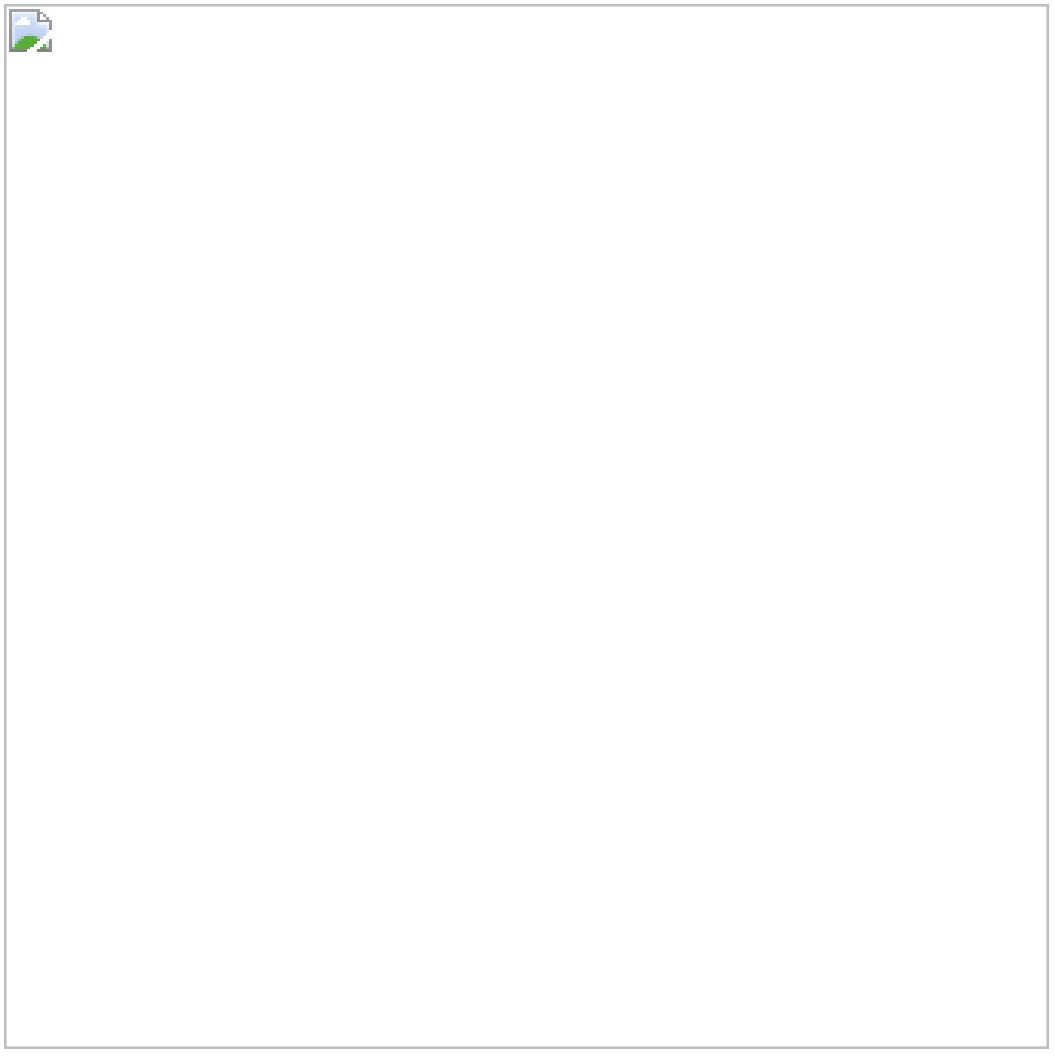

Induction and Characterization of Mutations at the b Locus of the Medaka, Oryzias latipes

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Induction and characterization of mutations at the *b* locus of the medaka, *Oryzias latipes*

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ABSTRACT—The *b* locus is one of the most familiar pigmentation loci in the medaka, but its biochemical function is still unknown. Here we report induction of new mutations at the *b* locus by radiation and ENU. We also characterized all these mutations and previously isolated spontaneous ones on the phenotypic basis. Unexpectively, all the 18 induced mutations reduced melanin contents in both eyes and skin correlatively, although degree of reduction was varied from mutations to mutations. Moreover, presumed null mutants (b^{s8}, b^{g8}, b^{c2}, b^{d3}, b^{d6}, b^{g13}, b^{g19}, b^{g24}) had slightly melanized (dark red) eyes. These results suggest that the *b*-locus product plays an important but not a critical role in melanogenesis. The spontaneous mutants were divided into two types: one (b^{dl2}, b^{dl3}, and b^{p}) had similarities with the induced mutants in that they had slightly colored eyes and skin, the other (b^{v}, *B*', b^{d}, b^{dl1}, and *b*) exhibited normally black eyes but lightly colored skin. The present study supports our recent results (Fukamachi *et al*., 2001) that mutational changes were found in the coding region of the *b* gene in some of the mutants which reduced both eyes and skin melanogenesis, while the mutational change for the *b* allele could not be found there. We speculate that the b^{v}, *B*', b^{d}, b^{dl1}, and *b* alleles might arise by the mutations in the regulatory region for skin melanogenesis.

Key words: medaka, *b* locus, induced mutations, melanogenesis, specific-locus test

INTRODUCTION

In the last decade, a number of pigmentation genes in mammals have been cloned and their role in biological pathways have been elucidated (Jackson, 1994). However, studies for pigmentation in lower vertebrates, which have four kinds of pigment cells, leucophores, xanthophores, iridophores and melanophores, have not yet progressed very much. In the medaka, about 70 spontaneous pigmentation mutants have been isolated from wild populations and fish farms (Yamamoto, 1975; Tomita, 1989). Thus, the medaka is a good material for studying pigmentation in lower vertebrates.

The *b* locus is one of the most familiar pigmentation loci in the medaka. The phenotype of the *b* mutant, which is so called orange-red variety or "Himedaka," has been well documented; the homozygous *b* fish have slightly melanized skin but normally melanized retinal pigment epithelium and choroidal melanophores in the eyes (Hirose and Matsumoto, 1992). Efforts to elucidate biochemical function of the *b* locus have been made for many years (Tomita and Hishida, 1961; Hishida *et al*., 1961; Sugimoto *et al.,* 1985; Hirose and Matsumoto, 1992), but it still remained unknown. Inagaki *et al*.(1994) showed that the skin of the *b* mutant expressed normal tyrosinase mRNA. In addition, Hyodo-Taguchi *et al.* (1997) documented that transgene of the mouse tyrosinase could not rescue the *b* phenotype. These two studies indicated that the *b* locus does not encode, at least, tyrosinase gene. Tomita (1989) reported eight other spontaneous alleles of the *b* locus, but they are not yet characterized in detail.

During the course of the specific-locus test experiments, where males were treated with radiation or ENU (ethylnitrosourea) then mated to tester females homozygous

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Table 1. Viability of the primary and homozygous induced mutants at the *b* locus.

Mutagen	Treated male germ cells	Observed[a]	Primary mutant (*b*/*b**)				Homozygote (*b**/*b**)		
			TM[b]	VM[c]	Matured	Matured /TM (%)	Observed	Lethal	Viable
γ-rays	Postmeiotic[d]	108,337	521	27	15	2.9	5	3	2
	Premeiotic[e]	261,060	100	6	3	3.0	2	1	1
ENU	Postmeiotic	36,264	20	2	1	5.0	NE	–	–
	Premeiotic	101,517	36	30	30	83.0	15	0	15

a. Number of F1 progeny obtained from the crosses between treated wildtype males and T3 females.
b. Total mutants: mutants detected during embryonic development.
c. Viable mutants: mutants which hatched and survived at least 4 days.
d. Sperm and spermatids
e. Spermatocytes and spermatogonia
NE; Not exaimined;

Table 2. Degree of melanization in the homozygous *b*-locus mutants relative to wildtype and albino (i^1/i^1) fish by visual observation.

Origin	Skin color of primary mutant	Allele	Eye			Skin			Tyrosin reaction
			Onset (Stage)	4-day embryo	Adult	Onset (Stage)	4-day embryo	Adult	
Control	Wildtype	b^+	25	++++	++++	21	++++	++++	ND
	Albino	i^1	–	–	–	–	–	–	–[a]
γ-ray-induced	Orange-red	b^{s8}	34	–	+	38	–	–	+[b]
		b^{s14}	34	–	+	ND	–	+–	+
		b^{g8}	34	–	+	38	–	–	+
ENU-induced	Intermediate	b^{g21}	25	++	++++	24	+++	++++	ND
		b^{d4}	26	+	++++	26	++	+++	ND
		b^{d2}	27	+	+++	27	++	++	ND
		b^{t17}	27	+	+++	26	++	++	ND
		b^{g12}	27	+	+++	26	++	++	ND
		b^{g15}	30	+–	+++	30	+	++	ND
	Orange-red	b^{d8}	ND	+–	++	ND	+	+–	ND
		b^{c1}	30	+–	++	34	+–	+–	+
		b^{d5}	30	+–	+	34	+–	+–	ND
		b^{c2}	34	–	+	38	–	–	+
		b^{d3}	34	–	+	38	–	–	+
		b^{d6}	34	–	+	38	–	–	+
		b^{g13}	34	–	+	38	–	–	+
		b^{g19}	ND	–	+	ND	–	–	+
		b^{g24}	ND	–	+	ND	–	–	+
Spontaneous		b^v	25	++++	++++	23	+++	++++[c]	ND
		B^i	25	++++	++++	27	+	++[d]	ND
		b^d	25	++++	++++	27	+	+++	ND
		b^{dl1}	25	++++	++++	27	+	++	ND
		b^{dl2}	26	+	++++	27	+	++	ND
		b^{dl3}	26	+	++++	27	+	++	ND
		b	25	++++	++++	27	+	+	+
		b^p	27	+	++	27	+	+–	+

Eyes: ++++, degree of melanization could not be distinguished from wild-type; +++, slightly lighter than wild-type; ++, ligher than wild-type; +, slightly melanized; +–, very slightly melanized; –, not melanized. These criteria were confirmed by melanin content of eyeballs in some of the mutants (see Fig. 3).
Skin: ++++, degree of melanization could not be distinguished from wild-type; +++, slightly lighter than wild-type (aggregated melanophores on the scale had the same color as the wild-type); ++, ligher than wild-type; (aggregated melanophores on the scale showed slightly lighter than wild-type); +, slightly melanized; (aggregated melanophores showed light color); +–, very slightly melanized (aggregated melanophores showed slightly melanized color); –, not melanized (aggregated melanophores had no color).
a. Melanin was not observable at all.
b. Melanin was observable (degree of melanization was not shown).
c. Black skin on the back.
d. Variegated.
ND, Not determined.

for the recessive alleles of the *b, lf,* and *gu* loci (Shima and Shimada, 1991; Shimada and Shima, 1998), many induced mutations at the *b* locus were generated. We thought that analysis of phenotypes of the induced mutations, including presumed null ones, is indispensable for prediction of the function of the *b* gene. We also thought that these mutants were very useful for cloning of the gene. Indeed, we have recently succeeded in identifying the cDNA of the *b* gene by utilizing these induced mutants (Fukamachi *et al*., 2001) and shown that the gene encodes an unknown transporter protein. Here we report induction of novel alleles of the *b* locus by radiation and ENU, and, in addition, analyze phenotypic characteristics of all the induced and already isolated spontanoues mutants in order to speculate a possible role of the *b* gene in melanogenesis.

MATERIALS AND METHODS

Induction of mutations

Adult males to be mutagenized were obtained from our wild population stocks, Sakura (stock #20), originally collected at Sakura, Chiba Prefecture (Shima *et al*., 1985). They were exposed in water to γ-rays (0.3–95 cGy) from a 80TBq ^{137}Cs source of University of Tokyo, or treated with 0.5 and 1.0 mM of ENU for 2 hr at 27°C. After treatment, the males were mated with non-treated female tester stock, T3 (*b/b; lf/lf; gu/gu*) (Shimada and Shima, 1988). The resulting F1 embryos were incubated in 96-well microtiter plates. The primary *b*-locus mutants with light colored skin (*b/b**; *b** represents induced mutant alleles with any type of genetic change that results in a full or partial loss of *b*-locus function) were detected at 6 day after fertilization (total mutants: TM). Among them, hatched viable ones (viable mutants: VM) were reared to adults. All the fish and embryos were cultured at 27±2°C under 14-hr light/10-hr dark cycle.

Mating scheme to establish the homozygous induced mutants

The primary mutants (*b/b**) were crossed with the T3 stock (*b/b*) which was used as a tester in the specific-locus test. In the case the primary mutants had orange-red skin, each of the resulting F1 progeny was mated with b^p/b^p fish in order to check whether it was a carrier (*b/b**) or not a carrier (*b/b*) of an induced mutation. [The b^p/b^p fish had light colored eye. If an F1 progeny was a carrier (*b/b**), about half of the F2 progeny showed light colored eye (b^*/b^p), and the remaining ones showed black eyes (b/b^p). If an F1 progeny was not a carrier (*b/b*), then all the F2 progeny showed black eyes (b/b^p).] In the case skin of the primary mutants was darker than orange-red, dark colored F1 progeny were considered as the carriers. The carriers thus obtained were then crossed with each other to establish homozygous mutant fish (b^*/b^*).

The homozygous induced mutants, b^{g8}, b^{g21}, b^{d4}, b^{d2}, and b^{c1}, sakura strain (b^+), and the spontaneous mutants, b^{dl2} and b^p were further crossed twice with the inbred strain Hd-rR (*b/b*), followed by being established as homozygotes again. This process made the genetic background of the mutants more homogeneous with each other than among the original ones. The Hd-rR was served as a homozygote for the *b* allele.

Estimation of melanin contents of eyes

Newly hatched fry were fixed with 5% TCA (trichloroacetic acid) for 10 min. A pair of eyeballs was dissected out and used as materials. As for adults, fish in 22–25 mm in length were fixed with 5% TCA for 30 min. A pair of eyeballs was dissected out, lens and cornea were removed, and the remaining choroid and retinal layer were used as materials. The quantitative colorimetric method developed by Foster and Stamas (1956), modified by Whittaker (1963) was applied to measure the melanin content. Briefly, nonmelanic substances were removed by repeated extractions; three extractions with 5% TCA, two extractions with ether-ethanol (1:3), and one extraction with absolute ether. The dried extracted residue was dissolved in 0.5 M NaOH (100 μl for a pair of fry eyes, 500 μl for an adult eye), and boiled for 10 min. Optical density was measured at 400 nm. For fry, optical densities of albino (i^1/i^1) eyes were subtracted from those of the specimens, followed by estimation of melanin amount (ng) from densities of pure synthetic melanin (Sigma). For adults, optical densities of the specimens were directly converted to the melanin amount.

Estimation of skin melanization

Degree of skin melanization was estimated by visual observation of embryonic and adult skin as well as melanophores on the scales of the adults. For the latter observation, three scales located between dorsal and lateral part of the body were picked up with tweezers and equilibrated in Ringer's solution (dispersed condition) and 0.1mM noradrenalin (aggregated condition).

Tyrosinase reaction

Six-day embryos or newly hatched fry were immersed with Yamamoto Ringer (100 parts of M/7.5 NaCl, 2 parts of M/7.5 KCl, and 2 parts of M/11 $CaCl_2$, adjusted pH 7.2 by N/10 $NaHCO_3$), and then incubated in the reaction mixture (6 parts of 0.1% tyrosine + 0.04% $NaHCO_3$ in M/7.5 NaCl, 3 parts of M/10 phosphate buffer [pH7.3], and 1 part of M/10 iodoacetamide) for 24 hr at 37°C (Hishida *et al*., 1961).

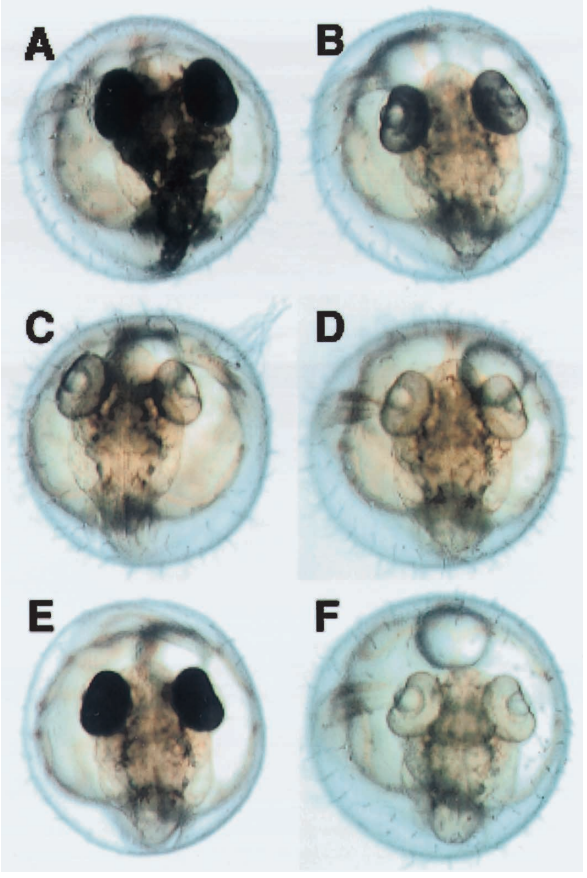

Fig. 1. Four-day homozygous mutant embryos. A, wildtype; B, b^{g21} (ENU-induced) ; C, b^{d2}(ENU-induced); D, b^{g8} (γ-ray-induced); E, *b* (spontaneous); F, i^1 (shown as control).

RESULTS

Yield of radiation- and ENU-induced mutations at the *b* locus

We screened a total of 369,397 F1 progeny of γ-irradiated males and 137,781 F1 progeny from ENU-treated males, and then obtained 621 and 56 primary mutants (*b*/*b**), respectively. However, most of the primary mutants derived from ENU-treated postmeiotic cells and γ-irradiated post- and premeiotic cells died during embryonic development as well as after hatching. In a sharp contrast, 30 out of 36 primary mutants derived from ENU treated premeiotic cells grew to mature adults (Table 1).

Among the matured primary mutants, we randomly chose some and made them homozygous. In the seven homozygous mutants induced by γ-rays, three were viable, while four were lethal (one died at stage 20, two died at stage 25, and one died at stage 27). In contrast, all the homozygous mutants induced by ENU were found to be viable (Table 1).

The final yield of mutations (the number of homozygous viable mutations obtained / F1 progeny screened) were ~7 $\times 10^{-5}$ for γ-rays exposed at postmeiotic germ cell stage, ~6 $\times 10^{-6}$ for γ-rays exposed at premeiotic stage, and ~3 $\times 10^{-4}$ for ENU treated at premeiotic stage, respectively. From these results, it was indicated that treatment of premeiotic male germ cells with ENU was the most efficient way to obtain the new mutations at the *b* locus.

Phenotypic characterization of the *b*-locus mutations

We examined degree of melanization on eyes and skin of each induced homozygous mutant relative to wild-type and albino (i^1/i^1) fish. We also examined for spontaneous mutants previously isolated, for comparison. Table 2 summarizes the results. Melanin deposition on the outer retinal layer (pigmented retina) of the wild-type eyes began to be recognized at around stage 25 (52 h at 26°C). However, in all the 18 induced mutants, onset of deposition was delayed, and melanin content was kept lower than those in the wild-type eyes even when they grew into adults (Figs. 1–3). Melanophores in skin of the wild-type fish began to appear on both yolk sac and lateral edge of the embryonic body at around stage 21 (36 h at 27°C). Like the eyes, mutant melanophores in the skin began to be observed with delay

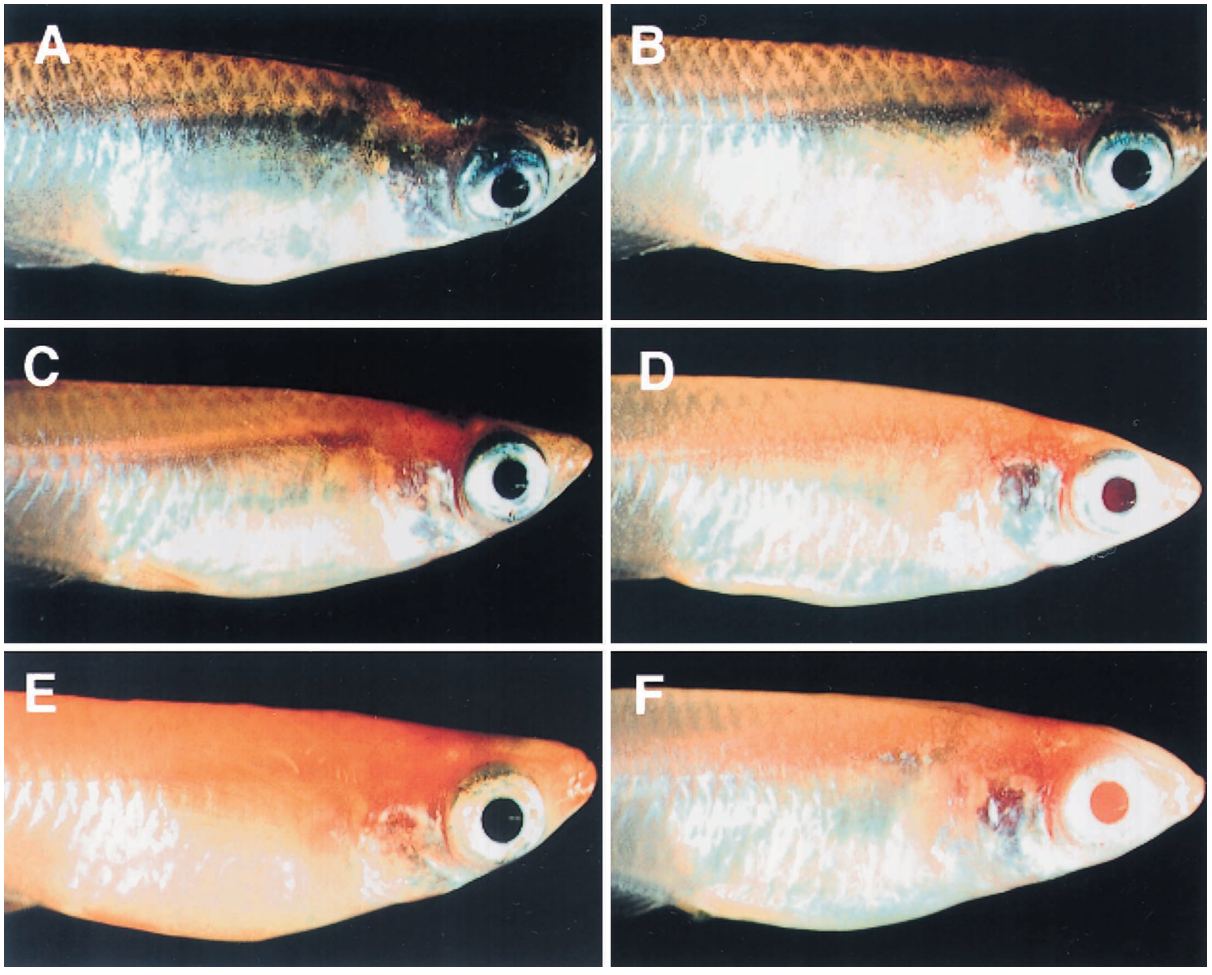

Fig. 2. Adult homozygous mutants. A, wildtype; B, b^{g21} (ENU-induced) ; C, b^{d2}(ENU-induced); D, b^{g8} (γ-ray-induced); E, *b* (spontaneous); F, i^1 (shown as control).

(Table 2) and degree of melanization of the body was kept lower than wild-type throughout their life (Table 2, Figs. 1, 2). Fig. 4 shows an example of b^{d2} melanophores on the scale. Under the dispersed conditions in Ringer's solution (Fig. 4B), color of melanophores was distinctly lighter than wild-type (Fig. 4A). On the other hand, under the aggregated conditions in 0.1 mM noradrenalin solution (Fig. 4D), color was slightly lighter than wild-type (Fig. 4C). Such degree of melanization was judged to be "++" in Table 2 (see the footnotes of Table 2). It is interesting to note that, in the individual mutant, degree of reduction of melanin content in the eyes and skin was strikingly correlated, although degree of reduction was varied from mutations to mutations (Table 2). We did not obtain any induced mutation that changed pattern of distribution of melanosomes nor melanophores. From these results, we infer that *b*-locus function

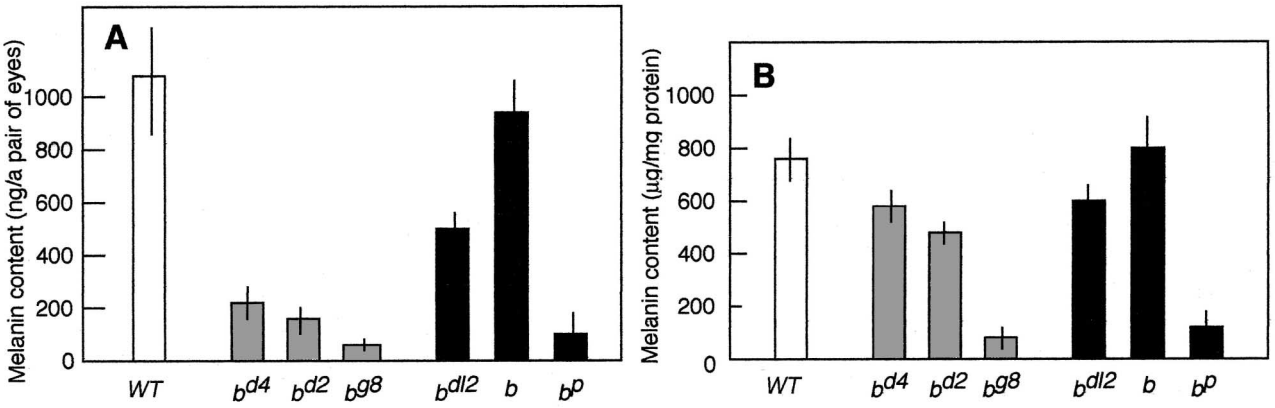

Fig. 3. Melanin content of the mutant eyes. (A) Fry, (B) Adult. b^{d4} and b^{d2} , ENU-induced; b^{g8}, γ-ray-induced (gray column); b^{dl2}, *b*, and b^{p}, spontaneous (black column).

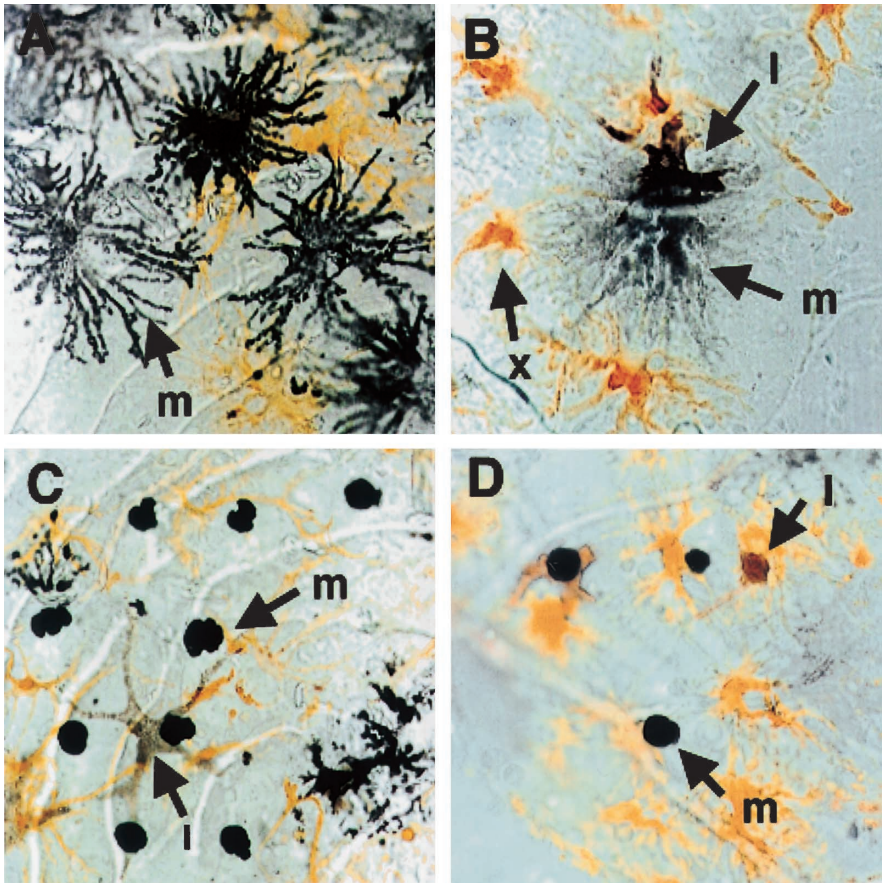

Fig. 4. Melanophores on scales of the wild-type (A, C) and b^{d2} (B, D) fish. A and B, equilibrated in Ringer's solution; C and D, treated with 0.1 mM noradrenalin. m, melanophore; l, leucophore; x, xanthophore.

might not be involved in regulation of melanosome movement nor differentiation of melanophores, but in melanogenesis itself.

The b^{s8}, b^{g8} (γ-ray induced) and the b^{c2}, b^{d3}, b^{d6}, b^{g13}, b^{g19}, b^{g24} (ENU-induced) fish shared the severest phenotype (Table 2), suggesting they were null mutants. They looked like albino fish. However, they had slightly melanized (dark red) eyes (Figs. 1–3). Moreover, they showed positive tyrosinase activity (Table 2).

In contrast to the induced mutants, phenotypes of the spontaneous mutants were not simple. The b^{v} fish had distinct black skin on the back. Melanophores of the *B'* fish exhibited variegated pattern. The skin of the b^{d} fish turned darker as they grew. Most interestingly, while skin of the b^{v}, *B'*, b^{d}, b^{dl1}, and *b* fish were lighter than the wild-type, melanization in their eyes appeared normal (Table 2, Fig. 3). However, the b^{dl2}, b^{dl3}, and b^{p} fish had similarities with the induced mutants in that degree of hypomelanization in the eyes was correlated with that in the skin.

DISCUSSION

Unexpectedly, all the radiation- and ENU-induced mutations at the *b* locus reduced melanin content in both eyes and skin, whereas, five out of eight spontaneous mutations had no effect on melanization in the eyes. We have recently isolated the *b* gene by positional cloning method. It was shown that one radiation-induced (b^{g8}) and four ENU-induced mutants (b^{g21}, b^{d4}, b^{d2}, b^{d8}) and spontaneous mutants, b^{p}, actuary had mutational changes in the ORF of the *b* gene. However, we failed to find any mutation in the cDNA from the *b* fish (Fukamachi *et al*., 2001). These results and the present phenotypic study suggest a schematic model for *b*-locus structure and the regions of the mutational changes: Mutations within a coding region might be responsible for hypomelanization in both eyes and skin of all the induced as well as three spontaneous mutations. On the other hand, mutations in a presumed regulatory region for skin melanogenesis might be responsible for the hypomelanization or variegated melanization in the skin but for normal black eyes of the b^{v}, *B'*, b^{d}, b^{dl1}, and *b* spontaneous mutants. At any rate, this model could be verified only after mutational changes of the b^{v}, *B'*, b^{d}, b^{dl1}, and *b* will be found in the regulatory region of the *b* locus.

We did not obtain any "*b* type" (black eyes and light colored skin) induced mutations (0/18), suggesting that the regulatory region for the skin melanophores, where the "*b* type" mutations are thought to be located, might be restricted within short DNA sequences relative to the coding region. However, five out of eight spontaneous mutations were "*b* type" (significantly higher proportion than 0/18 in the case of induced mutations). Furthermore, from the present results and the fact that the all the "*b* type" mutations (b^{v}, *B'*, b^{d}, b^{dl1}) were isolated from "Himedaka"(*b*) farms (Yamamoto, 1975), it is possible that these spontaneous mutations might not arise by independent rare mutational events but some sequential allelic changes from the *b* mutation like in the case of the mouse *agouti*-locus mutations, where the *black-and-tan* (a^{t}) or the *white bellied agouti* (A^{w}) are suggested to be generated from the *nonagouti* (*a*) by homologous recombinations (Siracusa, 1994).

The presumed null mutants (b^{s8}, b^{g8}, b^{c2}, b^{d3}, b^{d6}, b^{g13}, b^{g19}, b^{g24}) had slightly melanized eyes. This indicates that the *b*-locus product is important for melanogenesis but does not play a key role like tyrosinase whose null mutants exhibit complete albinism. Our preliminary experiments indicated that excess addition of tyrosine into tyrosinase reaction medium always resulted in excess melanization of embryos or fry tested (data not shown). Taking account of the fact that the deduced B protein consists of 12 transmembrane domains (Fukamachi *et al*., 2001), we would speculate that the *b*-locus function might relate tyrosine transportation whose gene has not yet been identified in vertebrates.

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