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Technical Report

INDUCTION OF MUTATIONS BY ENU IN THE MEDAKA GERMLINE

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Here, we report a method for chemical mutagenesis of the medaka (*Oryzias latipes*) using N-ethyl-N-nitrosourea (ENU).

Since the chorions and embryonic structures are transparent in the medaka and zebrafish (*Danio rerio*), the morphological phenotypes can be examined throughout the entire period of development in these fishes. This advantage has enabled investigators to successfully perform large-scale mutagenesis in the zebrafish to study genes controlling development in vertebrates (Solnica-Krezel *et al.*, 1994; Mullins *et al.*, 1994; Haffter *et al.*, 1996; Driever *et al.*, 1996). However, no such studies have yet been performed in medaka.

According to Nelson (1994), the teleosts contain four major subdivisions, the Osteoglossomorpha, Elopomorpha, Clupeomorpha, and Euteleostei (the most recently developed teleosts). Both medaka and zebrafish belong to the Euteleostei. The medaka (an order of the Beloniformes) belongs to the superorder Acanthopterygii, the most numerous and recently developed group in the Euteleostei (Nelson, 1994; Naruse, 1996). On the other hand, the zebrafish (belonging to the order Cypriniformes) does not belong to the superorder Acanthopterygii but to the Ostariophysi, a relatively "old" group, in the Euteleostei.

The ostariophysan teleosts including zebrafish belong to the most derived group of teleosts and may have had a unique and specific evolutionary history among the Euteleostei (Northcutt and Wullimann, 1988; Striedter and Northcutt, 1989). It is well known that many ostariophysans, such as *Carassius auratus* and *Cyprinus caprio,* have tetraploidic origins (Ojima, 1983; see also Fujii and Ojima, 1983). Postlethwait *et al.* (1994) estimated the entire length of the zebrafish genome to be approximately 1700 Mb, which corresponds to about twice that of the medaka genome (Uwa and Iwata, 1981; Tanaka, 1995). It is reported that the zebrafish has seven *Hox* gene clusters probably as a result of entire genome duplication (Amores *et al.*, 1998). On the other hand, the pufferfish (Acanthopterygii) has four, as do typical terrestrial vertebrates (Amores *et al.*, 1998; see also Wittbrodt *et al.*, 1998). If many developmental genes have two nonallelic copies that are functional, it may be difficult to recover all classes of developmental mutations in the zebrafish.

It is of interest to determine what classes of developmental mutations are recovered in the non-ostariophysans, such as the acanthopterygians. We have studied genetic malformations induced by environmental mutagens in the medaka, and started a pilot screen of medaka developmental mutants (Ishikawa, 1996; 1997; Ishikawa and Hyodo-Taguchi, 1995; 1997; Ishikawa *et al.*, 1997). A method for the induction of mutations by X-ray irradiation was reported previously (Ishikawa and Hyodo-Taguchi, 1997). Here, we report a mutagenesis method using ENU. According to this method, visible mutations are recovered at the frequency of about 0.82 mutations per single mutagenized spermatogonia.

Preparation

1. ***Fish and breeding:*** A medaka inbred strain, HO4C (Hyodo-Taguchi, 1980, 1990; Hyodo-Taguchi and Sakaizumi, 1993) is used. This orange-red variety strain has been maintained by full sibling mating for 80 generations, and the probability of homozygosity within the strain is greater than 99% (Hyodo-Taguchi, 1980, 1990; Hyodo-Taguchi and Sakaizumi, 1993). Fish are bred and raised under standard conditions: about 10 fish are kept in 3 liters of

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still water in a 6 ℓ-plastic vessel, and maintained under constant water temperature (26–29°C) and photoperiod (14-h light/10-h dark cycle). The fish are given powdered fish food (Tetra-min, Tetra Werke Co., Mells, Germany) once a day. Under these conditions, the fish mature sexually 3–6 months after hatching and begin to display mating behavior at the beginning of the light period. Several single-pair crosses are required to be tested in advance, and only successful pairs are used in the experiment.

2. A dissection microscope (for example, Nikon SMZ-10).
3. An isopac bottle of N-ethyl-N-nitrosourea (N-nitroso-N-ethylurea, ENU, Sigma, N3385, 1 g).
4. Methylene blue.
5. Distilled water.
6. A 100-ml syringe (Terumo).
7. Several 10-ml syringes (Terumo).
8. Ten 300-ml grass vessels.
9. A 3-ℓ beaker.
10. A fish net (made of a fine nylon mesh).
11. Six-ℓ plastic vessels for keeping fish.
12. Six-cm-diameter petri dishes.
13. Paper towels.

Chemical treatment

All work should be performed in a chemical hood with appropriate protective clothing. After use, solutions containing ENU and the glassware used to hold ENU should be inactivated by treatment with 5% sodium hydroxide solution according to the Safety Data Sheets for Carcinogenic Substances (Safety Data Sheets for the DHEW Guidelines for the Laboratory Use of Chemical Substances Posing a Potential Occupational Carcinogenic Risk, 1979).

1. Inject distilled water (100 ml) into an ENU bottle with a 100-ml syringe and incubate for 1 hr with occasional shaking to dissolve the ENU.
2. Remove 2.4 ml of the ENU solution from the sealed bottle with a 10-ml syringe and add to 97.6 ml of distilled water in a 300-ml glass vessel. The final concentration of the ENU is 2 mM, and the solution should be used immediately. Typically, 10 ENU solutions are prepared in glass vessels in advance.
3. Transfer a male fish into the 300-ml glass vessel containing 2 mM ENU and let it swim for 2 hr. Typically, 10 fish are individually treated in this manner.
4. At the end of the treatment, remove the mutagenized fish from the vessel with a fish net.
5. Wash the mutagenized fish several times with distilled water.
6. Transfer the treated fish to water (1 ℓ) in 6-ℓ plastic vessels and let them swim and recover for 4 hr. Until this step about 25% of the treated fish may die due to the toxic effects of ENU. Only healthy male fish are used for three-generation crosses.
7. At the end of the experiments, pour all solutions containing ENU into concentrated sodium hydroxide solution in a 3 ℓ-beaker to inactivate the ENU.

Three-generation crosses

A schematic illustration of the three-generation crosses is shown in Fig. 1.

1. Pair-mate each mutagenized male fish with an untreated female fish to produce F_1 eggs. According to Egami and Hyodo-Taguchi (1967), the treated stage of spermatogenic cells is predicted from the number of days after ENU treatment. Eggs laid at 1–3 days, 4–9 days and 30–36 days after ENU treatment are considered to have been fertilized by ENU-treated sperm, spermatids and spermato-

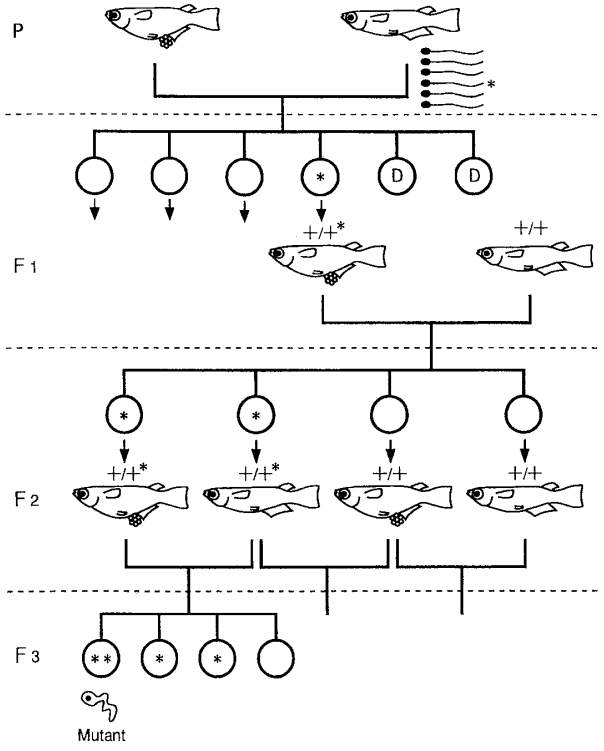

Figure 1. Schematic illustration of three-generation crosses. A recessive mutation (asterisk) in a male germ cell in the parental generation is driven to homozygosity in the F_3 generation. Dominant lethals are indicated by D.

gonia, respectively. A high mutation rate is recovered in eggs fertilized by ENU-treated spermatogonia.
2. Raise the F_1 eggs to adult fish.
3. Pair-mate each F_1 fish with an untreated partner to obtain F_2 eggs.
4. Raise the F_2 eggs to adult fish.
5. Perform several single-pair crosses between siblings for each F_2 progeny.
6. Collect eggs (F_3) seperately.

Detection of morphological mutations

1. Rub egg clusters between two small pieces of paper towel to isolate single eggs.
2. Place single eggs in a 6-cm petri dish containing 3 ml of distilled water supplemented with 0.00001% methylene blue.
3. Observe embryos once a day under a dissection microscope to score the phenotypes, until normal fry hatch at about 7–10 days after fertilization.
4. A phenotype is considered to represent a recessive morphological mutation if about 25% of all embryos develop a consistent set of defects before death or exhibit comparable abnormalities at hatching.
5. The spontaneous malformation frequency is 5.6% (Ishikawa and Hyodo-Taguchi, 1997).

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