underdeveloped or degenerate tecta, and may partly also have anterior-posterior mapping defects. Topographical defects could be secondary to a general tectal developmental impairment. However, during the screen, we have often observed mutants having a miniature tectum with a precise map compressed onto it. (These mutants have not been kept.) Consequently, reduced availability of target space does not necessarily lead to an abnormal map.

### DISCUSSION

To better understand the processes that lead to formation of the retinotectal map, we have undertaken a systematic genetic approach in the zebrafish, *Danio rerio*. A large-scale mutant screen was devised and a double-fluorescent axon tracing assay was developed. The mutations found in our screen affect the pathfinding of axons from the eye to the tectum including axon behavior in the optic tract (82 mutations in 19 genes; Karlstrom et al., 1996) and/or the retinotopic map on the tectum including arborization patterns (33 mutations in 10 genes; Trowe et al., 1996). The degree of overlap between individual phenotypic classes needs to be investigated further. Also, inherent to the genetic approach, it is not clear whether the individual mutations affect axon guidance directly, or disrupt upstream genetic pathways necessary for the development of retina, tectum, or the optic pathway. However, it is obvious that mutations affecting axon navigation to the tectum do not lead to mapping defects for those axons that reach the tectum. This indicates that the two processes employ basically independent mechanisms.

We isolated on average 3.7 alleles per locus. However, about 50% of the genes are represented with only a single allele in the collection of mutants. It is therefore likely that several genes have been missed in our screen. The frequency distribution of mutant alleles does not represent a binomial function as would be expected from random mutagenesis (see Appendix). This result is not due to a systematic error particular to the retinotectal part of the screen, since the frequency distribution of retinotectal mutants closely resembles that of the general Tübingen screen (Haffter et al., 1996; see Table 3



**Fig. 5.** Fluorescent labelling of retinal axons for screening mutants. Labelling patterns in three wild-type larvae (+; B–D) and one mutant (*mut*; E) are shown. A shows a semi-schematic representation of the fish in B. DiI (red) and DiO (green) were injected at two poles into the left eye (bottom in these photographs), as indicated in Fig. 1A, labelling two distinct subsets of retinal axons. In the wild type, red axons project invariably along stereotyped routes to the anterior-dorsal region of the tectum, and green axons to the posterior-ventral region. In the mutant (*boxer*; E), green axons project abnormally along a dorsal pathway, but terminate in the proper region. A counterstaining was performed with the nuclear stain DAPI (blue) to visualize the outline of the tectum. The tectal neuropil is relatively free of cell bodies and therefore not labelled by DAPI. The optic nerve between eye and tectum is outside the focal plane. Melanophores (m) are indicated. Scale bar: 100  $\mu$ m.

in the Appendix). It is likely that different loci were not mutated at comparable rates. Additional interpretations are given by Haffter et al. (1996). Mechanistic models of how the retinotectal projection forms would predict certain mutant phenotypes. Since saturation was not achieved in the screen, the absence of a predicted phenotype in the collection of mutants does not disprove the underlying model.

On average more alleles were found for pathfinding and fiber-sorting genes (4.3) than for mapping genes (1.25). One explanation is that our screening assay was differentially sensitive for the two phenotypic aspects: it is easier to detect a misrouting of fibers than topographic errors in a relatively small target area. Alternatively, map formation could be less susceptible to point mutations than pathfinding, because redundant or regulatory mechanisms may exist that compensate for loss of single gene products. (Redundancy implies the existence of two or more independent mechanisms that work in parallel leading to the same phenotypic feature. Regulation involves a change in gene expression in response to the loss of a gene product.) In this scheme, only a subset (or strong alleles) of mapping genes would have been discovered.

Another general result of our screen is that there appear to exist only few, if any, genes solely dedicated to the retinotectal projection. Most (perhaps all) of the mutants found in our screen display associated phenotypes, often in other parts of the nervous system. This suggests that individual genes are used more than once and at different places during development. In line with this observation, we must assume that some

genes necessary for the retinotectal projection could not be discovered because their mutations lead to premature death of the embryo. The finding of multiple gene use is not surprising given the situation in *Drosophila* where different developmental processes share common signalling cascades or components thereof (such as Notch, Ruohola et al. (1991); or hedgehog, Ingham (1994)). We observed particular phenotypic syndromes in defined groups of mutants. For instance, dorsal-ventral fiber sorting in the optic tract is always associated with jaw defects. Another group of mutants display defects in both xanthophore pigmentation and axon navigation to the tectum. The corresponding gene products could be individual components of common signalling cascades used in otherwise unrelated processes.

It should be stressed that our screen uncovered a collection of clear-cut and often unique mutant phenotypes derived from single-gene mutations. The fact that mutants in the retinotectal system can be found at all is an important result and was not guaranteed from the beginning. Transgenic mice mutated in previously cloned genes sometimes display no major defects. This result is often attributed to redundant or regulatory mechanisms. For instance, it is assumed that the phenotype of *en-2* gene knockouts is alleviated by the presence of *en-1* in regions where the expression of both genes overlap (Joyner et al., 1991). A clear example of redundant mechanisms in axon guidance has been found in *Drosophila* (Elkins et al., 1990). In most cases where reverse genetics is applied, however, redundancy of gene action cannot be easily distin-



guished from the possibility that the gene of interest does not play the functional role ascribed to it. In contrast, when using forward genetics, a mutant phenotype indicates from the outset that the mutated gene is important – however remotely – for the biological process under investigation.

Large-scale mutant screens can now be performed in zebrafish. In the past, a systematic mutational approach as a functional strategy to identify new genes was only applicable to invertebrates, like *Drosophila* or *C. elegans*. We have shown that the genetic approach can be targeted to relatively late and circumscribed developmental processes in the vertebrate brain, uncovering a comprehensive collection of mutants that display distinct defects in retinotectal axon guidance. We hope that further analysis and, eventually, cloning of the genes involved will shed light on long-standing questions of map formation and neuronal specificity.

**APPENDIX**

We calculated the output of a mutant screen as a function of the number of fish screened. All calculations are based on binomial statistics of independent events with Equ. 1 as the basic equation:

$$P(N) = 1 - q^N, \tag{1}$$

with P(N) being the total probability of a positive result after N trials and a chance of q for a negative outcome in a single trial. In a simple mutant screen, P would be the chance of finding a mutant, N would be the number of organisms screened, and q would be the (usually very large) probability that a randomly chosen organism does **not** carry the mutation of interest.

In the breeding scheme of the Tübingen screen, not all events are independent from each other, e.g., the chance of coming across an F<sub>2</sub> family carrying the mutation of interest and then the chance of selecting two heterozygous carriers of

the mutation for mating. Therefore, Equ. 1 cannot be simply applied. The following equation (Equ. 2) gives the chance W of isolating at least one mutation of a particular locus. In case of a purely random, Poisson-like mutagenesis, this probability (W) is equivalent to the fraction of loci that show up in mutant form out of all mutable loci. For reasons that become more obvious below, W will be named the ‘saturation function’ here.

$$W(p, r, t, l, m, n, E) = 1 - \{1 - 2p [1 - (1 - r(1 - (1 - E \cdot t)^l))^m]\}^n \tag{2}$$

with n: the number of F<sub>2</sub> families; m: the number of crosses within a family; l: the number of individual fish per cross; p: the mutation rate for a particular locus; r: the chance of picking two F<sub>2</sub> heterozygous carriers of the mutant gene for mating (0.25 for Mendelian inheritance); t: the chance of picking a homozygous mutant among the F<sub>3</sub> larvae (0.25 for Mendelian inheritance); and E: the efficiency of the screening assay (ability to detect a mutant).

The saturation function W ranges between 0 (no mutant alleles will be found) and 1 (a particular gene will certainly be identified with at least one allele). When drawn, W takes the form of a saturation curve (provided that all its variables are >0). The mutation rate p is multiplied by 2 in Equ. 2, simply because two mutagenized F<sub>1</sub> fish (carrying mutations with a frequency of p) were mated to give rise to one particular F<sub>2</sub> family, thus doubling the original mutation rate (valid approximation for p << 1). E is a measure of the average quality of the screening procedure. It can in principle range between 0 (no evaluation possible) and 1 (perfect). E is multiplied by t (0.25) because, for successful identification of a mutant, one not only needs to come across a mutant among the individual fish in a clutch (0.25), but its phenotype must also be detectable.

As an independent control of this analytical equation, Monte Carlo simulations were performed. A program (written in BASIC) simulated the sequence of steps of the procedure with their respective probabilities, i.e. the mutagenesis, the generation of F<sub>2</sub> families, the selection of mating partners within a family, the selection of F<sub>3</sub> larvae for analysis, and its effi-

**Table 2. Saturation function W, as a direct measure of the probability of identifying a mutation in a gene of interest**

No. of crosses per family	No. of fish per cross												
	0	1	2	3	4	5	6	7	8	9	10	11	12
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.22	0.36	0.46	0.53	0.58	0.62	0.64	0.66	0.68	0.69	0.70	0.71
2	0.00	0.38	0.58	0.69	0.75	0.80	0.83	0.85	0.86	0.87	0.88	0.89	0.89
3	0.00	0.51	0.71	0.81	0.86	0.89	0.91	0.92	0.93	0.94	0.94	0.95	0.95
4	0.00	0.60	0.80	0.88	0.91	0.93	0.95	0.96	0.96	0.96	0.97	0.97	0.97
5	0.00	0.68	0.85	0.91	0.94	0.96	0.97	0.97	0.97	0.98	0.98	0.98	0.98
6	0.00	0.73	0.89	0.94	0.96	0.97	0.98	0.98	0.98	0.98	0.99	0.99	0.99
7	0.00	0.78	0.92	0.96	0.97	0.98	0.98	0.99	0.99	0.99	0.99	0.99	0.99
8	0.00	0.82	0.93	0.97	0.98	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99
9	0.00	0.84	0.95	0.97	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
10	0.00	0.87	0.96	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

A ‘standard’ locus with a mutation rate of  $p=10^{-3}$  was assumed. The simulated screen employs 2746 lines and a variable number of crosses per family and individuals per cross. Screening efficiency is  $E=0.72$ . The boxed value indicates the result predicted for the conditions of the retinotectal screen. The broken line separates values below and above 0.9.

ciency. Then the program was run repeatedly (more than 15,000 iterations) and the average of all results was calculated. This type of statistics did not only confirm the analytical calculation, but also provided additional predictions about the hypothetical frequency distribution of mutant alleles.

A systematic analysis of Equ. 2 reveals that the saturation function is most sensitive to two parameters: the mutation rate and the number of F<sub>2</sub> families screened. To a much lesser extent, it depends on the number of crosses within the family and, to an even lesser extent, on the number of individual fish screened. Importantly, this qualitative result is valid independent of the actual parameter values, i.e. whether the mutation rate varies for different loci, as seems to be the case in the present screen.

An example: given the choice between screening 1,000 families thoroughly, i.e. with 10 individual fish for each cross, and 10,000 families superficially, i.e. with only 1 fish per cross, one should rather go for the latter. Of course, this reasoning does not take into account the large amount of work associated with raising families. This aspect, however, did not apply to the retinotectal screen as an appendage to the general Tübingen screen. Therefore, we decided to screen all families and crosses available and save work at the level of F<sub>3</sub> fish (only 8 per cross). Table 2 shows that this reduction does not decrease the output significantly (*W* was calculated here for a 'standard gene'; see below). The chance of missing a mutant in a cross (0.1; see Equ. 1) was partly counterbalanced by the presorting of mutants in separate batches during the preceding screens (see Materials and Methods).

After the screen, the frequency distribution of alleles per gene suggested strongly that the mutation rate was not the same for all loci: for particular loci up to 15 alleles were discovered whereas about half of the loci were hit only once. The frequency distribution of alleles therefore does not conform to the theoretical expectation of a binomial distribution (Table 3). Without knowledge about individual mutation rates the total degree of saturation of the screen cannot be calculated. But we can calculate *W* for a standard gene. The mutation rate *p*

**Table 3. Frequency of alleles: real and predicted distributions**

<i>n</i> alleles	% ( <i>n</i> ) genes retinotectal screen	% ( <i>n</i> ) genes Tübingen screen total*	% genes predicted $p=10^{-3}$	% genes predicted $p=5\times 10^{-4}$
0	? (?)	? (?)	2	14
1	46.4 (13)	59.3 (219)	7	26
2	14.3 (4)	16.5 (61)	13	27
3	7.1 (2)	7.6 (28)	19	18
4	3.6 (1)	4.1 (15)	20	10
5	3.6 (1)	3.3 (12)	17	4
6	3.6 (1)	2.4 (9)	11	1
7	3.6 (1)	1.9 (7)	6	0
8	3.6 (1)	0.5 (2)	3	0
9	3.6 (1)	1.1 (4)	1	0
10	3.6 (1)	0.3 (1)	1	0
11	0.0 (0)	0.8 (3)	0	0
12	0.0 (0)	0.5 (2)	0	0
13	0.0 (0)	0.0 (0)	0	0
14	3.6 (1)	0.3 (1)	0	0
≥15	3.6 (1)	1.4 (5)	0	0
<i>n</i> alleles/ <i>n</i> genes	3.75	2.4	4.1	2.0

\*Haffter et al. (1996).

(predicted from pilot screens for mutations in four known marker genes) ranged from  $1.3\times 10^{-3}$  to  $3.3\times 10^{-3}$  (Mullins et al., 1994). These genes probably represent loci that are more mutable than average (Haffter et al., 1995a). Assuming mutation rates of  $5\times 10^{-4}$  or  $10^{-3}$ , we calculate *W* to be 0.84 or 0.97, respectively (Table 2), with on average 2 to 4 alleles per locus (Table 3). This comes close to the empirical result of 3.7 alleles per locus.

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