Genet. Res., Camb. (1992), **59**, *pp.* 103–116 *With 4 text-figures Printed in Great Britain*

Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea

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(*Received 22 August 1991*)

Summary

Recessive lethal mutations and mutations at the *gol-1* locus were induced in the zebrafish by exposure of mature sperm to the alkylating agent ethyl nitrosourea (ENU). Embryonic lethal phenotypes were recognized among the parthenogenetic progeny of mutagenized animals or among the progeny of daughters of mutagenized animals. Novel specific locus mutations were identified by the failure of mutagenized chromosomes to complement pre-existing mutant alleles at the *gol-1* locus. Each mutagenized individual harboured approximately 10 embryonic lethal mutations in its germ line and about 1 in 500 mutagenized animals harboured a new mutation at the *gol-1* locus. Three lines of evidence indicate that the majority of mutations that were recovered following treatment of mature sperm with ENU were probably point mutations. First, the soma and germ lines of mutagenized animals were mosaic, as expected following simple alkylation of sperm DNA. Second, mutations induced by ENU at the *gol-1* locus affected pigmentation but not viability, unlike the majority of mutations induced at this locus with γ-irradiation. Third, the ratio of specific locus:recessive lethal mutations induced by ENU was approximately 50-fold lower than the ratio observed following mutagenesis with γ-rays. Comparison of the incidence with which embryonic recessive lethal mutations were induced with the incidence with which specific locus mutations arose indicates that there are greater than 5000 genes essential to the development and viability of the zebrafish embryo.

1. Introduction

Analysis of the normal pattern and regulation of development has profited greatly from the study of the effects of genetic alterations. In the accompanying report (Grunwald & Streisinger, 1991) methods were described for the induction of mutations in the zebrafish with ultraviolet (UV) light. To further extend the variety of genetic lesions that may be introduced into the germ lines of zebrafish, we have analyzed the ability of the chemical mutagen ethyl nitrosourea (ENU) to induce recessive lethal mutations affecting any gene essential to embryogenesis as well as mutations in pre-specified pigmentation genes.

Ethyl nitrosourea is at present the most potent mutagen known in mice and has been used successfully to induce mutations in genes of diverse functions (Russell *et al.* 1979; Johnson & Lewis, 1981; Bode, 1984; Moser *et al.* 1990). Mutation frequencies with this agent are sufficiently high in the mouse that substantial regions of the genome, such as the T-complex of chromosome 17, may be dissected genetically following saturation mutagenesis (Shedlovsky *et al.* 1988; King *et al.* 1989). The primary mutagenic action of ENU results from its ability to alkylate one strand of a DNA duplex, thereby generating base substitutions upon DNA replication (Richardson *et al.* 1987; Pastnik *et al.* 1989). Consistent with this interpretation, ENU has been found far more effective than γ-irradiation for the induction of isozyme variants in the mouse (Johnson & Lewis, 1981). Therefore ENU may be able to induce a variety of types of alleles, including hypomorphic, neomorphic, and null alleles, that would be useful for exploring the potential range of functions of a gene of interest (Clifford & Schupbach, 1989).

We present here results indicating that ENU is more effective than UV light as a mutagen in mature sperm of zebrafish. We have measured by several

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complementary means the approximate frequency with which heritable recessive lethal or specific locus mutations may be induced with ENU. The frequency of the induction of mutations at the *gol-1* locus relative to that of mutations at any essential locus is consistent with the interpretation that ENU primarily causes single-gene mutations in the zebrafish. Importantly, our results demonstrate that point mutations affecting the morphological development of the early embryo of this simple vertebrate can be recovered efficiently.

2. Materials and methods

(i) *Strains and mutations*

The origin of the standard strain and the clonal strains, C29 and C32, have been described previously (Chakrabarti *et al.* 1983). Three fully recessive mutant alleles at the *gol-1* locus (generically referred to as *gol-1*) were used in the present study: *gol-1(b1)*, *gol-1(b13)* and *gol-1(b6)*. *gol-1(b1)* was isolated as a spontaneous mutation from standard strain fish and at the time of this study had been crossed on to a clonal background for two generations. Fish homozygous for this allele were fully viable and expressed a defect in pigmentation easily observed at 2–3 days postfertilization in the pigmented retina of the embryo (Streisinger *et al.* 1989). *gol-1(b6)* and *gol-1(b13)* were induced independently following mutagenesis of (C29 × C32)F1 fish with γ-irradiation (Walker & Streisinger, 1983) and have since been maintained by sequential crosses with either C29 or C32 fish. The *b6* and *b13* mutations fail to complement *b1* for normal pigmentation. In addition, the two γ-ray-induced alleles are recessive lethal mutations, and each expresses a distinctive lethal phenotype recognizable at 2 days. The recessive pigmentation mutation, *alb-1(b4)*, which is unlinked to *gol-1*, arose spontaneously and does not affect viability (Streisinger *et al.* 1986). Homozygous albino embryos lack pigmentation and are easily distinguished from wild-type embryos at 2 days.

(ii) In vitro *fertilization and development*

Procedures for the collection of gametes and for fertilization *in vitro* have been described (Streisinger *et al.* 1981; Chakrabarti *et al.* 1983). Embryos were raised at 28·5 °C in embryo rearing water [ER water = glass-distilled water containing 0·06 g l^{-1} sea salts (Instant Ocean, Aquarium Systems, Inc.)]. Developmental times in the text refer to hours or days postfertilization at 28·5 °C.

(iii) *Preparation and handling of ethyl nitrosourea*

ENU is a proven mutagen and carcinogen and should be handled with special care. ENU (Pfalzer-Balz) was stored desiccated at −20 °C and was allowed to equilibrate with ambient temperature before dispensing. All manipulations were conducted over absorbent diapers in a chemical fume hood and manual pipetting aids were used. Potentially contaminated spatulae and glassware were immersed in 10% sodium thiosulphate. At the conclusion of an experiment the decontaminating solution was adjusted to *ca.* pH 12 by the addition of NaOH pellets. ENU was allowed to react with the sodium thiosulphate solution for at least 24 h before considering glassware decontaminated.

Shortly before use, 20 mM-ENU was prepared by dissolving ENU in Hank's-minus-bicarbonate (HMB = 137 mM-NaCl, 5 mM-KCl, 0·25 mM-Na_2HPO_4, 1·3 mM-$CaCl_2$, 1·0 mM-$MgSO_4$), adjusted to pH 5·95 ±0·05. ENU was further diluted in HMB to yield solutions containing 2× final desired concentrations. All solutions were maintained at 4 °C. Since ENU is somewhat unstable at −20 °C and since very small quantities of ENU were measured out for each experiment, it is likely that the true concentrations of the working solutions were only an approximation of the intended final concentrations of ENU. Small day-to-day differences in the actual concentrations of prepared ENU may have accounted for the variability observed between experiments measuring the lethal effects induced in sperm (see Fig. 1).

(iv) *Mutagenesis of sperm*

The procedures employed for treating zebrafish with the alkylating agent ENU were developed with concern for optimizing the mutagenic activity of ENU while minimizing the potential toxic effects experienced by sperm. As a simple and rapid measure of ENU activity, our initial experiments focused on the ability of ENU to induce histidine auxotrophy in the *Salmonella* strain TA 1535 (Ames *et al.* 1975; Lee *et al.* 1977) when 10^9 bacteria, in the presence of $1{\cdot}5 \times 10^6$ zebrafish sperm, were exposed to the mutagen. As reported previously (Loveless & Hampton, 1969; Hince & Neale, 1974) the mutagenic activity of ENU was highly dependent on pH, increasing more than 100-fold over the range tested, pH 6·0–8·5 (data not shown). 'Standard conditions' of mutagenesis were developed in which cells were exposed to mutagen at pH 8 for 10 min at room temperature. Sperm incubated under these conditions did not lose appreciable fertilizing capacity as compared with control sperm. In addition, under standard conditions of exposure the reversion frequency of TA 1535 was unaffected by the addition of $1{\cdot}5 \times 10^6$ sperm prior to treatment with 10 mM-ENU, indicating that the effective concentration of ENU was undiminished by the presence of the sperm.

Sperm were collected in Hank's-plus-Tris [HPT = HMB + 10 mM Tris(hydroxymethyl)aminomethane]

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adjusted to pH 8·00 ± 0·05. The final concentration of sperm was approximately 5×10^7 ml^{-1}. To mutagenize sperm, 25 μl ENU in HMB was mixed with an equal volume of sperm in HPT at room temperature for 10 min, after which time the sperm–ENU solution was mixed with freshly collected eggs. Sperm were activated with the addition of 0·5 ml ER water. After 0·5–1 min, 2 ml ER water was added, and eggs were transferred to 100 ml ER water in 250 ml glass beakers maintained at 28·5 °C in a water bath. After 2 h, the eggs were rinsed four times with 100 ml ER water, transferred to fresh beakers containing 100 ml ER water, and removed from the chemical hood for evaluation of further development.

(v) *Production of homozygous and partially homozygous parthenogenetic offspring*

To produce completely homozygous embryos (Streisinger *et al.* 1981): eggs were collected from individual females, activated with sperm rendered genetically impotent following exposure to a high dose of UV-irradiation, and incubated at 40 °C from 13 to 15 min postfertilization. This procedure, called Heat Shock (HS) treatment, yields diploid homozygous progeny by activating the replication of the maternal chromosomes and suppressing the first mitotic division.

To produce partially homozygous gynogenetic offspring (Streisinger *et al.* 1981, 1986): eggs were collected from individual females, activated with sperm rendered genetically impotent following exposure to a high dose of UV-irradiation, and subjected to a pulse of hydrostatic pressure from 1·4 to 6·0 min postfertilization. This treatment, called the Early Pressure (EP) treatment, eliminates any paternal genetic contribution to the offspring and suppresses the second meiotic division, which normally occurs following activation of eggs. Resulting gynogenetic embryos develop from meiotic half-tetrads, their chromosome complement being derived from sister chromatids present subsequent to the first meiotic division.

3. Results

(i) *Production of ENU-mutagenized zebrafish*

To establish the maximal concentration of ENU that could be used to mutagenize sperm under the standard conditions of treatment (defined in Materials and methods), sperm were exposed to varying concentrations of ENU and the ability of treated sperm to fertilize eggs and support normal development was examined. Exposure of sperm to $\leqslant 4$ mM-ENU had little effect on the ability of sperm to activate eggs to cleave normally (data not shown). However, eggs fertilized by ENU-treated sperm developed poorly following cleavage as compared with those fertilized by control sperm (Fig. 1). The lethal effects caused by ENU-treatment of sperm could be measured by the resultant decrease in the ability of embryos to develop swimbladders, an organ essential to survival and normally apparent by days 5–6. Following exposure of sperm to > 2 mM-ENU, less than 2% of the embryos survived to the swimbladder stage. Therefore, we chose to assess the mutagenic effects of ENU in individuals derived from sperm that had been exposed to 1 or 2 mM-ENU, conditions where 10–70% of the eggs developed normally following fertilization with treated sperm. Henceforth these individuals, exposed to ENU pre-zygotically, will be referred to as G0 mutagenized fish.

(ii) *Measurements of the presence of recessive lethal mutations in the germ lines of mutagenized zebrafish*

The induction of recessive embryonic lethal mutations was measured in three complementary ways (Fig. 2). In the first two series of experiments, the homozygous progeny of mutagenized females were examined for the expression of lethal mutations, using quantitative and qualitative criteria (Grunwald & Streisinger, 1991). First, the viability of the homozygous progeny of mutagenized females was compared with that of the offspring of control females. Second, homozygous progeny were inspected for distinctive aberrant phenotypes expressed by groups of sibling embryos. In the third series of experiments, mutagenized animals were outcrossed to wild type fish to generate G1 outcross families, members of which were progeny tested for the inheritance of recessive lethal mutations.

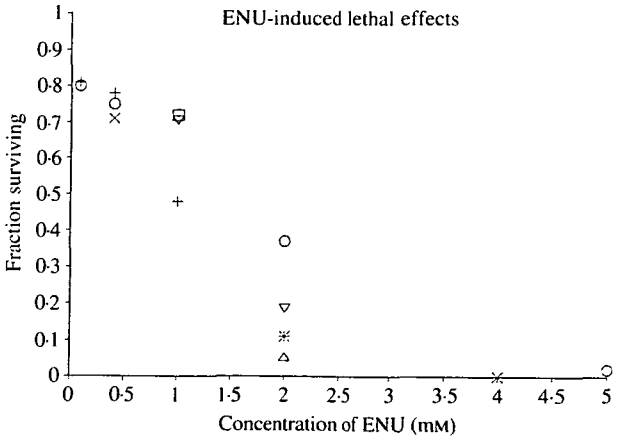

Fig. 1. ENU-treatment of sperm destroys the ability of sperm to support normal development. Wild-type eggs were fertilized with sperm that had been exposed to ENU under standard conditions. Eggs that failed to initiate cleavage were discarded. The fraction of cleaving eggs that developed into free-swimming larvae as measured by the appearance of the swimbladder by 7 days is plotted as a function of the concentration of ENU to which sperm had been exposed. Each symbol represents data from a single experiment in which sperm were treated with various concentrations of ENU. Several hundred cleaving eggs were inspected for each data point reported.

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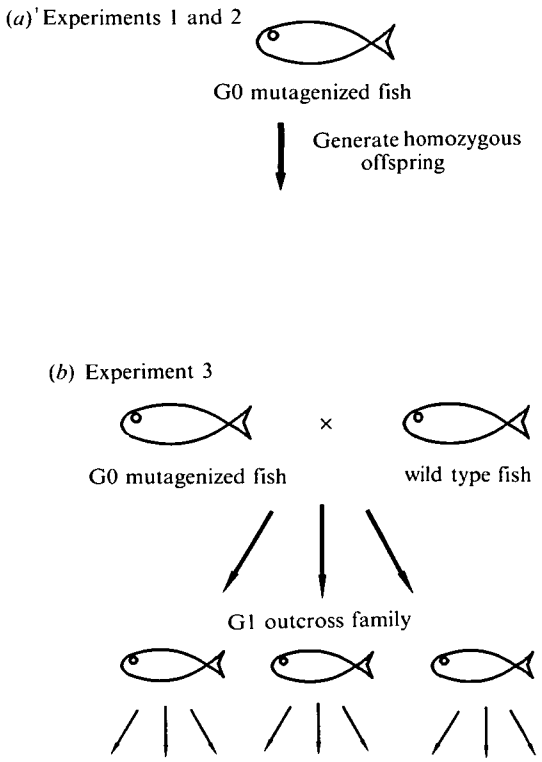

Fig. 2. Methods used for the detection of lethal mutations induced by ENU. To generate G0 mutagenized fish, wild-type eggs were fertilized with ENU-treated sperm. (*a*) Embryonic lethal mutations in the germ lines of G0 fish were detected by measuring the viability of their homozygous offspring relative to the viability of homozygous offspring of controls (experiment 1). Embryonic lethal mutations in the germ lines of G0 fish were also detected by recognizing morphologically aberrant phenotypes expressed by groups of developing homozygous embryos (experiment 2). (*b*) Recessive embryonic lethal mutations in the germ lines of G0 fish were detected among the grandchildren of mutagenized fish. G0 mutations were first propagated in heterozygous G1 offspring. To detect mutations inherited by G1 offspring, their partially homozygous, gynogenetic G2 offspring were examined for the expression of morphologically aberrant phenotypes (experiment 3).

(iii) *Induction of embryonic lethal mutations: analysis of the viability of homozygous progeny*

To analyse the induction of lethal mutations, eggs from individual mutagenized and control (C29 × C32)F1 females were activated and rendered homozygous by the HS procedure. Females with newly induced lethal mutations in their germ lines should yield homozygous progeny that fail to survive, and the magnitude of the difference between the viability of homozygous offspring from mutagenized and control groups may be used to estimate the average number of lethal mutations per gamete of a mutagenized individual (see Discussion). The fraction of each female's eggs that developed to the swimbladder stage was determined, and the mean survival of homozygous offspring from each group of females is presented in Table 1.

Homozygous embryos from mutagenized females exhibited poor viability relative to those from control females (Table 1). As observed in previous experiments (Streisinger *et al.* 1981; Grunwald & Streisinger, 1991), the HS procedure itself is damaging to the survival of eggs, and only 19–23% of the eggs from control females developed normally. Offspring of 1 mM-ENU-mutagenized fish survived about half as well as their control counterparts, and, in a small sample, all of the homozygous progeny of 2 mM-ENU-mutagenized females were inviable. In contrast, females that developed from sperm that had been exposed to lower concentrations of ENU (0·4 mM) yielded homozygous embryos whose viability was similar to that of controls.

It is likely that the relative failure of homozygous embryos of mutagenized females to survive was largely due to the expression of recessive lethal mutations present in those embryos and not due to maternal effects that generally inhibited egg maturation or development. Eggs from all of the mutagenized females initiated cleavage normally upon activation with genetically impotent sperm. Moreover, in several cases (see below) where mutagenized females were crossed with wild type males (including both 2 mM-ENU-mutagenized females represented in Table 1), their eggs developed normally and the survival of their heterozygous offspring measured at the swimbladder stage was not significantly different from that of control embryos (data not shown).

(iv) *Induction of embryonic lethal mutations: analysis of the phenotypes of homozygous progeny*

The homozygous offspring of control and mutagenized zebrafish were examined at days 1–3 for the expression of characteristic patterns of altered embryonic morphology or tissue degeneration (see Grunwald *et al.* 1988; Kimmel *et al.* 1989; Grunwald & Streisinger, 1991). At these stages, the overall body plan of the embryo has been established and segmentation has been completed. Aberrant developmental syndromes displayed by clusters of three or more homozygous embryos from a single female were recorded as evidence of an embryonic mutation in the G0 germ line.

Our goal in the present study was to establish that point mutations leading to embryonic defects can be induced in an efficient and reproducible manner in the zebrafish. Detailed descriptions of specific morphological defects will not be reported here. Five characteristic embryonic lethal phenotypes were identified among the homozygous progeny of four 2 mM-ENU-mutagenized females, and two distinctive clusters of mutant embryos were recognized among the progeny of three 1 mM-ENU-treated females (Table 2). These results indicate that at least one mutation in a gene essential to the establishment and maintenance of the normal embryonic body plan can be recovered from each 2 mM-ENU-mutagenized individual.

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Table 1. *Survival of homozygous progeny of control and mutagenized zebrafish*

	(1) Number of G0 females analysed[a]	(2) Number of homozygous eggs scored	(3) Mean fraction (±S.E.) of eggs that survived[b]	(4) Viability relative to controls[c]	(5) Average number of lethal mutations per G0 gamete[d]
Experiment 1					
Control	12	483	0·19±0·04	1·0	—
1 mM-ENU-treated	12	910	0·09±0·02[e]	0·46	0·8
Experiment 2					
Control	6	482	0·23±0·06	1·0	—
2 mM-ENU-treated	2	87	0 (⩽0·04)[e,f]	⩽0·17	⩽1·8
0·4 mM-ENU-treated	4	371	0·19±0·06	N.S.[g]	—

[a] G0 females arose from *in vitro* fertilization crosses between two homozygous clonal strains, C29 and C32. Prior to fertilization of eggs, ENU-treated sperm were exposed to 0·4, 1 or 2 mM-ENU under standard conditions described in the text. Control sperm were incubated without ENU. Experiments 1 and 2 were performed independently.
[b] Survival was measured at day 7 by the appearance of the swimbladder, a reliable indicator of prospective survival to adulthood. The fraction of each G0 female's progeny that formed a swimbladder was determined. The mean fraction is the non-weighted average of the fraction of each group of progeny that survived.
[c] Relative viability, calculated from the data of column (3), is simply:

$$\frac{\text{mean surviving fraction of one group}}{\text{mean surviving fraction of controls}}.$$

[d] This estimate, discussed in the text, is based on the application of the Poisson equation, $P(0) = e^{-m}$, to the survival data where m is the average number of lethal mutations per G0 gamete, and given the following interpretations: (i) that lethal mutations were distributed approximately normally among the gametes of the G0 female, and (ii) that the survival of homozygous offspring relative to controls [column (4)] indicates the fraction of a G0 mutagenized female's eggs that lacks ENU-induced lethal mutations.
[e] Significantly different from the survival of control eggs ($P < 0·05$).
[f] 95% confidence limits are indicated; none of the homozygous offspring survived (0/87).
[g] Not significantly different from the survival of control eggs ($P > 0·05$).

Table 2. *Identification of embryonic lethal phenotypes among homozygous progeny of mutagenized G0 zebrafish*

	Number of G0 females analysed	Number of characteristic lethal phenotypes	Estimated number of lethal mutations per G0 germ line
Control	4	0	0
1 mM-ENU-treated	3	2	0·67
2 mM-ENU-treated	4	5	1·25

Homozygous eggs from control and ENU-mutagenized fish were inspected at 1, 2, and 3 days for expression of distinctive aberrant embryonic phenotypes. The estimated number of lethal mutations per G0 germ line is simply:

$$\frac{\text{no. of characteristic lethal phenotypes}}{\text{no. of G0 females analysed}}.$$

(v) *Induction of embryonic lethal mutations: analysis of G1 outcross families*

To examine the possibility that many mutations had been induced in each germ line, a few G0 germ lines were analysed in detail. Several G0 mutagenized individuals were crossed with clonal strain partners to generate G1 outcross families. Each family represents the progeny of a single mutagenized fish (Fig. 2). Each G1 individual would have far fewer mutations per germ line than its mutagenized parent, simplifying the identification of developmental mutations. In addition, recovery of induced mutations from the germ lines of members of G1 outcross families would demonstrate the heritability of the embryonic lethal mutations induced in G0 germ lines.

To uncover potential embryonic lethal recessive mutations in the germ lines of G1 outcross individuals, eggs from G1 individuals were rendered partially homozygous by the EP treatment (see Materials and methods), and the resulting gynogenetic offspring were examined for the expression of lethal syndromes. Among offspring produced in this manner, genes that are proximal to their respective centromeres would

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Table 3. *Identification of heritable embryonic lethal syndromes recovered from individual G0 mutagenized zebrafish*

	(1) Number of G1 family members analyzed by progeny testing[a]	(2) Total number of mutant clusters observed among G2 gynogenetic progeny	(3) Mean number of mutant phenotypes transmitted per G0 gamete[b]	(4) Number of independent mutant phenotypes observed in a G1 family	(5) Number of independent mutant phenotypes inherited by several G1 members
Experiment 1 (2 mM-ENU-treated)					
G1 outcross family A[c]	8	10	1·25	5	3
G1 outcross family B	8	10	1·25	6	3
G1 outcross family C	4	4	1	3	1
Total	20	24	1·2	14	7
Experiment 2 (1 mM-ENU-treated)					
G1 outcross family D	3	2	0·67	2	0
G1 outcross family E	3	3	1	1	1
Total	6	5	0·83	3	1

[a] Eggs were collected from individual G1 females and were activated to develop gynogenetically (EP treatment). Developing embryos were examined microscopically at 1, 2 and 3 days for the expression of distinctive abnormal morphology or for evidence of distinctive patterns of degeneration. Clusters of sibling embryos that displayed a common abnormal phenotype were scored as a mutant phenotype.

[b] Calculated as:

$$\frac{\text{total no. of clusters of mutant embryos observed among the gynogenetic offspring of G1 family members [column (2)]}}{\text{no. of G1 family members that were progeny tested}}.$$

This number indicates the number of lethal mutations per G0 gamete transmitted to the next generation.

[c] Each G1 outcross family represents the progeny of a single G0 mutagenized individual that had been mated with a wild-type partner.

tend to be homozygous and genes that are relatively more distal would rarely be in homozygous state (Streisinger *et al.* 1986). Despite the limitation that only portions of the genome of G1 individuals could be canvassed for lethal mutations by this method, analysis of gynogenetic progeny for the expression of lethal syndromes is a useful tool. Since the majority of embryos produced in this way develop normally, it is simple to recognize groups of morphologically aberrant embryos among the gynogenetically produced offspring.

Almost every one of the 26 G1 individuals that were progeny tested (Table 3) had inherited a mutation that resulted in a distinctive pattern of embryo lethality. These data indicate that each gamete from a G0 mutagenized individual harboured, on average, at least one transmissible lethal mutation [columns (2) and (3)]. The identified syndromes ranged from major deformities to subtle alterations. For example, the three members of outcross family E each gave rise to clusters of gynogenetic 1-day embryos lacking heads. To demonstrate that the clusters of gynogenetic embryos reflected mutations present in heterozygous state in the G1 generation, eggs of two of the members of family E were also activated to develop as haploid embryos. Half of the eggs of each group displayed the headless phenotype. Other syndromes included improperly shaped body structures and several distinctive patterns of degeneration in the central nervous system, each specific with respect to timing and locale (see Grunwald *et al.* 1988; Grunwald & Streisinger, 1991).

Mutant embryonic lethal syndromes were found as single occurrences transmitted by an individual G1 family member, or as multiple occurrences transmitted by several members of a G1 family. Recovery of an embryonic lethal syndrome from several members of a G1 family is a clearcut indicator of the heritability of a mutation. In Table 3, we present separately the number of lethal syndromes recovered as multiple occurrences from 2 or more members of a G1 family. Among the descendants of three 2 mM-ENU-mutagenized G0 individuals (Table 3, Experiment 1), at least 7 embryonic lethal mutations were inherited by two or more members of a particular G1 outcross family. Counting only these we derive a minimum estimate that at least two embryonic lethal recessive mutations were induced per G0 germ line (7 syndromes per 3 G1 outcross families) following mutagenesis with 2 mM-ENU.

It is likely that each of the 17 syndromes that were identified among the G2 gynogenetic progeny resulted from inherited embryonic lethal mutations. The probability of recovering a G0 germ-line mutation in several G1 offspring was dependent upon both the

number of G1 family members sampled and the prevalence of the mutant allele in the germ line of the original G0 mutagenized individual. We present evidence below that, in general, any G0 germ-line mutation would have been present in at most one-fourth of the gametes. Given the number of G1 family members sampled in each case, it is not surprising that 9 of the 17 embryonic syndromes were recognized as single occurrences. If we regard each identified lethal syndrome as an heritable mutation, the germ lines of each 2 mM-ENU-mutagenized G0 individual harboured on average about 5 morphologically recognizable embryonic lethal recessive mutations (14 independent syndromes per 3 G1 outcross families), and the germ lines of 1 mM-ENU-mutagenized G0 individuals harboured 1–2 such mutations (3 independent syndromes per 2 G1 outcross families).

(vi) *Distribution of mutations in the germ lines of G0 ENU-mutagenized zebrafish*

We expected that mutations resulting from the initial alkylation of a single strand of the DNA duplex of a sperm chromosome might be borne often by only a subset of the paternally derived chromosomes in a G0 individual, as was found true for UV light-induced mutations (Grunwald & Streisinger, 1991). The actual prevalence of individual ENU-induced mutations in the germ lines of G0 zebrafish was measured in two ways. In the first experiment, eggs from G0 mutagenized individuals were made homozygous and diploid by the HS procedure, and the fraction of embryos from each female that displayed a common syndrome was determined. The six mutant syndromes that were recognized among the groups of 50 or more embryos were found present as 0·11, 0·22, 0·23, 0·26, 0·27, or 0·29 of the embryos analysed (Fig. 3). In addition, the fraction of G1 outcross family members that inherited each of the 17 lethal syndromes (Table 3) was determined and is presented in Fig. 3. Together, these data indicate that most of the mutations transmitted to the G1 offspring were represented in at most one-fourth of the gametes of G0 mutagenized fish. Thus the germ lines of the mutagenized fish were genetically mosaic.

(vii) *Induction of somatic mutations*

Given the high frequency with which recessive lethal mutations were induced in zebrafish following exposure of sperm to ENU, it seemed likely that newly induced mutations at pre-specified loci could be recovered following a similar mutagenesis regimen. The induction of somatic mutations affecting expression of the *gol-1* locus was measured in G0 individuals. Wild-type sperm were treated with ENU and used to fertilize eggs harbouring a recessive pigmentation mutation at *gol-1*. Embryos were inspected at 2 and 3 days for the presence of pigmentless

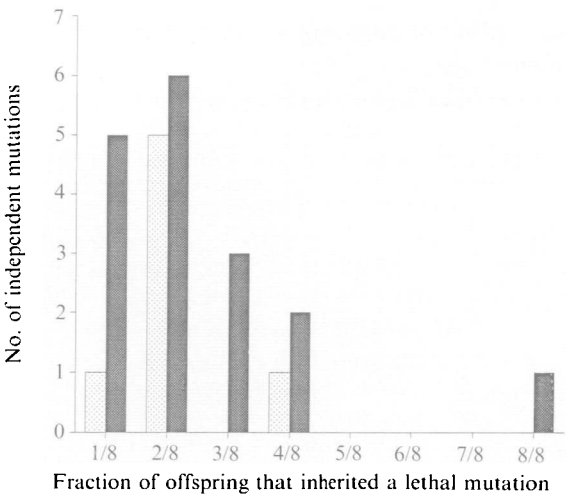

Fig. 3. Each newly induced lethal mutation is transmitted to less than half of the offspring of a G0 ENU-mutagenized fish. Results of two experiments, described in Fig. 2 and in the text, are presented. For the analysis of homozygous offspring (▨), homozygous diploid embryos from individual G0 mutagenized fish were inspected for expression of developmental syndromes. Each mutation recorded is presented as a function of the fraction of homozygous offspring that expressed a particular mutant syndrome. For the analysis of G1 outcross families (■), partially homozygous gynogenetic embryos from individual G1 family members were inspected for expression of developmental syndromes (Table 3). Each mutation recorded is presented as a function of the fraction of members of a G1 outcross family that inherited the mutation.

cells in the pigmented retinal epithelium (Grunwald & Streisinger, 1991). Fertilization of *gol-1* eggs with mutagenized sperm resulted in the development of embryos whose eyes were mosaic for pigmentation, consistent with the observation that mutagenized G0 individuals were genetically mosaic for recessive lethal mutations.

The incidence of somatic mosaicism was dependent on the dose of ENU experienced by sperm (Table 4). In some experiments the *gol-1* mutation resident in the egg (*b6* or *b13*) was also lethal when homozygous and thus necessarily was derived from heterozygous females. To compare the results of different experiments we have standardized the data and report the incidence of mosaicism as

$$\frac{\text{fraction of mosaic embryos}}{\text{average no. maternal } gol\text{-}1 \text{ mutations per egg}}.$$

Slight variation was observed in the incidence of mosaicism induced in eggs harbouring different maternally derived chromosomes. In general, when eggs harbouring any of three *gol-1* alleles were fertilized with sperm that had been treated with 2 mM-ENU, approximately 5% of the embryos that survived until day 3 were mosaic for pigmentation pattern. Following fertilization of *gol-1* eggs with 1 mM-ENU-treated sperm, roughly 2% of the surviving embryos were mosaic for pigmentation (Tables 4 and 5). The

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Table 4. *Effect of dose on the induction of somatic mutations*

ENU concentration	Incidence of mosaic individuals (× 100)
	(A) *gol-1(b1)* maternal allele
Control	0·06 (⩽ 0·22) (1663 embryos scored)
1 mM	3·3 ± 0·33 (n = 3; 882 embryos scored)
2 mM	6·1 ± 1·4 (n = 7; 4246 embryos scored)
	(B) *gol-1(b13)* maternal allele
0·67 mM	0·6 (0·06 – 3·2) (345 embryos scored)
1 mM	1·4 ± 0·32 (n = 5, 3505 embryos scored)
2 mM	4·4 ± 0·96 (n = 4; 2994 embryos scored)
	(C) *gol-1(b6)* maternal allele
1 mM	0·44 (0·04 – 2·5) (454 embryos scored)
2 mM	5·0 ± 2·7 (n = 3; 1736 embryos scored)

Data were derived from several independent experiments in which *gol-1*(+) sperm were exposed to buffer or buffer supplemented with ENU as indicated. Within each experiment eggs from several females, either homozygous or heterozygous for a mutation at *gol-1*, were pooled and fertilized with treated sperm. Mosaic embryos, recognized microscopically by the presence of pigmentless tissue in the eye, were identified at 2 days and re-examined at 3 days. The incidence of mosaic individuals was determined as:

$$\frac{\text{the number of mosaic embryos}}{\text{the number of scored embryos that harboured a maternal } gol\text{-}1 \text{ allele}}$$

Data presented: average incidence derived from independent experiments ± S.E. (n = number of independent experiments; total number of embryos that were examined). Where a single experiment was performed, the 95% confidence intervals are presented.

Table 5. *Induction of somatic mutations at different loci*

ENU concentration	Incidence of mosaic individuals (× 100)
	(A) All *gol-1(−)* alleles
1 mM	1·8 ± 0·36 (n = 11; 5381 embryos scored)
2 mM	5·4 ± 0·88 (n = 14; 8976 embryos scored)
	(B) *alb-1(b4)* allele
1 mM	2·2 (0·92 – 4·3) (371 embryos scored)
2 mM	2·4 (0·76 – 5·6) (210 embryos scored)

Data for the *gol-1* locus include all experiments performed with the *b1*, *b6*, or *b13* alleles and include all of the data from Table 4. Data for the *alb-1(b4)* locus were derived from a single experiment. Data presented: mean incidence of mosaic embryos per locus scored, ± *s.e.* or 95% confidence limits, (n = number of independent experiments; total number of embryos examined).

precise relation between dose and the induction of mosaicism is not clear. Results obtained within single experiments were consistent with a simple linear relation between dose and incidence of mosaicism, but did not exclude other possibilities (data not shown). Because of the lethal effects induced in sperm by exposure to ENU, the fraction of mosaic embryos observed at day 3 per treated sperm (mutational yield) was similar following mutagenesis with 1 or 2 mM-ENU.

The ability of ENU to induce somatic mutations was not restricted to events affecting the *gol-1* locus. Eggs bearing the recessive albino mutation, *alb-1(b4)*, were also fertilized with sperm that had been exposed to ENU. Mosaicism affecting the expression of the *alb-1* locus was induced with an incidence similar to that observed at the *gol-1* locus (Table 5).

As discussed in the accompanying report (Grunwald & Streisinger, 1991), mosaicism may be induced by a variety of recombinational or mutational mechanisms. For example, given that *gol-1* locus is genetically distant from its centromere (Streisinger *et al.* 1986), the high frequency of induced somatic events affecting the *gol-1* locus might reflect the ENU-induced initiation of mitotic recombination events anywhere between the centromere and the locus of interest. We reasoned that such events could be initiated on either the wild-type or mutation-bearing chromosome and still produce homozygous daughter cells. To examine whether the induction of symmetrical recombination events were a significant contributor to the ENU-induced somatic mosaicism, sperm bearing either the *gol-1(b1)* mutation or the wild type allele were mutagenized and tested for the ability to induce somatic mosaicism upon fertilization of eggs harbouring the opposing allele (Fig. 4). Only 0·1% of the embryos were mosaic following fertilization of wild-type eggs with mutagenized *gol-1* sperm. This incidence of mosaicism was similar to that observed among control, non-mutagenized *gol-1*/+ embryos (Table 4). In contrast, in the experiment presented in Fig. 4, approximately 3% of the embryos were mosaic following fertilization of *gol-1* eggs with mutagenized sperm. This result indicates that the induction of somatic mosaicism is not due in general to symmetrical recombination events but requires exposure of wild-type chromosomes to ENU. This result is consistent with the interpretation that the somatic test is a measurement of the induction of novel *gol-1* mutations.

(viii) *Induction of heritable* gol-1 *mutations*

To measure the frequency with which *gol-1* mutations might be induced in the germ lines of G0 mutagenized fish, we first generated G0 ENU-mutagenized individuals that would be good candidates for harbouring such mutations. Since all early blastomeres of the zebrafish embryo usually contribute some descendant cells to the pigmented retina (Streisinger *et al.*, 1989), it is likely that newly arising potential germ-line mutations of *gol-1*, if established by the 32-cell stage,

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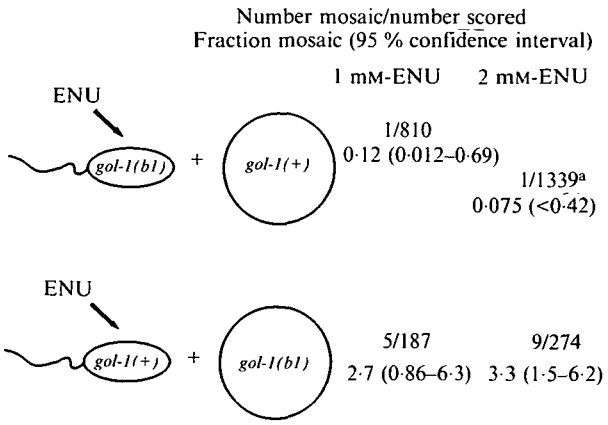

Fig. 4. Wild-type chromosomes must be exposed to ENU in order to induce somatic mosaicism in heterozygous embryos. Eggs harbouring the *gol-1*(+) wild-type allele were fertilized with *gol-1(b1)* mutation-bearing sperm that had been exposed to 1 or 2 mM-ENU. In the same experiment, eggs harbouring the *gol-1(b1)* mutant allele were fertilized with wild-type sperm that had been exposed to ENU. The fraction of developing embryos with pigmentless cells in the pigmented retina was recorded at embryonic day 2 and reconfirmed at day 3.
[a] The one potentially mosaic embryo that arose after exposure of *gol-1* sperm to 2 mM-ENU developed poorly and died before it could be reinspected at day 3.

would also be present in some of the cells of the pigmented retina. Thus, if wild-type sperm is mutagenized and used to fertilize *gol-1* eggs, the resulting G0 individuals with newly induced *gol-1* mutations in their germ lines should be a subset of those fish that displayed somatic mosaicism for pigmentation.

To identify heritable *gol-1* mutations induced by exposure of sperm to ENU, eggs harbouring the recessive lethal golden mutation, *gol-1(b13)*, or the viable golden mutation, *gol-1(b1)*, were fertilized with ENU-mutagenized wild-type sperm. Embryos with mosaic eyes were selected and were raised to sexual maturity. To detect ENU-induced *gol-1* mutations present in the germ lines of mutagenized individuals, each of the G0 mutagenized mosaics were crossed to fish heterozygous for the recessive lethal *gol-1(b13)* mutation. This test cross would reveal mosaic individuals that transmitted more *gol-1* alleles than wild type alleles, indicating an increase in the germ line of mutant alleles at the expense of wild-type alleles. In addition, this cross would reveal the presence of any ENU-induced viable *gol*-1 alleles in the germ lines of mosaic fish that harboured the maternal *gol-1(b13)* allele.

Two mosaic females that had arisen from the fertilization of eggs bearing the viable *gol-1(b1)* allele with ENU-treated sperm were crossed with *b*13/+heterozygous fish (Table 6). All of the resulting golden progeny were viable, and these were present in the expected proportions.

Of 10 G0 mosaic males that carried the maternal *b13* allele and were progeny tested in this manner (Table 6), 9 presented no indication of any ENU-induced *gol-1* alleles in their germ lines. All of the golden progeny resulting from crosses between these mosaic males and *b13*/+females exhibited the expected lethal syndrome associated with the *gol-1(b13)* mutation. In contrast, one mosaic male (♂5, Table 6)

Table 6. *Test cross to detect transmission of specific locus mutations*

Mosaic	Maternal allele	Total progeny scored	*b13/b13* golden progeny	Viable golden progeny	Fraction golden	Fraction of germ line that is *gol-1*	Fraction of germ line with a novel *gol-1* allele[a]
♂1	*b13*	259	70	—	0·27	0·54	—
♂2	*b13*	172	42	—	0·24	0·48	—
♂3	*b13*	413	107	—	0·26	0·52	—
♂4	*b13*	438	117	—	0·27	0·54	—
♂5	*b13*	550	134	48	0·33[b]	0·66	0·17
♂6	*b13*	133	31	—	0·23	0·46	—
♂7	*b13*	23	8	—	0·35[c]	0·70	—
♂8	*b13*	39	10	—	0·26	0·52	—
♂9	*b13*	141	40	—	0·28	0·56	—
♂10	*b13*	165	45	—	0·27	0·54	—
♀1	*b1*	53	15	—	0·28	0·56	—
♀2	*b1*	237	63	—	0·27	0·54	—

Mosaic adults, harbouring either the maternally derived recessive lethal *gol-1(b13)* allele or the maternally derived viable *gol-1(b1)* allele, were crossed to *gol-1(b13)*/+heterozygotes. Progeny of these crosses were examined at 2 days for expression of the wild type or golden pattern of pigmentation. Golden embryos were further subdivided into those that resembled the *b13*-associated embryonic lethal syndrome and those that appeared healthy. Healthy golden embryos developed swimbladders at day 7, whereas *gol-1(b13)* homozygotes never did.
[a] For mosaics harbouring the maternal *gol-1(b13)* mutations, the fraction of germ line with a novel *gol-1* allele is twice the fraction of offspring that are golden and viable.
[b] Significantly different from the expected 0·25 ($P < 0·01$).
[c] Not significantly different from 0·25 ($P > 0·05$).

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Table 7. *Segregation of* gol-1 *alleles among gynogenetic progeny of mosaic fish*

	Maternal *gol-1* allele	No. of embryos analyzed	Fraction of embryos that were golden
Control females			
gol-1(b1)/+	—	360	0·022
gol-1(b13)/+	—	543	0·024
Mosaic females			
♀3	*b1*	168	0·036[a]
♀4	*b1*	24	0·21[b]
♀5	*b13*	29	0·034[a]

Mosaic females arose from the fertilization of eggs harbouring either the *gol-1(b1)* or *gol-1(b13)* with ENU-treated wild-type sperm. Eggs from heterozygous *gol-1/+* or mosaic females were rendered diploid by the Early Pressure treatment. Resulting gynogenetic embryos developed from meiotic half-tetrads, their chromosome complement being derived from sister chromatids present subsequent to the first meiotic division. Gynogenetic embryos were examined at 2 days for the golden phenotype. Homozygous golden embryos arose either from meioses in heterozygous cells in which chromosome homologues bearing the *gol-1* locus failed to experience recombination or from meioses in mutant cells in which both homologues harboured a *gol-1* mutation.

[a] Does not differ significantly ($P > 0·05$) from values for control heterozygotes.

[b] Significantly different ($P < 0·01$) from values observed for heterozygotes. In addition, 34% of the gynogenetic progeny of mosaic ♀4 expressed a novel embryonic lethal syndrome that segregated independently from the *gol-1* locus.

proved to harbour a novel mutation at the *gol-1* locus. In a large sample, 33% of the testcross progeny between ♂5 and *b13/+* females were golden, significantly more than the expected 25%. In addition, almost 9% of the testcross progeny were viable golden embryos, indicating the presence of a non-maternal *gol-1* allele in the germ line of this individual.

The pigmentless cells of mosaic fish indicated the presence of *gol-1/gol-1* cells in the soma. To detect such *gol-1* cells in the germ lines of some of the somatically mosaic mutagenized females, we analyzed meiotic products, in the form of gynogenetic progeny. The genotype of gynogenetic progeny, generated by the EP treatment, is derived from sister chromatids that were present in the egg subsequent to the first meiotic division (half-tetrads). Because of the high frequency of single crossover events between the *gol-1* locus and its centromere (Streisinger *et al.* 1986) only a few per cent of the gynogenetic progeny of heterozygous females are *gol-1/gol-1*. In the present study (Table 7), only 2% of the gynogenetic offspring of either *gol-1(b1)/+* or *gol-1(b13)/+* females were golden. If somatic mosaics contained some germ cell precursors that lacked a wild type allele, we would expect greater than 2% of their gynogenetic offspring would appear golden.

Of three mosaic females whose gynogenetic progeny were analysed (Table 7), one (♀4) gave rise to significantly more golden embryos than would be expected from a true heterozygote. One-third of the gynogenetic embryos also displayed an independently segregating lethal syndrome, indicative of the fact that this female had been successfully mutagenized.

In sum, 2 of the 15 somatically mosaic fish showed evidence of a surfeit of *gol-1* alleles in their germ lines. Among the subclass of somatically mosaic fish that harboured a maternally derived recessive lethal *gol-1* mutation, 1 out of 10 was found to transmit a novel ENU-induced visible *gol*-1 mutation.

4. Discussion

The experiments reported here demonstrate that exposure of mature sperm to the alkylating agent ethyl nitrosourea is an efficient means of inducing germ line mutations in the zebrafish. Mutations affecting embryonic development as well as expression of pigmentation were recovered and could be propagated. Consistent with the anticipated action of ENU in sperm, zebrafish mutagenized in this manner were genetically mosaic in both their soma and germ lines. Most of the lethal mutations observed in G0 germ lines could be transmitted through normal appearing G1 offspring indicating that they were recessive. This may be because most ENU-induced point mutations truly are recessive. Alternatively, it may be that if dominant mutations were induced, their expression soon after fertilization might have had a culling effect on G0 mutagenized animals.

(i) *Induction of lethal mutations*

Fertilization of eggs with sperm that had been exposed to 1–2 mM-ENU generated mutagenized fish with germ lines that harboured many independently segregating recessive lethal mutations whose effects were

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Table 8. *Estimates of the number of independent mutations harboured in the germ lines of G0 2 mM-ENU-mutagenized zebrafish*

	Average number of embryonic lethal mutations transmitted per G0 gamete	Average number of independent lethal mutations per G0 germ line
1. Relative survival of homozygous progeny	$\geqslant 1{\cdot}8$[a]	$\geqslant 7{\cdot}2$[b]
2. Observed syndromes among homozygous progeny	—	1·25[c]
3. Inherited syndromes observed among gynogenetic G2 progeny	1·25[d]	4·67[e]

[a] Derived in Table 1.
[b] Calculated as 4 × (average number of lethal mutations per gamete) since most mutations would be present in only 0·25 gametes (Fig. 3).
[c] Derived in Table 2.
[d] Derived in Table 3.
[e] Calculated from data in Table 3. Fourteen embryonic lethal phenotypes were identified among offspring derived from 3 G0 2 mM-ENU-mutagenized founders.

expressed during embryogenesis. Because our assays for lethal effects associated with induced mutations were terminated after 7 days of development, it is not clear how many additional mutations that might have been present in the germ lines of mutagenized fish might have affected other characteristics such as growth to sexual maturity (approximately 3 months) or fertility.

Three experiments were performed to derive an estimate of the number of embryonic lethal mutations that were borne by 2 mM-ENU-mutagenized zebrafish (Table 8). In the first experiment the relative viability of the homozygous offspring of mutagenized G0 fish was determined. If the difference in the viability between the homozygous offspring of mutagenized and control females is attributed entirely to the presence of fully penetrant recessive lethal mutations in the germ lines of mutagenized fish, then about 46% of the gametes from the 1 mM-ENU-treated group did not harbour any lethal mutations and $\leqslant 17\%$ of the gametes from the 2 mM-ENU-treated were devoid of lethal mutations (Table 1). If we assume that most of the lethal mutations in the germ lines of G0 individuals were independently induced and were randomly distributed among the gametes of mutagenized individuals, then we can apply the Poisson equation to the survival data to obtain a rough estimate of the average number of lethal mutations per G0 gamete, as indicated in Table 1. This treatment of the data yielded an estimate that fish developing from sperm that experienced either 1 or 2 mM-ENU harboured approximately 0·8 or $\geqslant 1{\cdot}8$ embryonic lethal mutations per gamete, respectively (Table 1 and Table 8, first row).

Since the germ lines of mutagenized fish were mosaic and most of the recovered mutations were present in only one-fourth of the gametes, 4 × (average number of lethal mutations per gamete) becomes our working estimate for the true number of mutations per germ line of a G0 mutagenized individual. Hence, we estimate that at least 3 ($4 \times 0{\cdot}8$) independent lethal mutations were present in the germ lines of 1 mM-ENU-mutagenized fish and greater than 7 ($4 \times \geqslant 1{\cdot}8$) embryonic lethal mutations were induced in the 2 mM-ENU-mutagenized fish. We note that many of the induced mutations, such as the viable *gol-1* mutation, comprised less than one-fourth of the germ line of a G0 mutagenized fish. For this reason, we conclude that on the order of 10 independent embryonic lethal mutations were induced in each 2 mM-ENU-mutagenized G0 zebrafish.

Homozygous offspring of mutagenized individuals were also inspected microscopically for the expression of characteristic embryonic lethal syndromes. In these studies, clones of embryos displaying distinctively aberrant patterns of development were identified among the homozygous progeny of virtually every mutagenized female. We imagine that the poor development of HS-treated eggs together with the presence of several segregating lethal mutations might have obscured the expression of many syndromes. Thus our measurement of about 1·25 embryonic lethal mutations per mutagenized G0 fish (Table 8, second row) is potentially a significant underestimate of the true number of lethal mutations.

In a third measurement, we analysed only those mutations that had been transmitted to G1 offspring and that were associated with distinctive aberrant developmental syndromes. The presence of embryonic lethal mutations in the germ lines of G1 offspring was detected by inspecting the gynogenetic progeny of G1 individuals. By this method, 24 mutations, detected as

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clusters of mutant embryos, were transmitted to 20 G1 offspring, indicating that each G0 gamete harboured approximately 1·2 recessive lethal mutations (Table 3 and Table 8, third row). Moreover, 14 independent embryonic lethal mutations were found to be inherited by the G1 offspring of three 2 mM-ENU-mutagenized G0 progenitors (Table 3), indicating that about 4·67 embryonic lethal mutations could be recovered from each mutagenized G0 fish (Table 8).

Two factors make it likely that the majority of syndromes recognized in this assay were due to the inheritance of lethal mutations from the initially mutagenized G0 individuals. First, about half of the recognized syndromes were expressed among the progeny of 2 or more members of a single G1 family, but were not recovered among the progeny produced from other families. Second, in four out of five cases, when wild-type G2 siblings of mutant embryos were raised to maturity and progeny tested, the expected lethal syndrome was recovered.

By analysing gynogenetic G2 progeny, of which only a fraction would be homozygous at any particular locus, clusters of characteristic embryos could be recognized readily without much interference from independently segregating mutations. Clearly though, this method of analysis, which led to the recovery of about 5 embryonic lethal mutations per G0 mutagenized individual, is likely to yield an underestimate of the true number of mutations transmitted by G0 mutagenized fish. First, a portion of the genome, which is rarely present in homozygous state in gynogenetic offspring, was not thoroughly screened for recessive lethal mutations by this procedure. Second, it may be that many embryonic lethal mutations in the zebrafish would not be recognized as distinctive in an assay for morphological variants. Saturation point mutagenesis screens for identifying mutations that alter embryonic body pattern in *Drosophila* have indicated that only about 20% of embryonic lethal mutations result in a distinctive alteration in the embryo's morphology (Nusslein-Volhard *1984;* Jurgens *et al.* 1984; Wieschaus *et al.* 1984; Eberl & Hilliker, 1988).

In sum, mutagenesis of zebrafish by the treatment of sperm with 2 mM-ENU under the conditions utilized in this report results in individuals that harbour on the order of 10 recessive embryonic lethal mutations in their germ lines. These mutations affect both the establishment of the structure of the embryo and the maintenance of its integrity, and should provide useful materials for the study of the early development of this organism.

(ii) *Induction of specific locus mutations*

Our results demonstrate that mutations at the *gol-1* locus can be induced and recovered using the conditions of mutagenesis employed to induce recessive lethal mutations. Following mutagenesis with 2 mM-ENU, approximately 5% of the embryos alive at day 3 were mosaic in their expression of the *gol-1* locus (Tables 4 and 5). It is likely that most of the somatic events that we measured were mutations (including perhaps partial or complete chromosome loss). This interpretation is supported by experiments demonstrating that it was necessary to expose wild type and not mutation-bearing sperm to ENU in order to generate somatic mosaics from heterozygous zygotes. It is unlikely that the *gol-1* locus is especially sensitive to the mutagenic effects of ENU since somatic events affecting the *alb-1* locus were induced as readily as those affecting the *gol-1* locus.

We note that only 2 of the 15 somatically mosaic adult fish were likely to have harboured newly arising *gol-1* mutations in their germ lines. A likely reason for the discrepancy between the incidence of somatic and germ line mutations may be that *gol-1* mutations that did not become fixed immediately after fertilization (e.g. arose after the 4-cell stage) would have been easily measured in the somatic test but may have only rarely entered the germ line, since relatively few cells found the germ line (Walker & Streisinger, 1983). It is also possible that some mutations affecting the *gol-1* locus may have been tolerated in the soma of G0 mutagenized mosaic fish, but would have had dominant deleterious effects in the G0 germ line or in developing G1 embryos.

The frequency with which germ line mutations were induced at the *gol-1* locus may be used to estimate how difficult it would be to induce a new mutant allele at any locus. Mosaic embryos did not survive as well as their pigmented siblings, possibly because some mosaics may have arisen due to non-viable non-disjunction events affecting the *gol-1* chromosome. On average, mosaics survived 40% (range 15–70%) as well as their pigmented sibs. Correcting for the diminished survival of this population, we would expect that under standard conditions of mutagenesis with 2 mM ENU, approximately 2% (5% somatic mosaics at day 3, ×0·4 survival index) of a surviving mutagenized G0 population would have incurred a somatic mutation affecting expression of the *gol-1* locus. Among the somatically mosaic fish whose germ lines were analysed (Tables 6 and 7), we found that between 9% (conservative estimate based solely on *b13*/+mosaics) and 13% (2 of the 15 total mosaics tested) carried new ENU-induced *gol-1* mutations in their germ lines. Therefore, in an unselected mutagenized population where 1 in 50 of the wild-type-appearing individuals carry somatic mutations at a locus of interest, approximately 1 in 500 mutagenized G0 individuals would harbour a new heritable mutation at this locus. Our estimate of the incidence of the induction of new mutations at any specific locus in the zebrafish following the mutagenesis procedures described in this report is strikingly similar to the

optimal specific locus mutation incidence obtained in the mouse (Russell *et al.* 1979; Johnson & Lewis, 1981).

(iii) *Estimate of the genetic complexity of embryogenesis*

In the present study we utilized one mutagenesis protocol to induce mutations either at a specific locus, *gol-1*, or at any locus that contributed to the viability of the organism through the embryonic period up to the swimbladder stage (day 7). Comparison of the incidence with which these two types of mutations arose can give us a very rough estimate of the number of genes that are essential for viability during the embryonic period of the zebrafish. Our data indicate that for every mutation induced at the *gol-1* locus (1 *gol-1* mutation per 500 mutagenized G0 fish), approximately 5000 mutations affecting embryonic viability would be induced [(10 lethal mutations per 1 mutagenized G0 fish) × 500 mutagenized G0 fish]. Therefore, we suggest that about 5000 genes are required for normal embryonic developing the zebrafish. It is interesting to note that, in an independent series of experiments utilizing mutagenesis of zebrafish sperm with UV light (Grunwald & Streisinger, 1991), the ratio of the incidence of embryonic lethal mutations to the incidence of specific locus mutations was remarkably close to that found for ENU-mutagenesis.

Previous estimates, based purely on genetic arguments, of the total number of genes that contribute to an organism's viability have been made only for *C. elegans* (Brenner, 1974) and for *Drosophila* (Muller & Altenburg, 1919). These prior estimates, now generally considered to have been undervalued by only a few-fold (see Wood, 1988), represent considerably fewer essential genes than we suggest contribute to the embryogenesis of the zebrafish.

(iv) *ENU-induced mutations are probably point mutations*

Three aspects of our results indicate that the majority of ENU-induced mutations in the germ lines of G0 mutagenized zebrafish were point mutations. First, ENU-induced mutations arose as a subset of the paternally derived chromosomes of G0 individuals. This finding is consistent with the presumed mode of action of ENU in which alkylation of a single strand of the sperm DNA duplex leads to base substitutions upon DNA replication. Second, the recovery of two mutations that affected the *gol-1* locus but did not affect other linked essential genes indicates that the ENU-induced mutations tended to be small. This finding is in distinction to the majority of γ-ray-induced mutations at the *gol-1* locus, which were found to affect essential genes in addition to the *gol-1* gene (Walker & Streisinger, 1983). Last, for every 5000 lethal mutations induced with ENU, only one new mutation at the *gol-1* locus was recovered. In contrast, following mutagenesis with γ-rays (Chakrabarti *et al.* 1983; Walker & Streisinger, 1983), approximately 1 in every 100 lethal mutations affected expression of the *gol-1* locus. We interpret this difference as indicating that most of the ENU-induced mutations were small, affecting the expression of only one or a few genes whereas most of the γ-ray-induced mutations affected the expression of many genes.

This work could not have been performed without the long-standing and sometimes wistful encouragement and support from members of the Institute of Molecular Biology, University of Oregon. D.J.G. is indebted to Charles Kimmel for his patience and encouragement. Tana Ebaugh, Fred Singer, and Charline Walker assisted with all of the experiments described, and provided critical insight into their interpretation. Thom Montgomery provided invaluable assistance with the maintenance of fish stocks. The authors are grateful to Ralph Greenspan, Charles Kimmel, Eric Wieschaus, and Ellen Wilson for their helpful comments. This work was supported by NIH Grant GM 22731 to G.S. and ACS Grant NP-693 to D.J.G. D.J.G. acknowledges support as a Sloan Fellow and as a recipient of the Basil O'Connor Starter Scholar Award from the March of Dimes.

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