Efficient Recovery of ENU-Induced Mutations From the Zebrafish Germline

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ABSTRACT

We studied the efficiency with which two chemical mutagens, ethyl methanesulfonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU) can induce mutations at different stages of spermatogenesis in zebrafish (*Brachydanio rerio*). Both EMS and ENU induced mutations at high rates in post-meiotic germ cells, as indicated by the incidence of F_1 progeny mosaic for the *albino* mutation. For pre-meiotic germ cells, however, only ENU was found to be an effective mutagen, as indicated by the frequencies of non-mosaic mutant progeny at four different pigmentation loci. Several mutagenic regimens that varied in either the number of treatments or the concentration of ENU were studied to achieve an optimal ratio between the mutagenicity and toxicity. For the two most mutagenic regimens: 4 × 1 hr in 3 mM ENU and 6 × 1 hr in 3 mM ENU, the minimum estimate of frequencies of independent mutations per locus per gamete was $0.9–1.3 \times 10^{-3}$. We demonstrate that embryonic lethal mutations induced with ENU were transmitted to offspring and that they could be recovered in an F_2 screen. An average frequency of specific-locus mutations of 1.1×10^{-3} corresponded to approximately 1.7 embryonic lethal mutations per single mutagenized genome. The high rates of mutations achievable with ENU allow for rapid identification of large numbers of genes involved in a variety of aspects of zebrafish development.

SATURATION mutagenesis screens for embryonic lethal mutations led to the identification of a large number of genes that control the development of invertebrate animals and plants (*e.g.*, HIRSH and VANDERSLICE 1976; NÜSSLEIN-VOLHARD and WIESCHAUS 1980; JÜRGENS *et al.* 1991; MAYER *et al.* 1991). Saturation mutagenesis screens are feasible only in experimental systems that fulfill several criteria. First, a mutagenesis method has to be available that is both very efficient and causes predominantly intragenic lesions. This minimizes the number of genomes that have to be screened to reach saturation levels and assures that observed defects can be linked to lesions in single genes. Second, the efficient transmission of mutations to subsequent generations should be feasible without seasonal variation and in reasonably short time periods. Finally, convenient selection or screening procedures for phenotypes have to be available. The mouse is the only vertebrate in which large scale screens for visible morphological traits or metabolic defects have been carried out (JOHNSON and LEWIS 1981; MCDONALD *et al.* 1990; MOSER *et al.* 1990). However, in the mouse, screening for embryonic lethal mutations, and especially for those affecting the earliest stages of development, is difficult due to the intrauterine mode of embryogenesis. Thus far, the majority of embryonic lethal mutations have been found in mouse genetic screens by virtue of linkage to visible recessive mutations. This limits saturation mutagenesis to small portions of the murine genome (SHEDLOVSKY *et al.* 1986, 1988).

Among the vertebrate species in which the mechanisms of embryogenesis are currently investigated, zebrafish offers several advantages for saturation mutagenesis screens (STREISINGER *et al.* 1981). This small tropical fish has a generation time of 3 months, and mature females lay hundreds of eggs at weekly intervals, facilitating genetic analysis. A major asset of zebrafish is the accessibility of its embryonic development to visual inspection. The embryos develop synchronously outside the mother and are transparent, such that detailed aspects of embryogenesis and organogenesis can be monitored microscopically (STREISINGER *et al.* 1981; KIMMEL 1989). Several interesting mutations have been identified following γ-irradiation mutagenesis of pregonial cells (KIMMEL *et al.* 1989; FELSENFELD *et al.* 1990; WESTERFIELD *et al.* 1990; HATTA *et al.* 1991).

Chemical mutagens are preferentially used for saturation mutagenesis as they can efficiently induce lesions that are primarily limited to single genes (SINGER and GRUNBERGER 1983). Among them, two alkylating agents are most frequently used for mutagenic purposes. Ethyl methanesulfonate (EMS) is the mutagen of choice in *Drosophila melanogaster* and *Caenorhabditis elegans* (ASHBURNER 1989; WOOD 1988). *N*-Ethyl-*N*-nitrosourea (ENU) is the most potent mutagen of the mouse germline (RUSSELL *et al.* 1979), and a preliminary report suggests that ENU might be effective in mutagenesis of spermatogonia of the small teleost fish medaka (*Oryzias latipes*) (see discussion in SHIMA and SHIMADA 1991).

Zebrafish spermatozoa can be efficiently mutagenized

with ENU *in vitro* (GRUNWALD and STREISINGER 1992). Upon fertilization by mutagenized spermatozoa, F_1 individuals develop that are most frequently mosaic for the induced mutations. Several methods for genetic screening have been developed to recover embryonic lethal mutations among progeny of F_1 females derived from mutagenized sperm, or of females mutagenized at the blastoderm stage. Mutations are manifested in haploid gynogenetic progeny of adult females by fertilizing their eggs with sperm rendered genetically inactive by UV irradiation (FELSENFELD *et al.* 1990). Haploid embryos, however, start to develop abnormally on the second day after fertilization (STREISINGER *et al.* 1981). This hampers possibilities for screening for phenotypes affecting processes in later embryogenesis. Mutations have also been recovered among diploid parthenogenetic embryos produced by treatments of activated eggs during the first cell cycle with early pressure (EP) (STREISINGER *et al.* 1986) or with heat shock (HS) (STREISINGER *et al.* 1981) (Figure 1, A and B). Both these procedures, however, decrease survival of the eggs, so that only a fraction of diploid embryos develop normally (STREISINGER *et al.* 1981; GRUNWALD and STREISINGER 1991, 1992). Therefore, although these methods are advantageous as they involve only limited breeding (one generation), analysis of subtle or late phenotypes in parthenogenetic embryos would be difficult.

A classical three-generation of crosses scheme (F_2 screen), similar to the one performed in mice (HALDANE 1956) (Figure 1, C and D), in which mutations induced in the parental generation are driven to homozygosity in the F_3 generation, could circumvent this problem. If spermatozoa were mutagenized, however, recovery of mutations would require a large number of crosses between F_2 siblings, due to the mosaic character of the germline of F_1 progeny of mutagenized sperm (Figure 1C) (GRUNWALD and STREISINGER 1992). The mosaicism of the F_1 germline can be circumvented by mutagenizing pre-meiotic germ cells such as spermatogonia in adult males (RUSSELL *et al.* 1979). In this case mutations are fixed via subsequent rounds of DNA replication, before differentiation into mature sperm cells. Hence, the F_1 progeny of the mutagenized parental animals are non-mosaic heterozygotes each carrying one or several unique mutations. Subsequently, F_1 animals become founders of F_2 families in which 50% of progeny are heterozygous for the mutation(s) inherited from the F_1 founder. These mutations are manifested during screening the F_3 progeny of F_2 siblings (Figure 1D). An important advantage of this method is that each phenotype is revealed on average in every fourth cross, reducing the number of crosses needed to detect the majority of mutations harbored by a given F_2 line.

To initiate a saturation mutagenesis screen for embryonic lethal mutations in zebrafish we searched for an efficient method to mutagenize the germline. Here we report studies on the mutagenic activity of EMS and ENU on different cellular stages of the male germline. We demonstrate that ENU but not EMS can induce locus-specific mutations at high rates in pre-meiotic germ cells of zebrafish males and that induced recessive embryonic lethal mutations can be efficiently recovered in an F_2 screen.

MATERIALS AND METHODS

Fish tank facilities: Most of the experiments described depend on the ability to keep large numbers of zebrafish under conditions optimal for breeding. Given limited space, we had to adopt and develop methods to keep zebrafish at high population densities. We employed a closed circulating water system that guarantees a low water load of compounds deleterious to zebrafish and provides conditioned water of consistent chemistry.

We use 20-liter tanks (acrylic, custom-made: about 15 cm wide, 60 cm long and 25 cm high) to keep up to 100 adult fish; 4-liter tanks (acrylic, custom-made: about 12 cm wide, 30 cm long and 15 cm high) to keep up to 20 adult fish or up to 100 fish at the age of 1–2 months; and 1-liter tanks (Nalgene storage containers from clear plastic 5700–1000; 127 × 73 × 175 mm) to keep single adult fish. Four- and 20-liter tanks are equipped with standpipes and/or strainers to control the water level. One-liter boxes have three 2-mm wide holes drilled into the back that serve as drains. One-liter boxes sit in large plastic trays that are drained into a common reservoir. All tanks are constantly supplied with filtered water through inlet valves at a rate so that water in the tank is exchanged about three times per hour. Four-liter and 20-liter tanks are aerated at rates of about 1 liter and 3–5 liters of air per minute, respectively. Pressurized air for the entire fish facility is generated by three 1/2 hp regenerative blowers (Sweetwater Blower S-31; Aquatic Eco Systems, Apoka, Florida).

The water filtration system serves about 65,000 zebrafish in tanks with a total volume of about 16,000 liters. Water from reservoirs (total volume about 5,400 liters) is recirculated for biological filtration. A 3/4 hp. centrifugal pump (Max-E-Glass, Sta-Rite Industries, Inc., Delavan, Wisconsin) pumps water from the reservoir through a sand pool filter (hydro rate HRV-36; Baker-Hydro Inc.; Augusta, Georgia) and up onto a wet-dry trickle filter (ammonia tower with about 1 m^3 of plastic balls as substratum for denitrifying bacteria; Aquatec, Brockton, Massachusetts). Filtered water flows back into the reservoirs.

Five subsystems draw water from the main reservoirs to serve tanks with a total volume of about 3,000–3,500 liters for each subsystem. For each subsystem, a 1/2 hp. centrifugal pump (Max-E-Glass, Sta-Rite Industries, Inc., Delavan, Wisconsin) drives water at about 10 p.s.i. through particle filter cartridges (Jacuzzi CFR 100; Jacuzzi Bros. Div., Little Rock, Arkansas; filters are exchanged every 6 months) to remove debris and potential parasites. The filter cartridges are loaded with bagged carbon (Darco activated carbon casno 7,440–440; American Norit Co., Atlanta, Georgia; about 1 kg, exchanged monthly) to remove dissolved organic waste from the water. Water is sterilized while flowing through a 120 W UV sterilizer (Aquanetics 120 IL, Aquanetics Systems, Inc, San Diego, California). Water is distributed to the tanks and drains back through prefilter pads (cleaned weekly) into the main reservoirs.

Every day, about 10% of the total volume of water in the system is exchanged with fresh water. Under automatic control, 28° water (mixed hot and cold tap water, TempControl thermostatic water controller, Symmons Industries, Braintree, Massachusetts) flows six times a day for a few minutes through

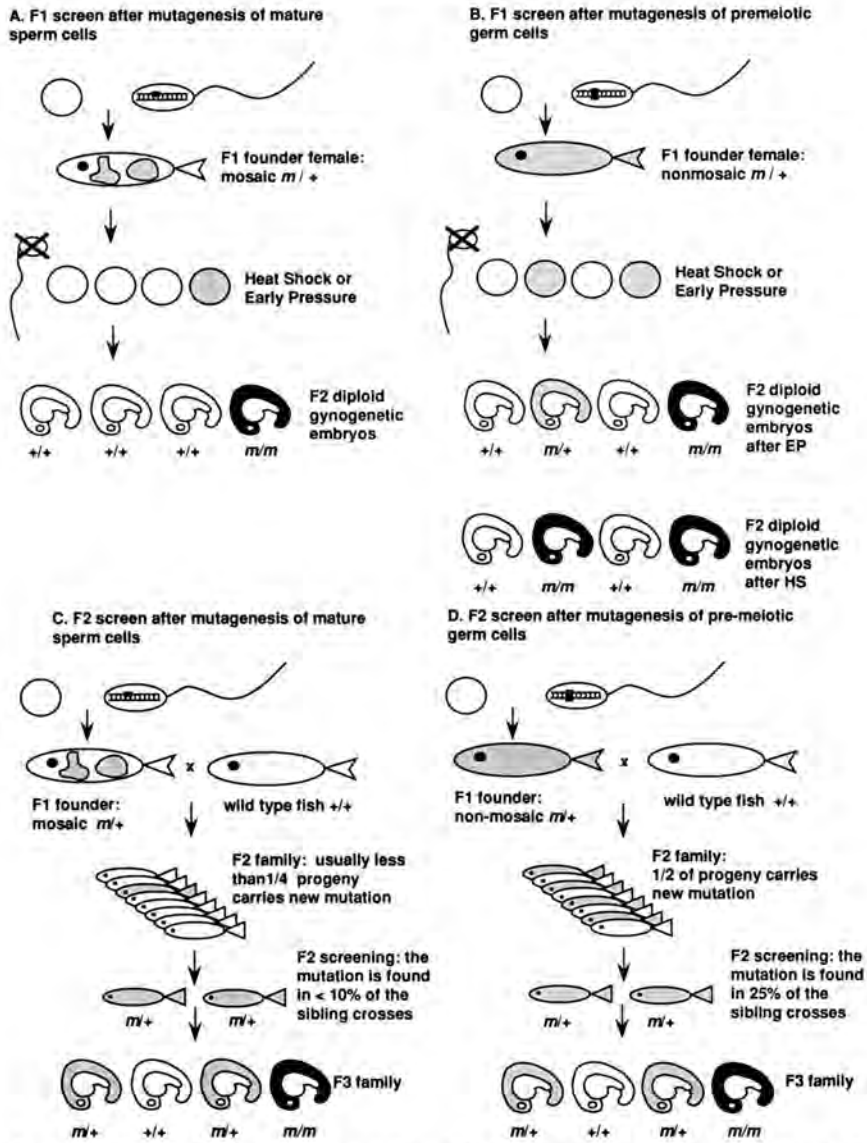

FIGURE 1.—Strategies for induction and recovery of mutations. (A) F_1 screening strategy after chemical mutagenesis of mature sperm (KIMMEL 1989; GRUNWALD and STREISINGER 1992). Upon fertilization with mutagenized sperm, mutations are fixed during early development and F_1 individuals develop that are most frequently mosaic for the induced mutations. Mutations can be revealed in gynogenetic progeny of F_1 females. First, development of eggs of F_1 females is initiated by sperm rendered genetically impotent by UV light treatment. Second, diploid embryos are produced by treating haploid activated eggs during the first cell cycle with early pressure (EP) or heat shock (HS) treatments (STREISINGER *et al.* 1981). Since F_1 females are mosaic for carried mutations, less than expected 50% and usually less than 20% of surviving F_2 parthenogenetic progeny exhibit mutant phenotypes (GRUNWALD and STREISINGER 1992). For the EP treatment proportion of homozygous mutant embryos is also inversly correlated with the locus-centromere distance (STREISINGER *et al.* 1986). (B) F_1 screening strategy after chemical mutagenesis of spermatogonia. Spermatogonial stem cells are mutagenized by treatment of adult males and mutations are fixed before differentiation into mature sperm cells. F_1 females derived from such mutant sperm are non-mosaic heterozygotes for the induced mutations. Consequently, up to 50% (for the EP usually less than 50% depending on the locus-centromere distance) of developing parthenogenetic progeny produced as described in (A) exhibit mutant phenotypes. (C) F_2 screening strategy after chemical mutagenesis of mature sperm. As in (A) F_1 progeny of mutagen-treated sperm are mosaic heterozygotes for induced mutation(s). Subsequently, F_1 animals become founders of F_2 families. Due to mosaicism of F_1 founders usually less than 25% of F_2 fish are heterozygous for the inherited mutations. Consequently, during screening a large number of crosses between F_2 siblings is needed to recover the mutation(s). In a cross in which mutation(s) are manifested, 25% of F_3 embryos exhibit each mutant phenotype. (D) F_2 screening strategy after chemical mutagenesis of spermatogonia. As in (B), after mutagenesis of spermatogonia, sperm cells contain fixed mutations. Hence, the F_1 progeny are non-mosaic heterozygotes for the mutation(s) carried by the sperm. Due to the non-mosaic character of F_1 founder fish, 50% of the F_2 siblings carry the inherited mutation(s). Consequently, the mutation(s) should be manifested in 25% of F_2 sibling crosses. As in (C), 25% of F_3 embryos in positive crosses exhibit each mutant phenotype.

a filtration unit (CUNO water filtration system: A124 and A110 dirt/rust filters and A117 activated carbon filter, CUNO Inc., Meriden, Connecticut; filters were exchanged every month) into the main reservoir. Simultaneously, a metering pump dispenses a defined amount of a saline solution (3% w/v Instant Ocean Salt mix, Aquarium Systems, Mentor, Ohio) to a salt content of 0.03% w/v in the water. The pH in the system is buffered by introducing cotton bagged crushed coral (Florida Crushed Coral 150, CaribSea Inc., Miami, Florida) into the particle filter cartridges.

System parameters are: temperature 28.5–29.5° (controlled by room air temperature); salinity 0.03–0.04% (conductivity: 0.9 mS); pH between 6.7 and 6.9; ammonia and nitrite below 0.5 ppm; and nitrate between 30 and 60 ppm. Fish are kept on a 14-hr light/10-hr dark cycle (8.30 AM to 10:30 PM). Tanks are kept clean by catfish (*Ancistrus* and *Plecostoma* sp.), and debris is siphoned off every 2–4 weeks.

Genetic crosses: Crosses between individual male and female zebrafish are set up in "breeding traps" in order to prevent the parents form eating the freshly laid eggs. The design of the breeding traps is based on a similar one by C. NÜSSLEIN-VOLHARD (Tübingen, Germany) described in CULP *et al.* (1991). The bottom of size A polypropylene mouse cages (Nalgene) is cut off and a stainless steel (grade T316) mesh of 1/8-inch mesh size is melted to the bottom of the breeding trap. The steel is coated with polyurethane. Such traps are placed inside size A polycarbonate mouse cages (Nalgene), and filled to 2/3 volume with water from our fish facility ("system water"). A small piece of a green plastic plant placed in the breeding trap serves as a "hideout" in case the behavior of one of the fish becomes too aggressive. Eggs fall through the mesh, and successful crosses can be identified by visual inspection of the clear polycarbonate bottom part of the trap.

Crosses are set up from the late afternoon until evening. The fish usually lay eggs during the first hour after the light is turned on. At noon, the eggs are collected and washed using plastic sieves. Fertilized eggs are sorted using a dissecting microscope and transferred into plastic Petri dishes with "egg water" (0.03% Instant Ocean salt mix in Millipore MilliQplus deionized water). To inhibit fungal and bacterial growth, the egg water is supplemented with 0.5 mg/liter methylene blue (Argentum Laboratories, Redmont, Washington).

Raising and feeding zebrafish: Five days after fertilization, larvae are transferred from Petri dishes to 1-liter beakers and fed with paramecia daily. Paramecia are grown in an axenic medium (SCHÖNEFELD *et al.* 1986) until it becomes clear. Then paramecia are harvested and washed using filter paper (Whatman No. I) or centrifugation (Hermle basket centrifuge SIEVA, setting 50). Every day paramecia from 40 ml of culture are fed to stocks of 50–100 larvae. Ten days after fertilization, the larvae are transferred to "larvae rearing tubes": acrylic tubes 10 cm in diameter with a 0.5-mm nylon mesh attached to the bottom with silicone glue. The mesh allows an easy water exchange without loss of the larvae. The larvae rearing tubes are placed in 2-liter beakers and aerated at about one to two air bubbles per second. Paramecia from 80 ml of culture are fed per 50–100 larvae daily. Around day 15–25 after fertilization, we start to feed freshly hatched brine shrimp *Artemia* sp. (small size: Argentemia Platinum, Argentum Laboratories), and larvae are transferred, within the tubes, into fish tanks. This allows continuous flow of fresh system water through the tubes. At this stage larvae are fed with freshly hatched brine shrimps *Artemia* sp. (San Francisco Bay Brand, Newark, California) and Spirulina algae powder (Argentum Laboratories). At the age of 1 month, fish are transferred to 4-liter tanks and fed with brine shrimps and flake food (Aquarian Growth Food, Mardel Laboratories, Glendale Heights, Illinois). At the age of 2 months, fish are transferred to 20-liter tanks and fed with a mixed diet of brine shrimp, freeze dried and X-ray-sterilized krill (Argentum Laboratories), Spirulina algae powder and flake food (TetraMin Flake Food and Tetra Conditioning Flakes, Tetra Werke, Melle, Germany). Using these conditions zebrafish grew to sexual maturity between 3 and 4 months of age. Within a fish population, the first breeding could be observed at an age of 10–12 weeks, and at the age of 4 months, about 30–50% of crosses were successful and yielded mostly more than 50 fertilized eggs.

Strains: Our standard wild-type strain was derived from the "AB line" from the zebrafish laboratories at the University of Oregon, Eugene, and has been described by CHAKRABARTI *et al.* 1983. These fish have been maintained in our laboratory since spring of 1991 by inbreeding. Every new generation is screened for the presence of embryonic lethal mutations: at least 10 sibling crosses within one stock are performed, and embryonic development is observed until day 7 after fertilization. If more than 95% of the larvae from each cross develop swim bladders on or before day 7, and none of the embryos shows developmental abnormalities, the stock is considered to be free of zygotic lethal mutations. Tracing the lineage allows us to conclude that most of our stocks should also be free of embryonic lethal maternal-effect mutations.

For specific locus tests, strains carrying recessive mutations at four different unlinked loci affecting body pigmentation were used. At the 3rd day of development mutant embryos homozygous for the mutation *albino*^{b4} (*alb*; STREISINGER *et al.* 1986) exhibit melanocytes that are very lightly pigmented compared to the black melanocytes of wild-type embryos. Embryos homozygous for the mutation *golden*^{b1} (*gol*; WALKER and STREISINGER 1983) develop light pigmentation of the body and the retina with a distinct brown tint. Embryos mutant at *brass*^{t} and *brass*^{b2} are characterized by almost wild-type pigmentation of the retina, but light pigmentation of the body (*bra*^{t} obtained from a dealer in Tübingen, Germany) does not complement the locus *bra*^{b2} described as *golden-2* by STREISINGER *et al.* (1986). A recent decision on gene nomenclature in zebrafish resulted in renaming the *golden-2* locus *brass* (MULLINS *et al.* 1993; WESTERFIELD 1993). Finally, the embryos homozygous mutant for the mutation *sparse*^{b5} (*spa*; STREISINGER *et al.* 1986) exhibit melanocytes of wild-type color, but their distribution on the body deviates from that of the wild type. In *spa/spa* 3-day-old embryos melanocytes are missing from the anterior portion of the head, accumulate posterior to the ears and are smaller.

We constructed a quadruply heterozygous stock *alb*^{b4}/+, *bra*^{b2}/+, *gol*^{b1}/+, *spa*^{b5}/+ by crossing a triply mutant stock (*alb*^{b4}/*alb*^{b4}, *gol*^{b1}/*gol*^{b1}, *spa*^{b5}/*spa*^{b5}) with *bra*^{b2}/*bra*^{b2} mutant fish. We used this heterozygous stock for testcrosses because its fertility proved superior to that of any multiply homozygous stocks we have generated.

Handling and inactivation of EMS and ENU: Both EMS and ENU are highly mutagenic and carcinogenic and were handled with extreme caution. All work with these substances was performed in a chemical hood with appropriate protective clothing. Solutions containing EMS or ENU and any equipment or accessories contaminated with these substances were inactivated by incubation in a 10% solution of sodium thiosulfate (inactivating solution) for at least 24 hr (SHEDLOVSKY *et al.* 1986). For EMS the inactivating solution was held at 37°. For ENU the inactivating solution was held at room temperature and adjusted to pH ~10 with sodium hydroxide.

EMS and ENU were purchased from Sigma in sealed bottles. Bottles containing EMS were opened and the liquid was transferred using a pipetman. Isopac bottles containing ENU powder were never opened. To dissolve ENU, 0.03% Instant Ocean

solution buffered with 10 mM sodium phosphate, pH 6.5, was injected into ENU bottles which were subsequently shaken on a rocking platform for 1.5–2.0 hr. The isopac bottles contained approximately 1 g of ENU, yielding a solution of final concentration of approximately 100 mM. The pH of the ENU solution has not been determined. The dissolved ENU solution was used within 2.5 hr from the moment of introduction of the buffer into the bottles. To achieve the desired final concentrations of the two mutagens, appropriate volumes of EMS or of the 100 mM ENU solution were transferred to 1-liter Erlenmeyer flasks containing 500 ml of 0.03% Instant Ocean and 1 mM phosphate buffer at pH 7 for EMS, and at pH 6.5 for ENU. The flasks were immersed in a 21° water bath.

Treatment of zebrafish males with EMS and ENU: Wild-type zebrafish males 4–8 months old were transferred to 1-liter Erlenmeyer flasks containing a mutagen solution at 21°. Up to three males were placed in each flask. At the end of a treatment the mutagen solution from Erlenmeyer flasks with mutagenized males was poured through a fish net held over a container filled with the alkaline sodium thiosulfate solution. The recovered males were immediately transferred to a tank filled with system water at room temperature. Recovery tank(s) containing up to 15 males each were placed in a water bath at 21°, and the temperature was gradually raised to 28°. Any animals that died were removed from the recovery tank and transferred into the alkaline sodium thiosulfate solution. On the following day mutagenized males were usually fed once with live brine shrimps, and their water was exchanged twice. Mutagen-containing water was transferred to the sodium thiosulfate container. At the end of the day the mutagenized males were transferred to a tank in the fish facility.

Testing efficiency of mutagenesis: The efficiency of mutagenesis was tested by two approaches. First, we determined the frequency of induced mutations at one or several pigmentation loci by the specific-locus test (RUSSELL 1951). In the second parallel experiment, the efficiency of mutagenesis was estimated by measurement of the frequency of zygotic embryonic lethal mutations in the progeny of mutagenized males by an F_2 screen. These tests are described in detail below.

Testcrosses with pigmentation mutant fish: The specific-locus tests were carried out by crossing mutagenized males with tester females carrying one or several pigment mutations. During the first 2–4 weeks after mutagenesis the treated males were crossed once per week with alb^{b4}/alb^{b4} tester females to determine the frequency of the newly induced mosaic and non-mosaic mutants at the *alb* locus. Usually 1 month after the completion of a mutagenic regimen females heterozygous for each of the four loci alb^{b4}, gol^{b1}, bra^{t} and spa^{b5} were used instead of the albino tester females. In these crosses we screened exclusively for non-mosaic mutants at these loci. Specific-locus tests were carried out once or twice per week, up to 4 months after mutagenesis.

Single pair matings were performed between mutagenized males and tester females. Resulting egglays were individually collected in Petri dishes and fertilized eggs were sorted out as described above. G_0 males from all EMS and one ENU (ENU4) mutagenesis regimens were kept in common tanks with other males from the same regimen. Males from the remaining ENU regimens, after the first testcross, were separated and kept in individual 1-liter boxes.

On the 3rd day of development larvae were anesthetized with 0.017% (w/v) 3-aminobenzoic acid ethylester (Sigma) in 20 mM Tris HCl, pH 7 (WESTERFIELD 1993). Using a dissecting microscope (Wild M5 and Zeiss Technival 2) we screened among testcross progeny of albino females for larvae with albino eye and body pigmentation, or for larvae with eyes mosaic for the albino phenotype. Progeny of crosses with quadruply heterozygous females were screened for larvae with one of the albino, golden, brass or sparse phenotypes. During the screening, each larva was viewed from the dorsal and lateral sides.

Larvae carrying putative allelic mutations at the tester loci were raised and kept individually in 1-liter boxes. To verify the mutations predicted by embryonic phenotypes, single pair matings were performed between the mature fish carrying the putative new mutant allele and a tester fish homozygous for a mutation at the tested locus. The embryonic pigmentation phenotype of the resulting progeny was examined as described above. If the embryos exhibited wild-type pigmentation, additional crosses with fish carrying the other tester mutations were performed.

Screening for embryonic lethal mutations: The frequency of embryonic lethal mutations induced in germ cells of mutagenized zebrafish males was assessed in a three generation of crosses screen (an F_2 screen).

Production of an F_2 screen: To generate F_1 founders from EMS mutagenesis, mutagenized males were crossed at weekly intervals with wild-type females within a period of 4 weeks following mutagenesis. F_1 founders from ENU regimens were generated in weekly crosses between mutagenized males and wild-type females, beginning 3 weeks after mutagenesis. When mature, each F_1 fish was crossed with either wild-type fish (F_1 × wild-type line), or alternatively with another F_1 fish (F_1 × F_1 line). F_1 fish that failed to produce more than 60 embryos were returned to tanks and reused for the production of F_2 lines. Embryos from crosses in which more than 60 embryos survived until day 1 of development were raised as described above.

Screening F_2 lines for embryonic lethal mutations: When F_2 fish reached 3–4 months of age, they were screened for embryonic lethal mutations. For each F_2 line, up to 25 single-pair crosses between siblings were performed. Eggs from successful crosses were collected in Petri dishes as described above. Up to 30 fertilized eggs from one cross were transferred to each of three wells of six-well tissue culture plates, so that a minimum of 20 and a maximum of 90 embryos from any given cross were screened. Embryos were viewed with a dissecting microscope at day 1, 2, 3 and 5 of development. At each time dead or degenerated embryos were removed from the dishes, and their numbers were recorded. A detailed screening protocol for monitoring various aspects of embryonic development will be provided elsewhere. We operationally defined an embryonic lethal mutation as leading to death or deviations from normal development until the end of day 5 of development. A phenotype was considered to represent a lethal mutation if approximately 1 of 4 (20–30%) of all embryos developed a consistent set of defects before death, or exhibited comparable abnormalities at day 5 of development, and the phenotype was found in separate wells.

Dominant lethal lesions in sperm of mutagenized males: The frequencies at which dominant lethal lesions were induced at different stages of male germ cell differentiation were determined by the analysis of the testcross progeny of mutagenized males. After 1 day of development degenerated embryos were counted and removed from the dishes. At day 3 of development embryos were anesthetized, and the number of both normal embryos and embryos with morphological abnormalities was determined.

Fertility of mutagenized males: To monitor the fertility of mutagenized males, we determined the fraction of fertilized eggs in egglays from the testcrosses. The collected egglays were inspected under a dissecting microscope 2–6 hr after mating. At that time an egglay was a mixture of fertilized eggs, unfertilized eggs and degenerated eggs. Degenerated eggs included abnormal eggs that could not be fertilized, fertilized eggs that

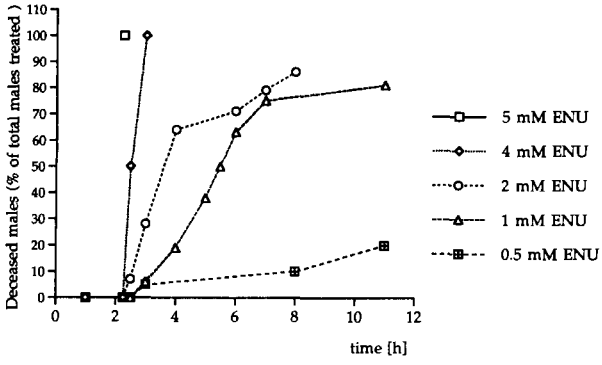

FIGURE 2.—Toxic effect of ENU on wild-type zebrafish males. Fraction of males deceased at different times of incubation in varied concentrations of ENU at 21°. Total number of males (N) for each experiment was: 0.5 mM ENU solution (N = 30); 1 mM (16); 2 mM (14); 4 mM (6); and 5 mM (6).

degenerated during the early divisions and degenerated unfertilized eggs. The various classes of degenerated eggs were not distinguished. However, as the defective eggs are mainly dependent on the females, their levels were constant in our experiments. Any changes in fraction of fertilized eggs in the testcrosses should reflect the fertilization ability of the sperm from mutagenized males.

Statistical analysis: Confidence intervals for the frequencies of specific-locus mutations were calculated assuming that the distribution of mutations can be approximated by the Poisson distribution, using tables of CROW and GARDNER 1959. All comparisons between mean values of different variables studied were performed using the t-test, assuming a significance level of 0.05 unless indicated otherwise. All statistical analyses were done using the computer programs StatView II (Abacus Concepts) and Microsoft Excel (Microsoft Co.).

RESULTS

Determination of the toxic effect of EMS and ENU on zebrafish males: As a method of *in vivo* delivery of EMS and ENU to zebrafish males, we used incubation of males in a mutagen solution (M. MULLINS and C. NÜSSLEIN-VOLHARD, Tübingen; A. SHIMA and A. SHIMADA, Tokyo, personal communications). Preliminary toxicity tests indicated that in 1-hr treatments at 21° EMS was lethal at concentrations higher than 50 mM. The toxicity of EMS was tested further by exposing zebrafish males to 10, 20 and 30 mM solutions of this compound and monitoring lethality at hourly intervals. This experiment indicated that dying started after 1 hr of incubation in 30 mM solution, after 3 hr in 20 mM and only after 6 hr in 10 mM solution of this compound.

The toxicity of ENU was studied by incubating males in a series of solutions from 0.5 to 5 mM at a temperature of 21° and monitoring deaths at hourly intervals. The results (Figure 2) indicated that zebrafish males die after 2 hr in 4 and 5 mM solutions of ENU, and only after longer periods at lower concentrations of this compound.

We then examined survival during and after 1-hr treatments at concentrations of 1–5 mM ENU, and 2-hr treatments with 1 and 2 mM ENU. As expected, all males were alive at the completion of each of these treatments, and none died following 1-hr treatments with 1–3 mM ENU (Table 1). However, all males from 2-hr treatments with 1 and 2 mM solutions of ENU died within 2 hr after the completion of a treatment. After 1-hr treatments with 4 or 5 mM solutions more than 50% of the males died within 2 hr. This indicated that the latter four treatments would not be practical for mutagenesis. The 1-hr treatments with 3 mM ENU performed at a higher temperature (25°) were also found to be too toxic, as less than 60% of treated males were alive 2 hr after treatment (data not shown).

Mutagenic regimens: Based on the toxicity studies we chose the following conditions for a single treatment with EMS: 3-hr incubation in 10 or 20 mM solutions, and 1-hr incubation in 40 mM solution. For ENU we chose 1-hr treatments with 2.0, 2.5, 3.0 and 3.5 mM solutions.

In mice, repeated doses of ENU allow adequate survival and yield higher specific-locus mutation frequencies than can be obtained with single doses at higher concentrations (HITOTSUMACHI *et al.* 1985). We therefore also tested repeated dose regimens for both mutagens.

For ENU, we tested six different mutagenic regimens. In one series, 1-hr treatment with 3 mM ENU was repeated two, four and six times. In the second series, treatments with 2.0, 2.5, 3.0 and 3.5 mM ENU solutions were repeated four times. It has been demonstrated for mice that applications of ENU spaced seven days apart resulted in recovery of fertility, whereas in regimens with shorter intervals, fewer males remained fertile (HITOTSUMACHI *et al.* 1985). We therefore chose the time interval between single treatments of ENU to be 1 week. For EMS, the treatments were repeated three or six times at 3–4-day intervals.

Survival and fertility of mutagenized males: Practically all males survived the EMS regimens and most of them survived for at least 3 months following the treatments (data not shown). The survival of ENU-treated males during consecutive mutagenic treatments is shown in Figure 3A. Only 50% of males survived four treatments with 3.5 mM ENU. Survival of males treated with 3 mM ENU was quite variable between regimens (Figure 3A and data not shown). Greater than 90% of males survived treatments with 2 and 2.5 mM mutagen solutions. The survival following the completion of a regimen, shown in Figure 3B, was poorest for the groups of males treated six times with 3.0 mM ENU where 70% of the males that survived the regimens died within the first 2 months. In contrast, only 15–40% survivors of the four treatment regimens died during the 3 subsequent months.

Male mice mutagenized with ENU go through a transient period of infertility (HITOTSUMACHI *et al.* 1985). Thus we monitored fertility of zebrafish males exposed

TABLE 1

Survival of males after completion of single ENU treatments

ENU concentration (mM)	1-Hour treatment		2-Hour treatment	
	No. of fish at the completion of a treatment	No. of survivors 2 hr after the treatment	No. of fish at the completion of a treatment	No. of survivors 2 hr after the treatment
1	9	9	NA[a]	NA
2	9	9	6	0
3	6	6	6	0
4	6	3	NA	NA
5	10	3	NA	NA

[a] Not assayed.

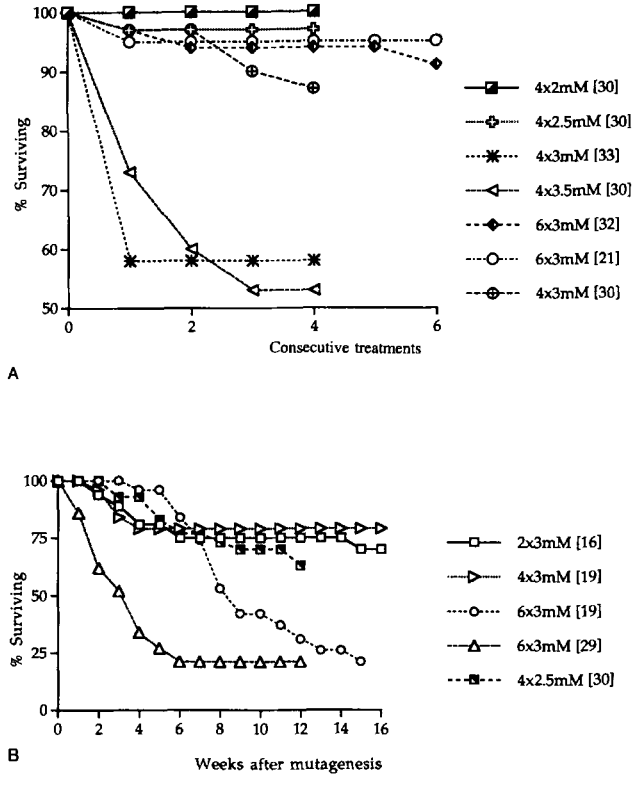

FIGURE 3.—Survival of males during and after mutagenic regimens. (A) Survival of males during consecutive mutagenic treatments as a fraction of the initial population in the ENU regimens: 4 × 2 mM; 4 × 2.5 mM; 4 × 3 mM; 4 × 3 mM; 4 × 3.5 mM, 6 × 3 mM, and 6 × 3 mM. All regimens were started with 30–33 wild-type males. (B) Survival of mutagenized males after the completion of a mutagenic regimen, as a fraction of the survivors of the regimen (N): 2 × 3 mM (16); 4 × 2.5 mM (30); 4 × 3 mM (19); 6 × 3 mM (19), and 6 × 3 mM (29).

to ENU by determining the fraction of fertilized eggs in egglays obtained in testcrosses performed at different times after the completion of mutagenesis (Table 2). Treated males were able to fertilize eggs at all times after mutagenesis. Their fertility, however, was significantly lower than that of untreated wild-type males of similar age (76.7 ± 25.9%) even 3 months after the last ENU treatment (ENU9, $P < 0.03$; ENU8, $P < 0.03$; ENU6, $P < 0.02$; ENU7, $P < 0.006$). Additionally, for all ENU regimens, the fertility during the first 2 weeks after completion of a regimen was significantly lower than the fertility in the 3rd month after mutagenesis (ENU9, $P < 0.003$; ENU6, $P < 0.02$; ENU7, $P < 0.001$; ENU5, $P < 0.0001$; ENU8, $P < 0.0001$). The reduction of fertility during the first 2 weeks after the treatments was dosage dependent. That is, males treated twice with 3 mM ENU fertilized eggs at significantly higher rates than those treated four times ($P < 0.09$); and males treated four times were more fertile then those treated six times ($P < 0.06$). The fertility of mutagenized males increased gradually over time reaching plateau values within 3 weeks, or in case of higher dosages, within 2 months of completion of treatments. Based on these observations we concluded that ENU treatments lead to a dosage-dependent severe reduction of fertility in the first 2 and up to 4 weeks after completion of mutagenic treatments.

Dominant lethal lesions induced by ENU and EMS: Chemical mutagens are known to induce dominant lesions, and their frequency is assumed to be an indicator of the mutagenicity of a given compound (EHLING *et al.* 1968). We therefore monitored embryonic death and morphological abnormalities among progeny of mutagenized males and tester females. We distinguished three categories of embryos: (i) embryos that died and degenerated during the first 24 hr of development and whose specific phenotype could not be assessed, (ii) monstrous embryos that developed body malformations until the 3rd day of development and (iii) embryos that were normal until at least the 3rd day of development. Table 3 shows changes in the frequencies of these three classes of embryos among the testcross progeny of males subjected to different EMS and ENU regimens. Among the progeny of males treated with EMS there was only a small reduction in the frequency of normal embryos during the first weeks after treatment, when compared to crosses performed at later times after mutagenesis or to control crosses. A much higher dominant lethality was observed among progeny of males treated with ENU. Among the testcross progeny obtained during the 1st week after treatments, less than 1% of embryos were normal, up to 70% of fertilized eggs died during the first

TABLE 2

Fertility of males after different ENU regimens

Regimen	1–2 Weeks after regimen		3–4 Weeks after regimen		2 Months after regimen		3 Months after regimen	
	No. of crosses	Percent fertilized (mean ± SD)	No. of crosses	Percent fertilized (mean ± SD)	No. of crosses	Percent fertilized (mean ± SD)	No. of crosses	Percent fertilized (mean ± SD)
ENU6 2 × 3 mM, 1 hr	23	38.0 ± 32.4	23	65.1 ± 22.4	42	65.5 ± 24.7	45	58.6 ± 29.3
ENU7 4 × 3 mM, 1 hr	22	24.0 ± 21.0	20	38.8 ± 29.8	53	56.6 ± 25.2	24	55.0 ± 25.0
ENU5 6 × 3 mM, 1 hr	22	12.9 ± 17.2	22	29.2 ± 24.6	26	54.5 ± 27.5	23	56.3 ± 25.6
ENU9 4 × 2 mM, 1 hr	38	40.7 ± 23.5	56	52.8 ± 27.4	111	54.7 ± 29.1	33	59.7 ± 28.0
ENU8 4 × 2.5 mM, 1 hr	38	26.9 ± 21.5	39	48.6 ± 20.5	63	48.0 ± 22.5	34	63.2 ± 19.7

Males were crossed with tester females at weekly intervals. The fraction of fertilized eggs in an egglay was determined as described in MATERIALS AND METHODS. A mean and the standard deviation of fraction of fertilized eggs was calculated for all egglays obtained from one set of mutagenized males during: 1–2 weeks; 3–4 weeks; 2nd month; and 3rd month time periods. The fertility of untreated wild-type males of similar age was 76.7 ± 25.9%.

TABLE 3

Changes in frequencies of dominant lethal mutants and normal embryos among progeny of EMS- and ENU-mutagenized males

Mutagenic regimen	Phenotypic category	Weeks after mutagenesis (frequencies given as a mean ± standard deviation)					
		1	2	3	4	8	12
EMS6 6 × 20 mM, 3 hr	Dead at 24 hr	0.16	0.15 ± 0.10	0.15 ± 0.16	0.27 ± 0.33	0.13 ± 0.10	NA
	Monstrous	0.04	0.04 ± 0.05	0.03 ± 0.02	0.02 ± 0.03	0.03 ± 0.02	NA
	Normal	0.80	0.81 ± 0.14	0.81 ± 0.18	0.71 ± 0.31	0.87 ± 0.15	NA
EMS7 6 × 40 mM, 1 hr	Dead at 24 hr	0.25	0.07 ± 0.07	0.08 ± 0.11	0.27 ± 0.33	0.13 ± 0.10	NA
	Monstrous	0.14	0.05 ± 0.02	0.15 ± 0.06	0.02 ± 0.03	0.03 ± 0.02	NA
	Normal	0.61	0.89 ± 0.09	0.77 ± 0.12	0.71 ± 0.32	0.84 ± 0.11	NA
ENU6 2 × 3 mM, 1 hr	Dead at 24 hr	0.65 ± 0.22	0.47 ± 0.27	0.23 ± 0.18	0.26 ± 0.25	0.17 ± 0.18	0.15 ± 0.13
	Monstrous	0.34 ± 0.22	0.09 ± 0.05	0.09 ± 0.07	0.05 ± 0.05	0.05 ± 0.04	0.05 ± 0.06
	Normal	0.01 ± 0.01	0.44 ± 0.24	0.68 ± 0.23	0.69 ± 0.24	0.78 ± 0.18	0.78 ± 0.18
ENU7 4 × 3 mM, 1 hr	Dead at 24 hr	0.77 ± 0.18	0.32 ± 0.27	0.12 ± 0.09	0.11 ± 0.08	0.10 ± 0.11	0.08 ± 0.07
	Monstrous	0.25 ± 0.15	0.17 ± 0.13	0.05 ± 0.05	0.08 ± 0.05	0.05 ± 0.05	0.05 ± 0.08
	Normal	0.01 ± 0.02	0.51 ± 0.31	0.84 ± 0.07	0.82 ± 0.09	0.85 ± 0.14	0.87 ± 0.12
ENU5 6 × 3 mM, 1 hr	Dead at 24 hr	0.83 ± 0.22	0.33 ± 0.16	0.16 ± 0.12	0.19 ± 0.10	0.07 ± 0.07	0.10 ± 0.10
	Monstrous	0.26 ± 0.20	0.11 ± 0.08	0.09 ± 0.09	0.05 ± 0.05	0.03 ± 0.02	0.04 ± 0.05
	Normal	0.01 ± 0.01	0.60 ± 0.14	0.75 ± 0.17	0.80 ± 0.09	0.90 ± 0.97	0.87 ± 0.15

Mutagenized males were crossed with tester females at weekly intervals. For each egglay, the fraction of fertilized eggs that (1) died during the first 24 hr of development, (2) exhibited morphological abnormalities at the 3rd day of development and (3) were normal on the 3rd day of development was determined, as described in MATERIALS AND METHODS. Mean and standard deviation reflect variation among egglays from one set of males in a given week. Normal embryos comprised 88.9 ± 11.6% of progeny from control crosses between wild-type males and quadruply heterozygous females. NA = not assayed.

24 hr of development, and most of the remaining embryos exhibited morphological abnormalities by the 3rd day of development. The fraction of normal embryos increased to 40–50% in progeny from testcrosses performed during the 2nd and 3rd week after treatments. Four weeks after mutagenesis normal embryos comprised 80–90% of the testcross progeny, a value not significantly different from that observed for control crosses between untreated wild-type males and quadruply heterozygous females (88.9 ± 11.6%).

Among progeny of ENU-, but not of EMS-treated males, phenotypically albino embryos were found that also exhibited morphological features characteristic of haploid individuals (STREISINGER *et al.* 1981). Haploid development is known to be induced by spermatozoa whose genetic material has been inactivated, by UV irradiation for example (STREISINGER *et al.* 1981). We observed haploid embryos predominantly in crosses performed during the first 2 weeks after completion of ENU regimens. During this time they were detected in 21% of crosses with males treated with the ENU9 (4 × 2 mM; 1 hr) regimen, in 38% of crosses after the ENU8 (4 × 2.5 mM; 1 hr) regimen, and in 46% of crosses following the ENU 7 (4 × 3.0 mM; 1 hr) regimen. In a total of 29 crosses with 10 or more fertilized eggs in which any haploids were observed, haploid embryos constituted 8 ±

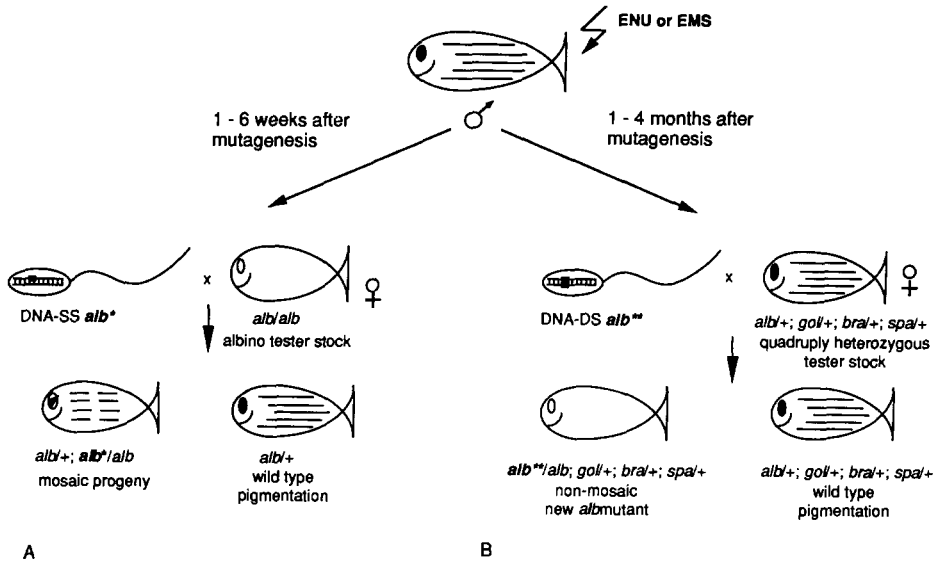

FIGURE 4.—Testing the efficiency of mutagenesis by specific-locus test. Fraction of mosaic and non-mosaic mutants among the progeny of mutagenized males and tester females was determined. (A) During the first 3–6 weeks after the completion of mutagenic treatments mutagenized males were crossed with albino tester females (alb^{b4}/alb^{b4}). All the resulting progeny are heterozygotes $alb^{b4}/+$ and phenotypically wild type, unless a sperm carries a modification of single strand of DNA [as in (A)] or a fixed mutation [as in (B)]. A modification of a single strand of DNA can cause a mutation during DNA replication in a developing embryo, which eventually is a mosaic of wild-type tissue $alb^{b4}/+$ and of mutant tissue alb^{b4}/alb^{*} (* indicated a newly induced allele). When a sperm cell carries a fixed mutation the resulting embryo is uniformly alb^{b4}/alb^{*} and is phenotypically albino. The frequency of mosaic (non-mosaic) mutants at the *alb* locus was calculated as: no. of mosaic (non-mosaic) mutant embryos/total no. of normal embryos (equal to the no. of tested *alb* chromosomes). (B) In the second part of the specific-locus test, the albino females were substituted with quadruply heterozygous females. Every egg produced by such a female carries on average any two of the four tester chromosomes. The resulting progeny are wild type unless sperm from a mutagenized male carries a mutation that does not complement one of the tester mutations. The mean frequency of mutations per locus per gamete in testcrosses was calculated as: no. of non-mosaic mutant embryos found/no. of normal progeny × 2 (average no. of tester chromosomes in each embryo).

14% of embryos. The peak of occurrence of haploid embryos coincided in time with the peak in frequency of dominant lethal lesions induced by ENU.

Frequencies of specific-locus mutants induced by EMS and ENU: EMS and ENU efficiently induce mosaic mutations at the *alb* locus: Drosophila males treated with alkylating agents initially produce F_1 progeny mosaic for the induced mutations, and only later non-mosaic mutant progeny (FAHMY and FAHMY 1957). Because our objective was to generate non-mosaic mutants, it was important to determine how long mosaic mutants would persist among the progeny after the mutagenic treatments of zebrafish males. Frequencies of mutations induced at a specific locus and the mosaic *vs.* non-mosaic character of the mutant progeny of mutagenized males were determined by crossing males with tester mutant females as illustrated in Figure 4. An example of a mosaic embryo recovered in such a testcross with alb^{b}/alb^{b4} homozygous females is shown in Figure 5. The alb^{*}/alb^{b4} (alb^{*} indicates a newly induced mutation) patches are visible both in the ($alb^{b4}/+$) eye retina (Figure 5A) and on the body (Figure 5B) of the otherwise normally pigmented embryo.

Table 4 shows the temporal changes in the frequency of mosaic mutants at the *alb* locus detected among progeny of males treated with different regimens of EMS and ENU. For ENU up to 1%, and for EMS up to 4% of sperm cells from mutagenized males yielded embryos mosaic at this locus. For both mutagens the frequency of mosaic mutants decreased as a function of time after completion of mutagenesis. The highest frequencies ($3–44 \times 10^{-3}$) were observed in the testcrosses performed within the first 2 weeks after completion of a regimen. The frequencies of albino mosaics were lower in the 3rd and 4th weeks after mutagenesis ($0.4–10 \times 10^{-3}$), and less than 0.6×10^{-3} beyond one month after completion of a regimen.

ENU but not EMS efficiently induces non-mosaic mutants: The frequencies of locus-specific non-mosaic mutants recovered from different mutagenesis regimens are shown in Table 5. For EMS regimens, only the *alb* locus was tested. For ENU regimens, both the number of *alb* chromosomes, as well as the number of chromosomes containing all four loci tested for each set of males are given. In cases when all four tester loci were used, the frequencies reflect average numbers of mutations per locus, although each locus was found to have a different mutability (see below).

The minimal number of independent mutational events shown in Table 5 reflects only the first mutation at each tester locus recovered from a single mutagenized male. The frequencies given in the last column of Table

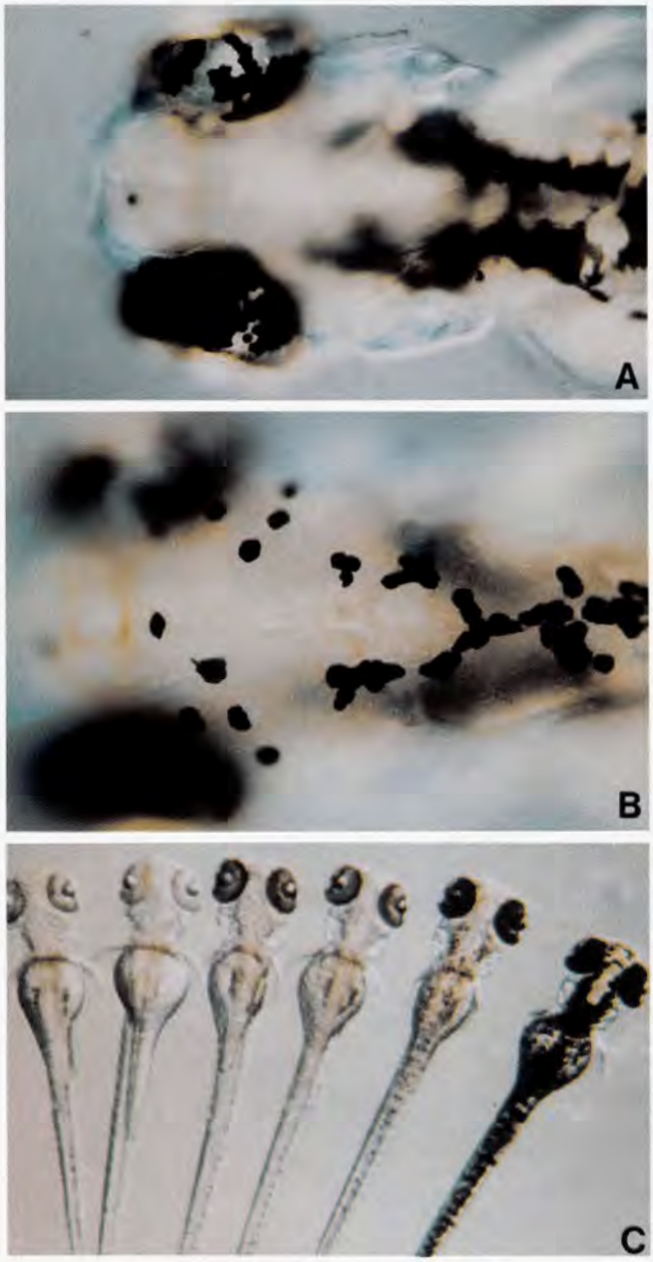

FIGURE 5.—Mosaic and non-mosaic carriers of the ENU-induced alleles at the *alb* locus. (A and B) Five-day-old embryo recovered in a cross between an ENU-treated male and alb^{b4}/alb^{b4} homozygous female. The embryo is mosaic for an ENU-induced mutation at the *alb* locus and exhibits patches phenotypically albino in the otherwise pigmented retina (A) and on the body (B). (C) Group of 3-day-old embryos. The rightmost embryo is wild-type (*alb/alb*) and exhibits dark pigmentation of the retina and of the body, while the leftmost embryo is a homozygous for the standard alb^{b4} allele. The four embryos in the middle contain each one alb^{b4} allele and one of the different, independently induced hypomorphic alleles of the *alb* locus.

5 are based on all mutations, including multiple mutations at the same locus, recovered from a single male. Multiple mutations recovered from the same male may have arisen during independent mutational events. Alternatively they could represent sibling clones derived from single mutational events that subsequently were multiplied during germ cell proliferation. Several observations supported the notion that some of the novel mutant alleles recovered from a single male originated from independent mutational events. First, we observed that two sibling mutant progeny carried novel *alb* mutations of different strengths. Second, of six cases of mutant alleles derived from a single male in crosses with the quadruply heterozygous females, five were mutations in *spa*, the most mutable of the tester loci (see below). Finally, several males produced progeny mutated in two or three different tester loci; these mutations were clearly of independent origin. Therefore, the true mutation rates were probably higher than our minimal frequency estimate, but may be lower than the total mutation frequencies.

The most important finding of the specific-locus tests was that all ENU regimens resulted in high frequencies of recovered non-mosaic mutants (0.5×10^{-3} and up to 3.9×10^{-3}). In contrast, only low mutation frequencies ($<0.1–0.2 \times 10^{-3}$) were observed for EMS-treated males.

Two additional questions were addressed by the specific-locus test. First, was the frequency of mutations induced by ENU dependent on the mutagenic dosage? Figure 6A shows the mean number of mutations per locus per gamete induced for one series of mutagenic regimens in which 1-hr treatments with 3 mM ENU were

TABLE 4

Frequencies of mosaic mutants at the *alb* locus

Regimen	1–2 Weeks after regimen: Mosaic mutants No.	1–2 Weeks after regimen: Mosaic mutants Frequency[a] × 10^3	1–2 Weeks after regimen: Chromosomes tested	3–4 Weeks after regimen: Mosaic mutants No.	3–4 Weeks after regimen: Mosaic mutants Frequency[a] × 10^3	3–4 Weeks after regimen: Chromosomes tested	>1 Month after regimen: Mosaic mutants No.	>1 Month after regimen: Mosaic mutants Frequency[a] × 10^3	>1 Month after regimen: Chromosomes tested
EMS4 1 × 10 mM, 3 hr 2 × 20 mM, 3 hr	14	44 (27–67)	315	4	10 (4–21)	414	1	0.3 (0–2)	2893
EMS5 3 × 20 mM, 3 hr	30	21 (15–28)	1408	2	2 (0.4–5)	1267	1	0.6 (0–3)	1589
EMS6 6 × 40 mM, 1 hr	2	8 (2–25)	240	0	<1.0 (<2)[b]	1019	0	<0.4 (<1)[b]	2287
EMS7 6 × 20 mM, 3 hr	5	14 (7–27)	359	1	1 (0.1–6)	736	0	<0.6 (<2)[b]	1610
ENU6 2 × 3 mM, 1 hr	1	3 (0.4–16)	286	0	<0.4 (<1)[b]	2027		NA[c]	
ENU7 4 × 3 mM, 1 hr	3	6 (2–15)	510	0	<0.6 (<2)[b]	1557		NA	
ENU4 6 × 3 mM, 1 hr	6	10 (4–18)	620	1	1 (0.1–4)	1183		NA	
ENU5 6 × 3 mM, 1 hr	1	6 (0.6–27)	171	2	3 (0.8–10)	627		NA	
ENU9 4 × 2 mM, 1 hr	7	6 (3–10)	1247	0	<0.5 (<1)[b]	1901		NA	
ENU8 4 × 2.5 mM, 1 hr	6	6 (2–11)	1047	0	<0.4 (<1)[b]	2493		NA	

Mutagenized males were crossed with alb^{b4}/alb^{b4} tester females at weekly intervals. The fraction of embryos mosaic for the albino phenotype was determined as described in MATERIALS AND METHODS. The data in the table are pooled from all males in a set and given time period.

[a] Frequencies are given as a point estimate (90% confidence limits).

[b] An upper confidence limit is given.

[c] Not assayed.

TABLE 5

Frequencies of non-mosaic mutations induced in four tester loci

Mutagen	Dosage	Locus tested	No. of chromosomes screened	Minimum of independent mutational events: No. of mutants observed	Minimum of independent mutational events: Frequency (×10^3) Point estimate	Minimum of independent mutational events: Frequency (×10^3) 90% confidence intervals	Total no. of mutational events: No. of mutants observed	Total no. of mutational events: Frequency (×10^3) Point estimate	Total no. of mutational events: Frequency (×10^3) 90% confidence intervals
EMS4	1 × 10 mM, 2 × 20 mM, 3 hr	*alb*	8,204	—	—	—	1	0.12	0.01–0.55
EMS5	3 × 20 mM, 3 hr	*alb*	7,897	—	—	—	1	0.13	0.01–0.57
EMS6	6 × 20 mM, 3 hr	*alb*	2,932	—	—	—	0	<0.34	<0.83
EMS7	6 × 40 mM, 1 hr	*alb*	3,546	—	—	—	0	<0.28	<0.69
ENU6	2 × 3 mM, 1 hr	*alb*	3,486	—	—	—	3	0.86	0.32–2.15
		alb, gol, bra, spa	12,360	5	0.40	0.20–0.79	6	0.49	0.20–0.92
ENU7	4 × 3 mM, 1 hr	*alb*	2,148	3	1.40	0.51–3.50	5	2.33	1.13–4.52
		alb, gol, bra, spa	13,910	17	1.22	0.82–1.76	24	1.73	1.15–2.40
ENU4	6 × 3 mM, 1 hr	*alb*	1,803	—	—	—	7[a]	3.88	1.99–6.95
		alb, gol, bra, spa	11,028	13	1.18	0.68–1.82	15[a]	1.36	0.86–2.02
ENU5	6 × 3 mM, 1 hr	*alb*	2,407	3	1.25	0.46–3.12	5	2.08	1.01–4.04
		alb, gol, bra, spa	2,966	3	1.01	0.37–2.53	6	2.02	0.82–3.82
ENU9	4 × 2 mM, 1 hr	*alb*	3,233	—	—	—	2	0.62	0.16–1.85
		alb, gol, bra, spa	17,520	12	0.68	0.43–1.05	13	0.74	0.43–1.14
ENU8	4 × 2.5 mM, 1 hr	*alb*	2,399	—	—	—	1	0.42	0.04–1.89
		alb, gol, bra, spa	27,224	19	0.70	0.46–0.99	21	0.77	0.50–1.10
ENU10	4 × 3 mM, 1 hr	*alb*	300	—	—	—	1	3.33	0.35–15.1
		alb, gol, bra, spa	12,204	16	1.31	0.80–1.95	17	1.39	0.93–2.00
ENU11	4 × 3.5 mM, 1 hr	*alb, gol, bra, spa*	5,744	—	—	—	8	1.39	0.79–2.36

Mutagenized males were crossed with tester females at weekly intervals, and mutant embryos were screened for as described in MATERIALS AND METHODS. The table presents data obtained from the whole set of mutagenized males.

[a] Males from this set were kept in a common tank. Therefore the minimal number of mutations was calculated as: (total no. of mutants) − (no. of multiple mutants in the same locus from one egglay). This number may be an overestimation of the real minimal number of independent mutants, as single mutants in egglays obtained at different time points could be derived from the same male.

repeated two, four and six times. There was more than a twofold difference in the frequency of mutations between the treatments repeated four and two times. However, only a small further increase of mutation frequencies was observed for one of the two regimens in which the number of treatments was increased to six. Figure 6B shows the frequency of specific-locus mutations as a function of concentration of ENU for a series of regimens in which 1-hr treatments were repeated four times. There was more than a twofold increase in mutation

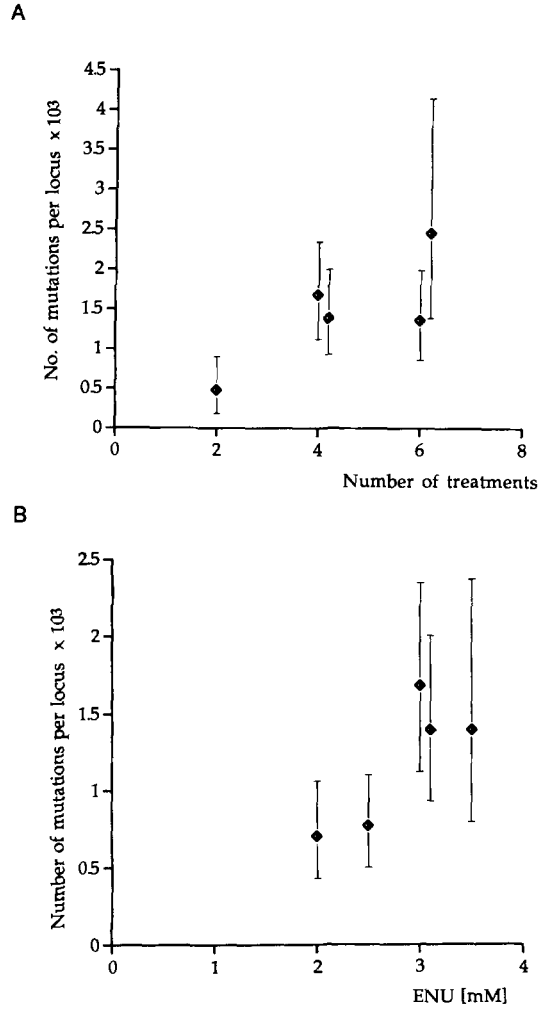

FIGURE 6.—Specific-locus frequencies of non-mosaic mutants as the function of a mutagenic dosage plotted with 90% confidence intervals. Given frequencies were based on the total number of mutational events (see text and Table 5 for details). (A) Frequencies after the 2 ×; 4 ×; 6 × 3 mM ENU regimens. (B) Frequencies after the 4 × 2.0; 2.5; 3.0 and 3.5 mM ENU regimens.

frequency between regimens utilizing 2.5 mM and 3 mM ENU. However, an increase of the ENU concentration from 3 to 3.5 mM has not raised the mutation frequency further.

Second, did the frequency of non-mosaic mutant progeny of mutagenized males change over time after the completion of a mutagenic treatment? This was an important issue for determining the time period during which mutagenized males could be used to generate F_1 founders for subsequent embryonic lethal screen. To answer this question, for each mutagenic regimen, we normalized the mean number of mutations per locus per gamete recovered during monthly intervals relative to the 1st month, and subsequently compared average monthly mutation rates. No significant differences were observed between mutation frequencies during a period of 4 months after mutagenesis, for neither the frequencies adjusted for clusters nor for the frequencies reflecting all mutations recovered in the specific-locus test (data not shown).

Testing the allelism of mutations recovered in the specific-locus test: Mutations detected in the specific-locus test by virtue of not complementing one of the tester loci could represent new allelic mutations at the given locus. Alternatively, they could represent dominant mutations at distinct loci, or non-allelic non-complementing mutations. To distinguish between these possibilities, we tested the fish carrying putative new mutant alleles in crosses with homozygotes at the tester locus in question. For example, a fish carrying a new mutant allele at the *alb* locus should yield exclusively albino progeny in crosses with *alb/alb* tester fish. The presence of wild-type embryos would indicate that the new mutation is at a locus distinct from *alb*.

A total of 137 mutants were detected in the specific-locus test. Neither of the two fish carrying mutations induced by EMS survived until testing. From the 135 fish carrying the ENU-induced mutations, 52 survived to adulthood, and one was sterile. The allelism tests were completed for 44 fish and confirmed that all of them carried a mutation at one of the tester loci. Table 6 shows representative examples of such tests. In most cases (37/40) the tests confirmed the allelic classification done at embryonic stages. Additionally, these tests indicated that some of the newly induced *albino* and *golden* mutations, represented weaker mutant alleles of these loci. When fish carrying such weaker *albino* alleles were crossed with alb^{b4}/alb^{b4} tester fish, two classes of embryos were observed among the progeny. 50% of the albino embryos were darker than the remaining alb^{b4}/alb^{b4} homozygotes and represented heterozygotes of an old alb^{b4} and a new weaker *alb** allele (Table 6). Figure 5C shows a series of embryos, each carrying a different, independently induced mutation at the *alb* locus. The existence of hypomorphic mutations at the *alb* and *gol* loci often made it difficult to identify new mutations unequivocally in embryos derived from the crosses of mutagenized males and the quadruply heterozygous females. In several ambiguous cases new mutations could not be classified at embryonic stages (M in Table 6). However, when adult fish carrying these M mutations were crossed with tester fish, they proved to harbor mutations at either *alb* or *gol* loci. In three of five cases embryos classified as carrying new mutations at the *bra* locus proved upon testing to carry mutations at one of the other three tester loci.

Based on the above tests, we concluded that most if not all animals recovered in the specific-locus test carried a mutation at one of the tester loci, or at a distinct but very closely linked gene. In addition, these tests demonstrated that the ENU-induced mutations at pigmentation loci were transmitted in the germline of the F_1 progeny of the mutagenized males.

TABLE 6

Testing allelism of the newly induced mutations *vs.* the tester loci

Mutation predicted by an embryonic phenotype	Mutation confirmed in test crosses	Results of a cross identifying a newly induced tested mutation			How closely linked if not the same gene (cM)
		No. embryos scored	No. mutant embryos	No. wild-type embryos	
alb	*alb*	639	639	0	0.2
alb	*alb*	906	906	0	0.1
*alb**	*alb**	310	164* + 146	0	0.3
gol	*gol*	342	342	0	0.3
*gol**	*gol**	273	133* + 140	0	0.4
bra	*bra*	260	260	0	0.4
spa	*spa*	951	951	0	0.1
spa	*spa*	232	232	0	0.4
M[a]	*alb**	592	290* + 302	0	0.2
M	*alb**	623	295* + 328	0	0.2
M	*gol**	117	117	0	0.9
bra	*gol*	247	247	0	0.4
bra	*alb**	338	159* + 179	0	0.3
bra	*spa*	483	483	0	0.2

* New allele of a different strength when compared to the canonical allele at this locus in the tester strain.
[a] *M*, in these cases the locus at which the newly induced mutations occurred could not be predicted by the embryonic phenotype.

TABLE 7

Comparison of relative mutability of the tester loci in the specific-locus test

Gene	Chromosomes screened	Minimum of independent mutational events			Total no. of mutational events		
		Alleles recovered	Frequencies ($\times 10^3$)	Frequencies $1/X$	Alleles recovered	Frequencies ($\times 10^3$)	Frequencies $1/X$
alb	25,739	19	0.74	1/1355	21	0.82	1/1225
gol	25,739	21	0.82	1/1225	22	0.86	1/1170
bra	25,739	11	0.43	1/2340	12 (6)[a]	0.47 (0.23)	1/2150 (4290)
spa	25,739	42	1.63	1/610	55	2.14	1/470
Total	102,956	93	0.90	1/1100	110	1.1	1/930

[a] Five out of 15 putative *bra* mutants survived and could be tested. Out of these five only two (40%) were confirmed as new *bra* alleles. Therefore, probably not all of the putative *bra* mutants carried a mutation in this locus. The numbers in parentheses are adjusted to 40% confirmed surviving mutants.

The distribution among the tester loci of mutations induced by ENU is shown in Table 7. The table includes mutations confirmed in the allelism tests as well as mutations for which carriers died. It can be seen that mutations were induced at all four loci, with the rates at least 10-fold higher at the *spa* locus than at the least mutable *bra* locus. The estimated mean mutation frequencies could be underestimated if some very weak alleles at the tester loci were not recognized. Furthermore, several mutants that exhibited a questionable phenotype subsequently died, and could not be tested, were excluded from our calculations. On the other hand, the frequencies at the *bra* locus could be overestimated. Just 5 out of 15 putative mutants survived to adulthood and only two proved to carry a new *bra* mutation. This 40% rate of confirmation of surviving putative *bra* mutants was extrapolated to all isolated putative mutants at this locus (Table 7).

Recovery of induced mutations in an F_2 screen: The numbers of embryonic lethal mutations induced in germ cells of mutagenized males were determined in an F_2 screen (see MATERIALS AND METHODS). An embryonic lethal mutation was operationally defined as leading to death or developmental abnormalities until the end of day 5 of development. This definition deviates from that of CHAKRABARTI *et al.* (1983) who included in the group of embryonic lethal phenotypes fish that did not show an inflated swim bladder at day seven of development. Our definition is based on two reasons. First, we found that individuals with a non-inflated swim bladder eat and swim at day 7. We therefore consider these fish to be in a larval, not embryonic, stage of development. Second, several mutations with the noninflated swim bladder phenotype that had been isolated from distinct F_2 lines did not complement one another. This indicated that they probably preexisted in the AB wild-type stocks used

for mutagenesis. A phenotype was considered to represent a lethal mutation if approximately one in four (20–30%) of all embryos developed a consistent set of defects before death, or exhibited comparable abnormalities at day 5 of development, and the phenotype was found in separate wells. This strict definition of embryonic lethal mutation could result in omission of potential hypomorphic mutations that lead to a variable phenotype. To confirm a specific phenotype, we repeated the cross in which a given phenotype was observed and embryos were rescreened. More than 95% of initially isolated mutations were confirmed in the rescreens.

In the case of EMS mutagenesis we screened 71 F_2 lines generated by crosses between two F_1 founders ($F_1 \times F_1$) and thus containing mutations from 142 mutagenized genomes. On average 10.5 crosses were performed during screening of each line. F_1 founder animals used for the generation of F_2 lines were produced by crossing the mutagenized males during the first 4 weeks after completion of mutagenesis. The high incidence of mosaic mutations in the *alb* locus was observed during this time (Table 4). Nevertheless, only 16 embryonic lethal mutations were detected during screening of these lines. This corresponded to 0.11 mutations per mutagenized genome.

For the ENU screen, F_2 lines were produced using F_1 founders generated only later than 3 weeks after completion of mutagenic treatments. Therefore, these lines were expected to harbor mutations induced in premeiotic germ cells. Of 116 F_2 lines screened, 66 were derived from crosses of F_1 founders and wild type fish ($F_1 \times$ wild type), and corresponded to 66 mutagenized genomes from the ENU4 regimen. The remaining 50 F_2 lines were produced by crosses between F_1 founders, and thus represented mutations from 100 mutagenized genomes. Screening a single F_2 line described here involved a minimum of 6 and on average 9.4 sibling crosses (90% confidence interval: 9.1–9.7). F_3 progeny were then screened for embryonic lethal mutations. A total of 214 embryonic lethal mutations was recovered. Each mutation was manifested on average in 22% of crosses (90% confidence interval: 21–25). This indicated that, as expected, most of the mutations were of the nonmosaic type. Most mutants in which specific aspects of development were affected exhibited unique phenotypes. They most likely represented new mutations in different genes. Complementation tests among mutants with similar phenotypes indicated that the majority of mutations affected distinct loci.

The results of screening $F_1 \times$ wild type lines indicated that sperm from males mutagenized in the ENU4 and ENU5 regimens (6 × 3 mM ENU, 1 hr), harbored on average 1.70 (90% confidence interval: 1.47–1.93) embryonic lethal mutations per single mutagenized genome. Interestingly, $F_1 \times F_1$ lines have not yielded the expected twofold increase in the average rate of lethal mutations relative to $F_1 \times$ wild-type lines. On average only 2.12 mutations per $F_1 \times F_1$ line were recovered which corresponded to 1.06 mutations per mutagenized genome (90% confidence interval: 0.93–1.19).

DISCUSSION

As the first step toward saturation mutagenesis screens for embryonic lethal mutations in zebrafish we compared the capability of two chemical mutagens to induce mutations in the germline of males.

Mosaic mutants–mutagenic effect on post-meiotic germ cells: Males treated with different EMS and ENU mutagenic regimens produced initially a high incidence of embryos mosaic for the albino phenotype in crosses with females homozygous for a mutation at the *alb* locus. The frequencies of mosaic mutant progeny of mutagenized males decreased following the 2nd week after mutagenesis. Mosaic mutants were also reported to be induced by EMS and/or ENU exposure of mature spermatozoa in Drosophila (Ondřej 1971), mouse oocytes (Lewis *et al.* 1985), postgonial germ cells in mice (Lewis *et al.* 1984, 1990; Favor *et al.* 1990a), and mouse zygotes (Russell *et al.* 1988). The formation of mosaic mutants in zebrafish via action of ENU on post-meiotic germ cells is consistent with earlier reports that fertilization with sperm exposed to ENU results predominantly in mosaic embryos (Golin *et al.* 1982; Grunwald and Streisinger 1992). In our studies, 1-hr treatments of males with 2–3 mM ENU solutions resulted in frequencies of mosaic mutants at the *alb* locus (Table 2) very similar to those observed for this locus in the case of sperm treated *in vitro* for 10 min with 1 and 2 mM ENU solutions (Grunwald and Streisinger 1992).

Appearance of mosaic mutant progeny after treatments of postreplicative germ-cell stages with alkylating agents like ENU or EMS has been explained by the fact that these mutagens can alkylate bases on one strand of DNA. In post-meiotic germ cells or a zygote, the modifications, if not repaired, become fixed as mutations only during divisions following fertilization. Resulting F_1 embryos would be genetically mosaic (Russell *et al.* 1988). The observed temporal changes in frequency of albino mosaics (Table 4) indicated that the majority of post-meiotic mutagen-sensitive cells were utilized within a two week period following mutagenesis, only a small fraction (10%) in the 3rd and 4th week. This is consistent with the studies of spermatogenesis in the related teleost, medaka (*O. latipes*). In medaka, it takes at least 12 days for DNA-replicating spermatocytes and 7 days for spermatids to differentiate into mature spermatozoa at 25° (Egami and Hyodo-Taguchi 1967). The rate of spermatogenesis is temperature-dependent (Egami and Hyodo-Taguchi 1967). Hence, the differentiation in zebrafish at 28.5° may be slightly faster. It has been suggested that in mouse germ cells, ENU-induced DNA lesions may persist through a number of cleavage divisions

before being eventually repaired or fixed as a mutation (FAVOR *et al.* 1990b). Therefore, some of the mosaic albino mutants, obtained at later time points after mutagenic treatments, could be caused by mutagenic lesions effected in still dividing germ cells. Alternatively, they could be derived from mutagenized post-meiotic germ cells utilized at later time points after completion of mutagenic treatments.

Non-mosaic mutants–mutagenic effect on pre-meiotic germ cells: DNA lesions formed by chemical mutagens in a pre-meiotic germ cell can be fixed as mutations before differentiation into spermatozoa. Embryos formed upon fertilization with such a sperm would be non-mosaic for mutations introduced by the sperm. Very low frequencies of non-mosaic mutants were detected after EMS treatments. The high mutagenic potency of EMS in the zebrafish on the later stages of spermatogenesis and only low activity on the earlier stages of germ cell differentiation is consistent with mutagenicity of EMS in Drosophila (FAHMY and FAHMY 1957; VOGEL *et al.* 1982) and in the mouse (RUSSELL *et al.* 1981; VAN ZEELAND *et al.* 1990).

In contrast, all ENU regimens tested resulted in high frequencies of non-mosaic mutants among the progeny of treated males. The spontaneous mutation rate of 3×10^{-5} reported for the *gol* locus in zebrafish (WALKER and STREISINGER 1983), is 50 times lower than the mutation frequency at this locus obtained in this study (*e.g.*, ENU7: 1.4×10^{-3}). The specific-locus test utilizing four pigmentation loci indicated that our most efficient ENU regimens yielded non-mosaic mutants at a rate of 1 per 700–800 mutagenized genomes scored. Such high rates are very similar to the average mutation frequencies at 7 tester loci in the mouse after the most efficient repeated dose experiments (1 mutation at 1 locus per less than 700 mutagenized genomes; HITOTSUMACHI *et al.* 1985). The comparison of the ENU dosage utilized in the fish and the mouse is not straightforward. In the zebrafish, ENU was delivered to animals by incubation of fish in the mutagen solution, whereas mouse males received intraperitoneal injections of ENU solution (RUSSELL *et al.* 1979).

Two observations indicated that apparent whole-body mutants carried mutations induced in pre-meiotic germ cells, most probably spermatogonia. First, mutant fish carrying newly induced putative mutations at tester loci, produced only mutant progeny in crosses with appropriate testers. This indicated that their germline was not mosaic. Second, non-mosaic mutants were observed at constant rates among testcross progeny even 4 months after completion of mutagenic treatments.

For all ENU regimens the frequencies of non-mosaic mutants at the *alb* locus were two to threefold lower than the frequencies of albino mosaics (Table 4). Similar differences between these two classes of mutants were observed in Drosophila (FAHMY and FAHMY 1957). In contrast, in mice ENU was found to be less effective in inducing mutations in post-spermatogonial germ cell stages than in spermatogonia (RUSSELL and HUNSICKER 1984; VAN ZEELAND *et al.* 1985). The lower ratio of mosaic *vs.* non-mosaic mutants in the mouse compared with fish is not likely to be the result of more efficient repair in the mouse, as murine mid-spermatids and mature spermatozoa are probably incompetent in DNA repair (SEGA 1974). Rather, during rapid mitoses in fish (and Drosophila) blastoderm, premutational lesions induced in spermatids and spermatozoa may have a higher fixation rate than those in very slowly developing mouse embryos.

Dominant lethal lesions: The peak in ENU-induced dominant lesions was observed during the first 2 weeks after mutagenesis. It corresponded to the peak in frequencies of mosaic mutants and coincided with occurrence of haploid embryos. There was no obvious correlation between the incidence of dominant lesions and non-mosaic locus-specific mutants. Although, the correspondence between specific-locus and dominant-lethals patterns is found for several chemical mutagens in the mouse (RUSSELL *et al.* 1989), this correspondence is not found for ENU (GENOROSO 1984), consistent with our results in zebrafish.

EMS treatments lead to very high frequencies of mosaic mutants; however, neither haploid embryos nor high numbers of dominant lethal lesions were observed among progeny of EMS-treated males. Consistent with these observations, no significant levels of dominant lesions were observed after exposure of medaka males to varied doses of EMS (SHIMADA and EGAMI 1984). Also in mice EMS proved to be less effective in inducing dominant lethality than other alkylating agents (EHLING *et al.* 1968).

Chemical basis for induction of different types of mutations by ENU and EMS: In post-meiotic germ cell stages both EMS and ENU efficiently induced locus-specific recessive mutations (mosaic mutants). Only ENU, however, induced a high level of dominant lesions. In pre-meiotic germ cells ENU induced locus-specific mutations at frequencies an order of magnitude higher than EMS. The basis for differences in effectiveness of ENU and EMS in induction of different types of mutations in the two cell types is not clear. However, the distinct spectra of DNA lesions effected by these two mutagens are likely to play a role (VAN ZEELAND *et al.* 1990). The high mutagenic potency of EMS in the late stages of spermatogenesis is explained by relatively high levels of *N*-alkylation and subsequent accumulation of apurinic sites (VAN ZEELAND *et al.* 1990). The DNA lesions induced in postreplicative cell stages leading eventually to mosaic mutants may be distinct from those leading to dominant-lethal lesions and genetic inactivation of the sperm. The latter would be effected rather by ENU than EMS.

The higher mutagenicity of ENU compared with EMS in spermatogonial cells is explained by the ratio of *O- vs. N*-alkylations which is higher for ENU than EMS (SINGER and GRUNBERGER 1983). It is not clear which of the *O*-alkylations effected by ENU is responsible for the high mutagenicity of this compound in spermatogonial cells. The O^6-ethylguanine has been suggested to be the relevant adduct which eventually should lead to GC → AT transitions (LOVELESS 1969). Indeed, the majority of mutations induced by ENU in *E. coli* were demonstrated to be GC → AT transitions, and less frequently AT → CG transversions (RICHARDSON *et al.* 1987). In the mouse, however, most of the ENU induced mutations analyzed at the molecular level have been shown to be transitions and transversions at AT sites, and less frequently GC → AT transitions (POPP *et al.* 1983; LEWIS *et al.* 1985; HARBACH *et al.* 1992). Thus, in mammalian cells DNA-alkylation products other than O^6-ethylguanine may be also relevant for ENU mutagenesis. It is not known whether the same DNA adducts are responsible for induction of all types of mutations brought about by ENU in different germ cell stages.

Nature of induced mutations: Three observations indicated that mutations induced in fish by ENU were probably either point mutations and/or small, intragenic lesions. First, induction of mosaic mutations by ENU is consistent with a proposed mode of mutagenic activity via alkylation of DNA, a process that generally produces point mutations (SINGER and GRUNBERGER 1983). Second, a large proportion of the new mutations induced at the *alb* locus were hypomorphic–consistent with an intragenic type of lesion. Finally, none of the four tested mutations induced by ENU at the *alb* locus was linked to lethal mutations, supporting the notion that the mutations were limited to single genes (data not shown). In other systems, as discussed above, point mutations have been demonstrated to constitute the majority of lesions produced by ENU *in vivo* (RICHARDSON *et al.* 1987; POPP *et al.* 1983; and see below). Additionally, it has been suggested that the germ cell stage of induction is the major determinant of the nature of induced mutations. Large lesions constitute only a small proportion of mutations induced by a variety of chemicals in stem cell or differentiating spermatogonia (RUSSELL *et al.* 1990).

Fertility of mutagenized males: Male mice treated with ENU go through a transient period of infertility, the length of which positively correlates with the dose of a treatment, and inversely with the length of intervals between treatments (HITOTSUMACHI *et al.* 1985). This transient period of sterility observed for mouse males is explained by ENU-dependent spermatogonial killing (OAKBERG, as cited in RUSSELL *et al.* 1979). In contrast, mutagenized zebrafish males have not exhibited even a short period of complete infertility following mutagenesis. We have observed, however, a period of severalfold reduction of fertility during the first two weeks after treatments, the degree of which was dosage dependent. Additionally, mutagenized males have not recovered to the fertility of control males in the 3 months following treatments.

Dosage dependence: The frequency of non-mosaic mutants generated by ENU was dependent on the dosage. When the concentration of ENU was varied from 2.0 to 3.0 mM, we observed a significant increase in the mutation frequency. However, there was no further increase in frequencies for the 3.5 mM ENU regimen. We were interested in the most effective treatments, and thus have not extended our studies to lower concentrations of ENU. Lower concentrations of ENU have been tested in medaka: a single 2-hr incubation of males at 27° with up to 1 mM ENU results in specific-locus mutation rates in spermatogonial cells lower than those induced by any treatments in this paper (A. SHIMA and A. SHIMADA, personal communication).

As observed with mice (HITOTSUMACHI *et al.* 1985), we observed an approximately twofold increase in the frequency of locus-specific mutations when the number of treatments in a mutagenic regimen was increased from two to four. However, only a small increase in mutagenic frequencies was observed for one of the two regimens in which the ENU treatment was repeated six times.

Which of the mutagenic regimens tested would be optimal for production of F_1 founder animals? Besides the dosage-dependence of mutation frequencies discussed above, for an F_2 screen the survival of mutagenized males is very important, since they are used for the generation of F_1 founders in the period between 3 weeks and 3 months after mutagenic treatments. Noticeably, the 4 × 3.5 mM regimen was very toxic during mutagenic treatments. In both 6 × 3 mM treatments, 50% of the animals that survived the treatments died during the first 2 months after the completion of mutagenesis. For these reasons, we have chosen the 1-hr treatment with 3 mM ENU repeated four times as our routine mutagenesis regimen. However, a measurement of frequencies of embryonic lethal mutations in F_2 lines produced from males after various mutagenic regimens is needed to decide which of them is best for saturation mutagenesis screens.

Our studies indicated that 3.5 mM is the maximal feasible concentration of ENU for 1-hr treatment at 21°. There are other variables of mutagenesis that have not been explored in this study. For example higher temperature or higher ENU concentrations with shorter treatments may be tested for increased frequency of induced locus-specific and embryonic lethal mutations.

Variable mutability of the tester loci: Among the four pigmentation loci used for specific-locus tests, mutation rates in the *spa* gene were more than two-fold higher compared to *alb* and *gol* loci, and more than 10-fold

higher than in the least mutable *bra* locus. Similar differences of mutability of the tester loci have been observed in mice (HITOTSUMACHI *et al.* 1985; RUSSELL *et al.* 1990). The low number of mutations isolated at the *bra* locus might be assigned to two factors. First, the *bra* locus might be a cold spot for ENU mutagenesis, *e.g.*, due to a small locus size. Second, embryos carrying hypomorphic *bra* alleles might have been scored as wild type at day 3 of embryogenesis.

Advantages of mutagenizing spermatogonial stem cells: These have been well recognized and proven in the mouse (RUSSELL *et al.* 1979; PETERS 1985; RINCHIK 1991). Here we demonstrated that they may also apply to zebrafish. A group of males treated with ENU can continue to produce mutagenized sperm derived from mutagenized spermatogonia until the males cease mating, or die. Because female fish produce large numbers of eggs, we were able to generate up to a thousand F_1 founder animals from a single mutagenized male. Thus, one group of males subjected to ENU mutagenesis can produce several thousand F_2 lines. This minimizes the handling of ENU.

The screening of F_2 lines for embryonic lethal mutations has revealed the main advantage of spermatogonial *vs.* sperm mutagenesis: on average a quarter of F_2 sibling crosses manifested a given mutation harbored by this line. This is very important for several reasons. First, only small numbers of crosses are needed to recover the majority of mutations carried in each line. Second, recovery of a mutation more than once during screening of a single line provides rapid confirmation of a mutant phenotype. Third, several carriers can be identified at once, which enables one to work immediately on the mutation, and facilitates its preservation via sperm freezing. Finally, the average number of mutations per genome is probably lower for non-mosaic than mosaic F_1 founders for screens detecting similar numbers of mutants. This makes further genetic analysis of mutants derived from non-mosaic F_1 less difficult.

An estimate of the number of genes that can mutate to a recessive embryonic lethal phenotype: The frequency of specific-locus mutations (1.1×10^{-3}; 90% confidence limits: $0.69–1.70 \times 10^{-3}$, an average rate of minimal independent mutational events for the ENU4 and ENU5 sets of mutagenized males used to generate F_2 lines, Table 5) was compared with that of embryonic-lethal mutations per mutagenized genome (1.70; 90% confidence interval: 1.47–1.93). This yielded an initial estimate of the number of genes that can mutate to embryonic lethality in the zebrafish to be around 1,500 (800–2,800). This estimate was based on four assumptions. First, mutations in embryonic lethal genes should be transmitted to subsequent generations with an efficiency equal to those in tester genes. Second, the average target for generating an embryonic lethal allele should be equal to that for producing an allele for the preexisting pigmentation mutation (DOVE 1987). In the latter case both the relative mutabilities of single loci, as well as frequencies of loci with different mutabilites in a class of genes are important. However, neither of these two variables between embryonic lethal and pigmentation loci in vertebrates have been rigorously compared. Therefore, our extrapolation of mutation frequencies from a small number of pigmentation loci to embryonic lethal genes could lead to under or overestimation of the number of embryonic lethal genes in zebrafish. Third, hypomorphic mutations in embryonic lethal genes should be detected as efficiently as hypomorphic mutations in the tester pigmentation loci. This might not necessarily be the case, as hypomorphic mutations in some genes might be manifested in a range of variable phenotypes. Such hypomorphic embryonic lethal mutations could be excluded in our screen due to the strict definition of the embryonic lethal phenotype. This strict definition is nevertheless necessary as the nonspecific lethal effects can be exhibited by up to 20% progeny of a screen cross, and often of the rescreen cross. Our fourth assumption was that the number of embryonic lethal genes per gamete is linearly dependent on the average rate of the specific locus mutations. We have not observed, however, the expected two-fold increase in the number of embryonic lethal mutations in $F_1 \times F_1$ lines *vs.* $F_1 \times$ wild-type lines. The basis for this phenomenon is not clear. One interpretation is that while the number of locus-specific mutations per genome increases linearly with the ENU dosage, the number of embryonic lethal mutations permitting a successful generation and screening of an F_2 family reaches a plateau. This could lead to an underestimation of the number of embryonic lethal genes.

The estimated number of 1,500 of embryonic lethal genes is threefold smaller than 5,000 of genes essential for development and viability of the zebrafish proposed by GRUNWALD and STREISINGER (1992) based on ENU mutagenesis of sperm cells. This discrepancy results probably from different operational definitions of embryonic lethal mutations. The number of essential genes in the mouse has been estimated at 5,000–10,000 by CARTER (1957). A similar number was reached after extrapolating results of saturation mutagenesis of the *T/t-H-2* region with recessive pre-natal lethal ENU mutations (SHEDLOVSKY *et al.* 1986). In *D. melanogaster* the number of genes that are required zygotically for embryonic development has been estimated at 1,500 (NÜSSLEIN-VOLHARD *et al.* 1984; JÜRGENS *et al.* 1984; WIESCHAUS *et al.* 1984). A comparison between the fish, fly and the mouse estimates is difficult as we do not know whether the phenotypic class of embryonic lethal genes in the fish or fly precisely corresponds to the genes essential for mouse development.

Toward saturation of zebrafish genome with zygotic embryonic lethal mutations: The results reported here

support the notion that zebrafish fulfills all the criteria required to perform saturation mutagenesis screens for embryonic lethal mutations (STREISINGER *et al.* 1981; KIMMEL 1989). First, ENU can induce in the zebrafish germline high rates of mutations affecting predominantly single genes. Second, mutations are efficiently transmitted to progeny of mutagenized males and segregate at Mendelian ratios in the progeny of F_2 sibling crosses. Screening is fast and even subtle defects in organogenesis can be detected using a dissecting microscope.

How many lines would have to be screened to detect 87% of all genes that can be mutated to a zygotic embryonic lethal phenotype? Our initial results allow us to make a rough approximation, based on two assumptions about the mutability and transmission of embryonic lethal mutations discussed above. If the distribution of mutations in the genome can be approximated by the Poisson distribution, about 87% of genes would be detected by a screen that obtained on average two mutations per locus. At an average locus-specific mutation rate per genome of 1.27×10^{-3} (Table 5, an average for the 4×3 mM, 1-hr regimens, now used routinely for the production of F_1 founders), 1,600 genomes would have to be screened. Since genes are not equally mutable, however, distribution of mutations in some genes deviates from that predicted by Poisson. For instance a saturation screen in Drosophila has revealed that at an average recovery of about four alleles per locus about 25% of all identified genes were represented by single alleles (WIESCHAUS *et al.* 1984) (also see LEFEVRE and WATKINS 1986). Consequently, in 1,600 genomes screened we would recover more than 95% of loci that are as highly mutable as *spa* (locus-specific mutation rate per genome 2.1×10^{-3}) but less than 50% of the loci that have as low a mutability as *bra* (locus-specific mutation rate of less than $0.2–0.4 \times 10^{-3}$). To isolate mutations in 87% of the loci that are as mutable as *bra* at least 5,000–10,000 genomes would have to be screened. Currently our laboratory is screening 160 F_2 lines per month, making the objective of detecting the majority of mutable embryonic lethal genes reachable within 2 years.

Our main goal is the identification of genes that determine the vertebrate body plan during embryogenesis. An initial assessment of mutant phenotypes indicated that mutations induced by ENU can affect both early embryogenetic processes: formation of the notochord, patterning of the neural tube, as well as specific aspects of later development and organogenesis: formation of the ear, jaw and heart. The availability of mutations in several genes affecting the same process will enable one to use the full power of genetic analysis in vertebrates and to establish hierarchies in which these genes work. The analysis of mutations affecting fish embryogenesis will complement and enrich the knowledge about mechanisms of vertebrate development obtained from current embryological and molecular genetic approaches.

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