The tyrosinase gene from medakafish, *Oryzias latipes*

メダカ *Oryzias latipes* のチロシナーゼ遺伝子の解析

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主論文

CONTENTS

SUMMARY

I have determined the 9.8 kb genomic nucleotide sequence of the tyrosinase gene and its 5' upstream region from a teleost, medakafish (*Oryzias latipes*), and shown that the coding region is composed of five exons and four introns, spanning 4.7 kb. While the number and sizes of the exons were found to be similar to those of mammalian tyrosinase genes, however, the total size of the coding region (4.7 kb) was demonstrated to be less than one tenth those of mouse (ca. 70 kb) and human (> 70 kb) genes. Primer extension analysis revealed that the transcription initiation site starts with a long untranslated leader sequence (340 nucleotide long) from the AUG start codon. A characteristic CATGTG sequence known as a putative regulatory motif in melanocyte-specific genes was present in the 131th base upstream from the initiation site, while other typical regulatory elements such as the TATA-box or M-box common to terrestrial vertebrates were lacking. Transgenic experiments were carried out by microinjecting two kinds of plasmid clones into fertilized eggs of the albino *i*^{1} mutant; one consisting of the genomic tyrosinase gene with the 10 kb 5' upstream region, and the other the tyrosinase cDNA with the 3 kb 5' upstream region. The results showed that 53 and 45 of 114 and 118 transgenic eggs, respectively, developed normally beyond

hatching and 15 and 10 exhibited a mosaic pattern of pigmentation. Despite the absence of typical regulatory elements like a TATA-box in both cases correct melanin pigmentation was obtained without ectopic expression. Thus, transgenic expression rescued from the albino-i^{1} mutation, and the *i* locus of the medaka genome can be concluded to encode the tyrosinase gene.

INTRODUCTION

Medakafish is a small egg-laying freshwater teleost that is widely used as a laboratory animal (see *Medakafish Homepage* on the Internet; URL is *http://biol1.bio.nagoya-u.ac.jp:8000/*). One of the best explored areas is the genetics of the body color variants, and more than 40 mutants have been isolated and maintained (Tomita, 1993). Among them, a mutant which has been intensively studied is an albino-*i1* having amelanotic red eyes and colorless melanophores in the skin. The recessive albino phenotype has been shown to be governed by a single autosomal locus (Yamamoto, 1969), and *in vivo* tyrosinase activity is absent in *i1/i1* fish (Tomita, 1975).

To clarify the molecular basis of melanin pigmentation, I have recently isolated authentic DNA probes specific for the tyrosinase gene, and determined the nucleotide sequence of the tyrosinase cDNA (Inagaki et al., 1994). Following this line, we analyzed the structure of the tyrosinase gene in the albino-*i1* and revealed that insertion of a transposon, designated *Tol1-tyr*, in exon 1 is responsible for the albino mutation (Koga et al., 1995; Koga and Hori, 1997).

In the present study, I first determined the genomic nucleotide sequence of the wild-type tyrosinase gene and its 5' upstream region; secondly

performed primer extension analysis of tyrosinase mRNA to identify the expression mechanisms of the gene. I also report here phenotypic rescue of the *i*^{1} mutation in transgenic medaka by means of microinjection of the authentic tyrosinase gene into albino-*i*^{1} *e*ggs.

MATERIALS AND METHODS

Fish Strains

Wild-type specimens of Japanese medaka (*Oryzias latipes*, Taxonomy ID 8090 in NCBI taxonomy database) were collected in Ichinomiya near Nagoya, Central Japan. The albino-*i1*, having red eyes and whitish skin, was originally obtained from Drs. Tomita and Wakamatsu, Laboratory of Freshwater Fish Stocks, Bioscience Center, Nagoya University. These fish were raised and maintained in our laboratory under standard conditions (Hyodo-Taguchi and Egami, 1985).

Cloning and Sequencing of the Tyrosinase Gene

Isolation of genomic DNA and construction of the genomic library were performed using the methods previously described (Koga et al., 1995). To isolate a genomic clone of the tyrosinase gene, about 200,000 phage plaques of a wild-type library were screened with a radio-labelled tyrosinase-specific probe, *TyrG* (Inagaki et al., 1994) and one of positive clones was subcloned into the pBluescript II plasmid vector (STRATAGENE). Several restriction endonucleases were used for further subcloning, and both strands of these subclones were sequenced by the dideoxy chain termination method.

Primer Extension Analysis

Primer extension analysis was performed using a standard method (Sambrook et al., 1989). Briefly, a primer 5'-AGTACACAGACAATGAAAA ATCCC-3', an antisense oligonucleotide corresponding to positions 92 - 115 in exon 1 of the medaka tyrosinase gene, was synthesized and used as an extension primer. About 5 μg of mRNA isolated from wild-type fish as described earlier (Inagaki et al., 1994) was hybridized with the end-labelled primer at 20 °C for 16 h in hybridization buffer (80 % formamide; 40 mM PIPES, pH 6.4; 1 mM EDTA; 0.4 M NaCl), and then reverse transcribed with Superscript II reverse-transcriptase (GIBCO BRL) at 42 °C for 45 min followed by at 50 °C for 15 min. After RNase A treatment, single strand DNAs were fractionated on 7 % polyacrylamide / 7 M urea gel with the sequencing reaction product using the same primer.

Plasmids

For microinjection, *pTyr-genome* and *pTyr-cDNA* constructs (see Fig. 7) were used. *pTyr-genome* contains an authentic medaka tyrosinase gene consisting of about 10 kb 5' upstream sequence, 5 kb coding region and 2 kb 3' downstream sequence cloned into 3 kb of a pBluescript II plasmid vector. The total size is about 19 kb. *pTyr-cDNA* is a medaka minigene consisting of 3.0

kb 5' upstream and tyrosinase cDNA sequences cloned into the same vector. This clone was constructed by fusing the 3 kb 5' upstream sequence from the tyrosinase genome (*W1* clone, see below) with the complete coding region of the cDNA clone isolated previously (Inagaki et al., 1994). These clones were provided for transgenic injection in the circular form or after linear conversion.

Microinjection

Plasmid DNA for injection was extracted using standard methods (Sambrook et al., 1989). Digested or undigested plasmids were then purified by QIAGEN (QIAGEN), diluted to a final concentration of 1 mg/ml in 10 mM Tris HCl (pH 7.0) buffer and 10 to 20 pl were injected into i^1 fertilized eggs at the one-cell stage following an established protocol (Ozato et al., 1989).

Analyses of Nucleotide and Amino Acid Sequences

The DNASIS program (Hitachi Software Engineering, Tokyo) was used to compile sequence data, to search reading frames and transcriptional control signals, and to obtain a G+C content profile of the nucleotide sequence. For alignment of amino acid sequences, I first searched the conserved regions by means of the package program in DNASIS, and then manually obtained the "best match alignment", with minimal gap insertions. Using the aligned

sequence, I calculated evolutionary distance (*Kaa* ; number of amino acid substitutions per site) in a pairwise manner (Zuckerkandl and Pauling, 1965). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the package program Clustal W version 1.4 (Thompson et al., 1994). The bootstrap method in the Clustal W package was also used to access the degree of support for internal branches of the phylogenetic tree. A similarity search of the sequence was performed by means of the BLAST program of the National Center for Biotechnology Information, available on the Internet network (http://www.ncbi.nlm.nih.gov/).

RESULTS

Isolation of the Medaka Tyrosinase Gene

I screened about 200,000 plaques of the wild-type genomic library with the probe *TyrG* for the tyrosinase gene, where 9 positive signals were isolated. From one of the clones, designated *W1*, the insert fragment of about 17 kb was subcloned into the pBluescript II plasmid. Analyses using several restriction endonucleases showed *W1* to be a tyrosinase clone. Using the *W1* plasmid, *pTyr-genome* and the 5' half of *pTyr-cDNA* were constructed. About 10 kb fragment of the *W1* clone (*W1HS9.8*) was sequenced and registered in the DNA databank (GSDB/DDBJ/EMBL/NCBI DNA database accession No. AB010101). Note here that medaka is the first vertebrate for which the genomic sequence for tyrosinase has been completely determined.

Organization of the Tyrosinase Gene

Comparing the tyrosinase genomic DNA sequence with the cDNA sequence described previously (Inagaki et al., 1994), I estimated the exon-intron structure of the gene. Our result indicates that the medaka tyrosinase gene is composed of 5 exons with a total length of 4.7 kb (Fig. 1A). The genome sizes for mouse and human are ca. 70 kb (Ruppert et al., 1988) and

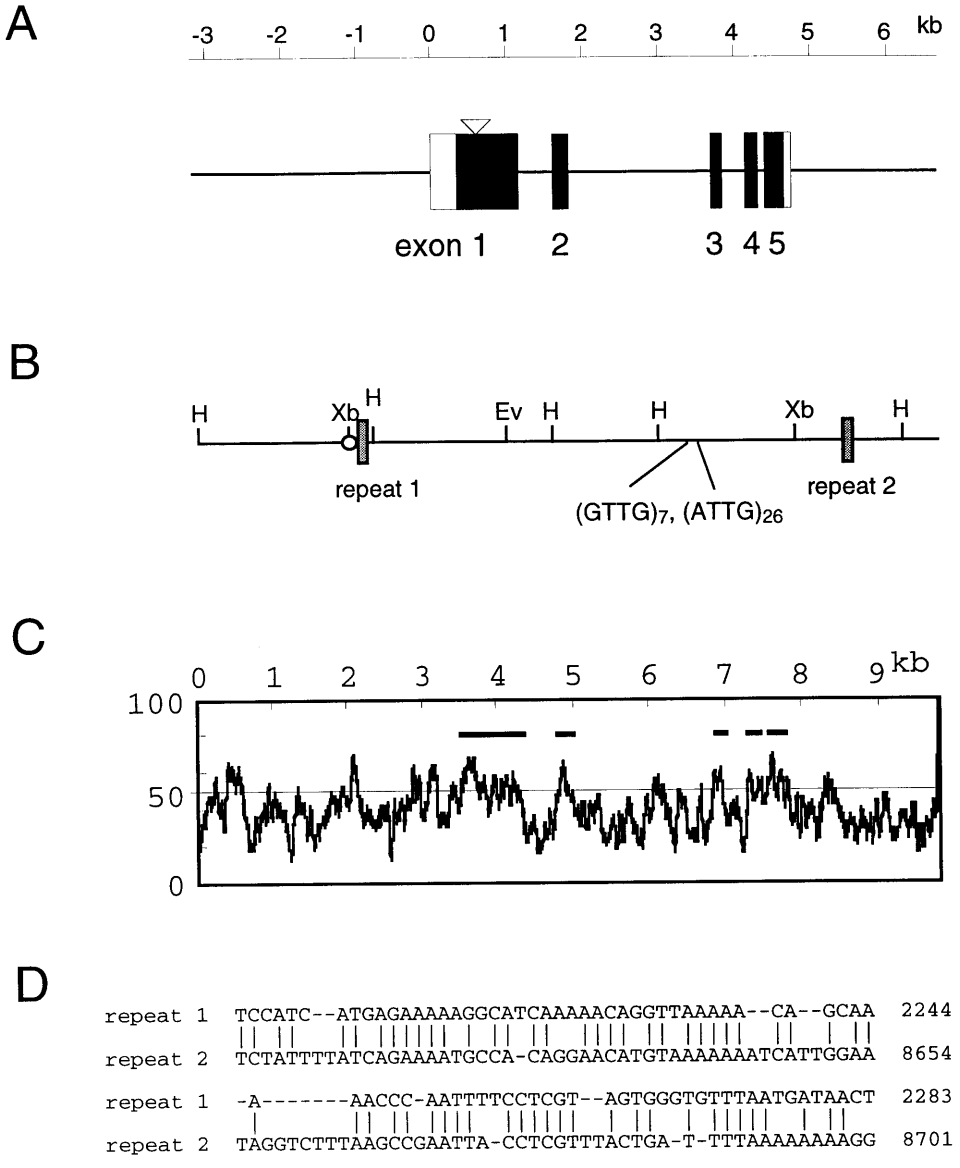

Figure 1. Genomic organization of the medaka tyrosinase gene.
(A) Exon-intron structure. Coding regions of the tyrosinase gene are shown by closed boxes, and non-coding exon regions by open boxes. The 5'- and 3'- flanking regions and introns are shown by straight lines. The open triangle represents the insertion point of the *Tol1* transposon in the gene from the albino-i^1 mutant (Koga et al., 1995). (B) The position of repetitive sequences. The open circle is a mermaid sequence (Shimoda et al., 1996) and the two shaded boxes indicate an unknown repetitive sequence about 50 nucleotides in length. Abbreviations: H, *Hin*dIII site; Xb, *Xba*I site ; Ev, *Eco*RV site. (C) G+C contents (%) of the tyrosinase gene. Bold lines indicate the coding regions. (D) Novel repeat sequences found in the tyrosinase gene. Repeats 1 and 2 correspond to those in Fig. 1B.

> 70 kb (Ponnazhagan et al., 1994), respectively, with similar numbers and sizes of the exons as the medaka. However, the medaka coding region was found to be only 4.7 kb, less than one tenth those of mammals (Fig. 2).

Though nucleotide sequences of human and mouse genomic tyrosinase genes have not yet been determined, sequences for exon/intron boundaries are available. Fig. 3 shows boundary sequences of the tyrosinase genes from medaka. All of the splice junctions match the human and mouse consensus sequences (Müller et al., 1988; Giebel et al., 1991), and are identical among vertebrates. Exceptional is the case of ascidian tyrosinase gene (Sato et al., 1997), which contains only one intron of about 100 bp in the sequence encoding the predicted cytoplasmic tail of the protein, and the insertion site of the intron does not correspond to those of vertebrates.

Fig. 1C shows a profile of the G+C contents of the tyrosinase gene. Average G+C % values for the coding regions, intron regions and whole 9.8 kb sequence were 52.8 %, 33.9 % and 39.2 %, respectively.

Repetitive Sequences

In the medaka intron regions, several repetitive sequences were found (Fig. 1B). In intron 2, two kinds of tetranucleotide repeats, $(GTTG)_7$ and $(ATTG)_{26}$ are present. Using these repeats as a probe, I carried out Southern

blotting analysis of the genomic DNA. Many hybridizing band signals at different positions became apparent, indicating that the repeats are one kind of microsatellite sequence spread throughout the medaka genome (manuscript in preparation). Such microsatellites are very useful for genome mapping and for elucidation of the evolution of eukaryotic genomes (Nadir et al., 1996). Another unique repetitive sequence was also found in 5' upstream and 3' down stream regions (Fig. 1D). Furthermore, a BLAST search of the gene against GenBank DNA database revealed a *mermaid* copy, one of the SINE sequences found in fish and human (Shimoda et al., 1996), in the 5' upstream region (Fig. 1B).

Phylogeny of the Tyrosinase Gene

It is well established that tyrosinase, tyrosinase related protein 1 (TRP1) and tyrosinase related protein 2 (TRP2) diverged by gene duplication, constituting a tyrosinase gene family (Jackson et al., 1992). I aligned the amino acid sequence of the medaka tyrosinase with those from other vertebrates and the ascidian, as well as mouse TRP1 and TRP2 (data not shown). On the basis of the alignment, 427 unambiguously aligned sites (gaps not included) were selected and used for comparison. The alignment required insertion of many gaps in some regions, *i.e.*, the region for signal peptides (corresponding to residues 1 - 19 in medaka tyrosinase), transmembrane

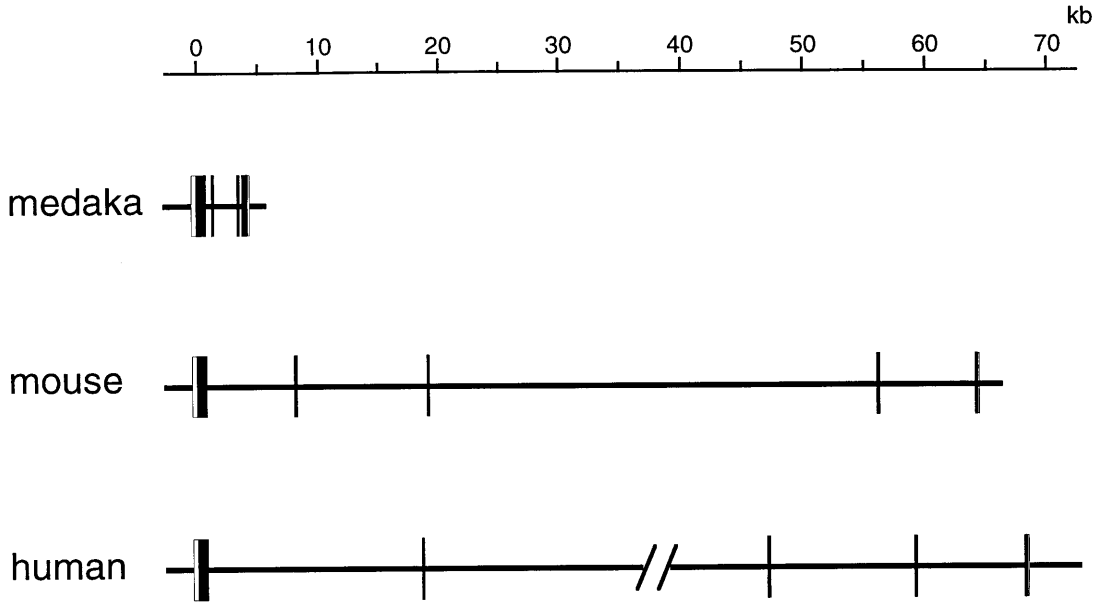

Figure 2. Comparison of the genomic organization of vertebrate tyrosinase genes. Closed and open boxes represent coding and non-coding exons, respectively. The size of human intron 2 has not been reported.

Intron No.	size	Exon-Intron boundary sequence		
		5′ Splice	intron sequence	3′ Splice
		SerTrpLys[276]		
		TCATGGAAG	gtaaga...	
1	439 bp		...cactaaagcttttgtgggccaaacacatgat	
			taaaattggtgtatttttgtttttatacctctag	GTAATCTGC
				ValIleCys
		ValLeuGlu[348]		
		TCTTAGAGG	gtagac...	
2	1859 bp		...tagccttgttttgctgttatcttgattgaaa	
			ctgtaacttcttcacctgcttctttttctttgtag	GTTTTGCCA
				GlyPheAla
		IleAspSer[398]		
		CATTGACAG	gtaaca...	
3	306 bp		...tttgtttaatttttttatcttatcttaacttt	
			catcttgcatatttgtttcttacatcatttctag	CATCTTTGA
				IlePheGlu
		LeuAspPro[458]		
		TGGACCCAG	gtcatt...	
4	87 bp		...gccctctgatctgcaaaagacgtgaatatct	
			gttcagacacccatatccactctgttccacacag	GTCAGAGGT
				GlyGlnArg

Figure 3. Exon-intron boundaries of the medaka tyrosinase gene.
The numbers in the first and second columns are the intron number and total nucleotide lengths, respectively. The number of each amino acid represents the position from the amino-terminal of the premature tyrosinase peptide. The consensus branch point sequence YNYTRAY (Ruskin et al., 1984) of each intron is underlined. Nucleotides of exons and introns are shown in upper and lower cases, respectively.

regions and cytoplasmic domains (453 - 540). *Kaa* values were calculated in a pairwise manner, and a phylogenetic tree was constructed by the neighbor-joining method. The tree indicated separation of fish first, then amphibians, birds and mammals with high bootstrap values (Fig. 4). This order is in accordance with the classical view of vertebrate phylogeny.

It may be premature to estimate the phylogenetic origins of tyrosinase family genes at the present time. However, the tree shown in Fig. 4 does cast some light on this problem. It is probable that tyrosinase, TRP1 and TRP2 diverged from an ancestral gene of the tyrosinase family before the ascidian diverged from the vertebrates. Further sequence data on TRP1 and TRP2 from lower vertebrates are highly desirable to help understand the early evolution of the tyrosinase gene family.

Primer Extension

To determine the transcription initiation site, I carried out the primer extension analysis. The result of eye mRNA showed that (Fig. 5A and B, column E), although several minor signals were seen, two major bands, designated site S1 for the strongest band and site S2 for the second strongest band, were predicted as the initiation sites of the medaka tyrosinase gene, respectively positioned 331 bp and 340 bp upstream from the AUG start codon. These two bands were not detected at the corresponding site of the

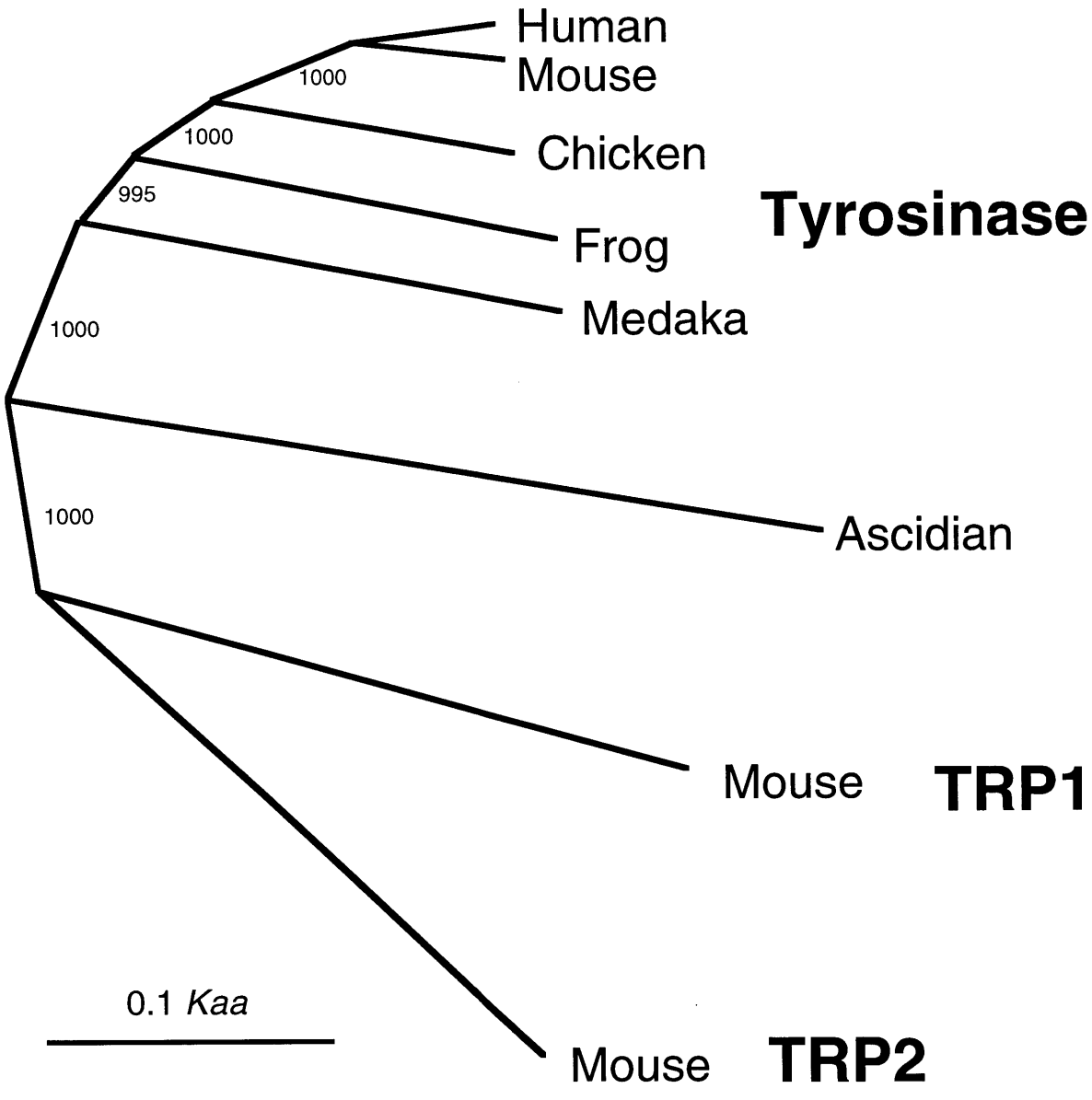

Figure 4. Phylogenetic tree of tyrosinase family in Chordata.
Phylogenetic tree was constructed by means of the neighbor-joining method (Saitou and Nei, 1987), using an alignment of the amino acid sequences of tyrosinases known to date. In the alignment, 427 unambiguously aligned sites (see text) were used for tree construction. The deepest root of the tree was determined with TRP1 and TRP2 sequences as an outgroup. Numbers adjacent to each node represent bootstrap values of 1,000 times. Abbreviation: *Kaa* is the evolutionary distance estimated by the standard method (Zuckerkandl and Pauling, 1965). Amino acid sequences were obtained from the SWISS-PROT data base. Accession numbers of the tyrosinase sequences from human, mouse, chicken and frog are P14679, P11344, P55024 and Q0404, respectively. The ascidian sequence was from Sato et al. (1997). Those for mouse TRP1 and TRP2 are P07147 and P29812, respectively.

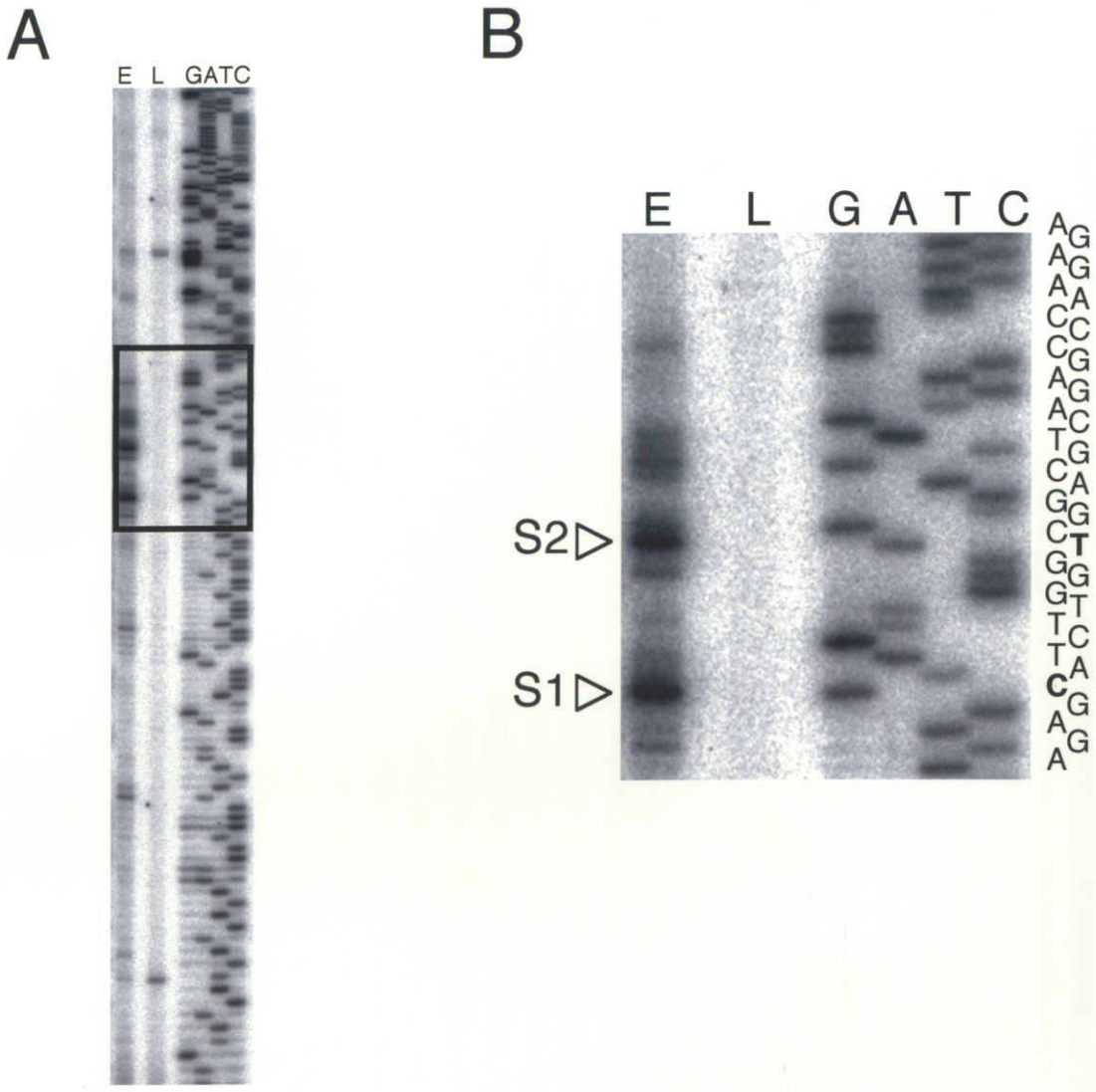

Figure 5. Primer extension analysis of the 5' region of the tyrosinase gene. Open triangles (S1 and S2) indicate the primary and secondary major transcriptional initiation sites, respectively. (A) Autoradiograph of a sequencing gel with the primer extension columns. (B) Enlarged part of A. Abbreviation: G; guanine column, A; adenine column, T; thymine column, C; cytosine column, E; extension analysis column of eye mRNA, L; extension analysis column of liver mRNA.

extension product from liver mRNA (column L). Furthermore, using a different primer and/or different mRNA isolated from the orange-red mutant (genotype *b/b, i+/i+* in *b* and *i* loci), I performed other primer extension analyses, and confirmed these two sites (data not shown). Previously, based on the cDNA sequence, I could estimate the 5'-end site of the mRNA (Inagaki et al., 1994). From these results, S1 and S2 are positioned 11 bp and 20 bp upstream from the 5' end of the cDNA, respectively.

Promoter Region of the Medaka Tyrosinase Gene

Fig. 6 shows an alignment of the sequences around the transcription initiation sites of vertebrate tyrosinase genes established to date. S1 and S2 are also indicated in the alignment. It is well known that a characteristic motif, CATGTG, is shared by all tyrosinase genes from vertebrates which might play an important role in the expression of melanocyte specific genes (see Ferguson and Kidson, 1997). In the medaka sequence, only one CATGTG motif exists, at position -131.

The M-box is a putative control element of the tyrosinase gene found in mammalian sequences (Lowings et al., 1992; Yamamoto et al., 1992), having CATGTG as a core motif (Fig. 6). Not only mammals, but the turtle, quail and chicken have been shown to have the M-box motif in their sequences. This box is a binding site for basic helix-loop-helix leucine zipper proteins, and a

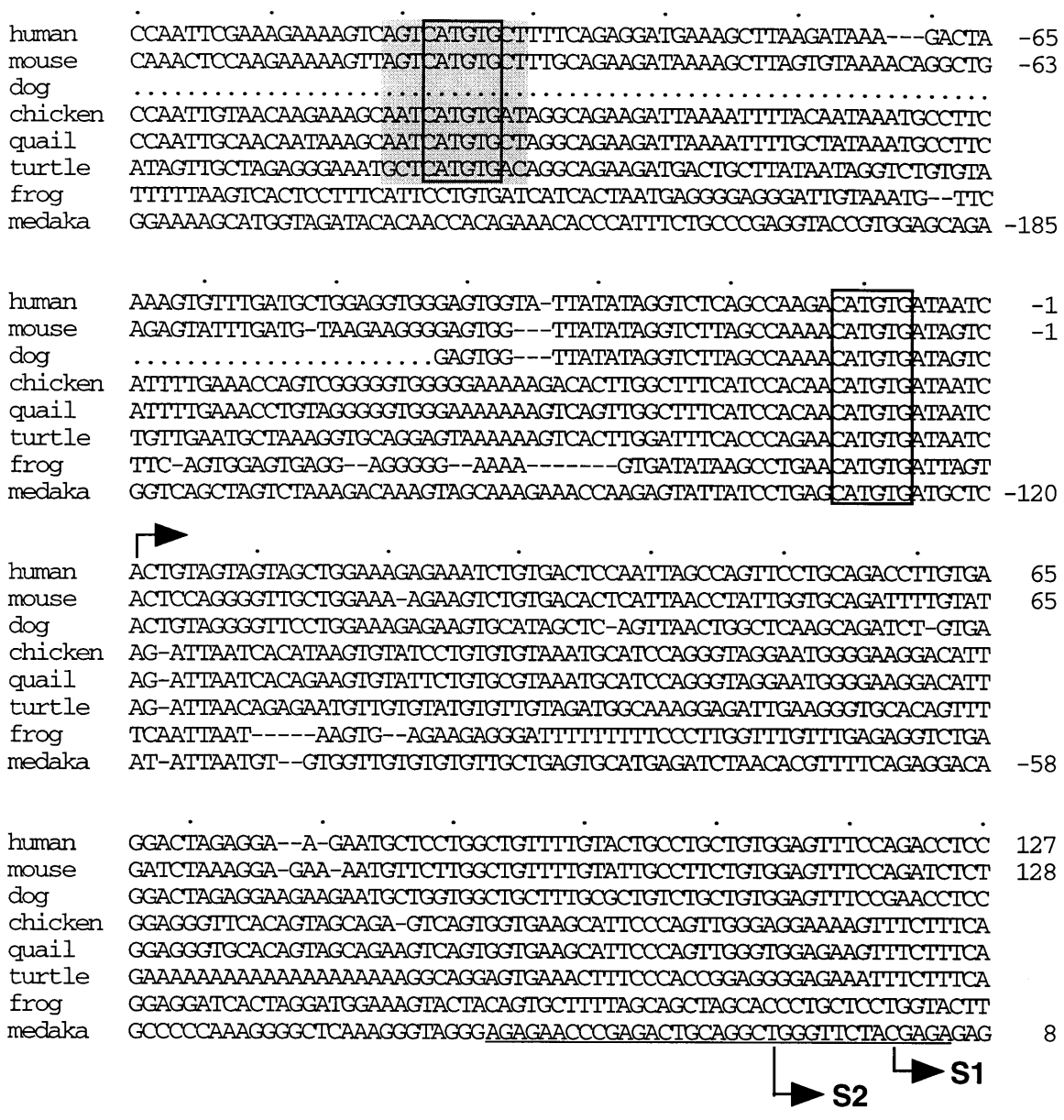

Figure 6. Alignment of the 5' upstream nucleotide sequences of vertebrate tyrosinase genes.

The shaded area indicates the M-box motif, and the boxes in the first and second columns enclose CATGTG motifs (see text). No M-box-like sequence was found in the medaka and frog genes. Arrows in the middle and lower columns show transcription initiation sites for the human and medaka species, respectively. The underlined sequence corresponds to that shown in Fig. 5. Numbers showing the nucleotide positions in the human sequence are counted from the putative transcription initiation sites for these sequences (Ponnazhagan et al., 1994; Ruppert et al., 1988; Yamamoto et al., 1989), and those shown for the medaka sequence are from the initiation site S1 determined in this study. Nucleotide sequences from human, mouse, dog, quail, snapping turtle and frog are cited from accession nos. X16073, D00439, U42219, S56788, S56789 and D37779 in the GenBank database, respectively.

candidate binding protein MITF has been identified (see Ferguson and Kidson, 1997). However, in the alignment (Fig. 6), the region from the corresponding medaka sequence shows no similarity to the mammalian M-box sequence. Thus, medaka lacks the M-box, like the frog, which has only one CATGTG motif and no M-box-like sequence in its 5' proximal region (Miura et al., 1995).

Tyrosinase Gene Expression in Transgenic Fish

We have shown that the *i* locus mutant, albino-i^1, has an insertional mutation in exon 1 (Koga et al., 1995). To confirm whether the *i* locus of medaka encodes the tyrosinase gene or not, I demonstrated the rescue of the albino phenotype of the i^1 mutant by introducing the wild-type tyrosinase gene into i^1 eggs. *pTyr-genome* is a 19 kb plasmid containing 10 kb 5' upstream sequence, the coding region of the tyrosinase gene, and 2 kb downstream sequence (Fig. 7). Linear or circular forms were introduced into fertilized eggs of the i^1 mutant. Table 1 summarizes the results. About 30 % of the embryos showed black melanin-containing cells in eye and skin. When the same assay was performed using the plasmid *pTyr-cDNA,* which has only 3 kb 5'-upstream region and tyrosinase cDNA sequence, equal phenotype recovery was observed (Table 1). As a control, eggs injected with no plasmids or plasmids without tyrosinase gene did not show phenotype recovery. Fig. 8

illustrates one such embryo, in which melanin-containing cells were limited to the eye and skin.

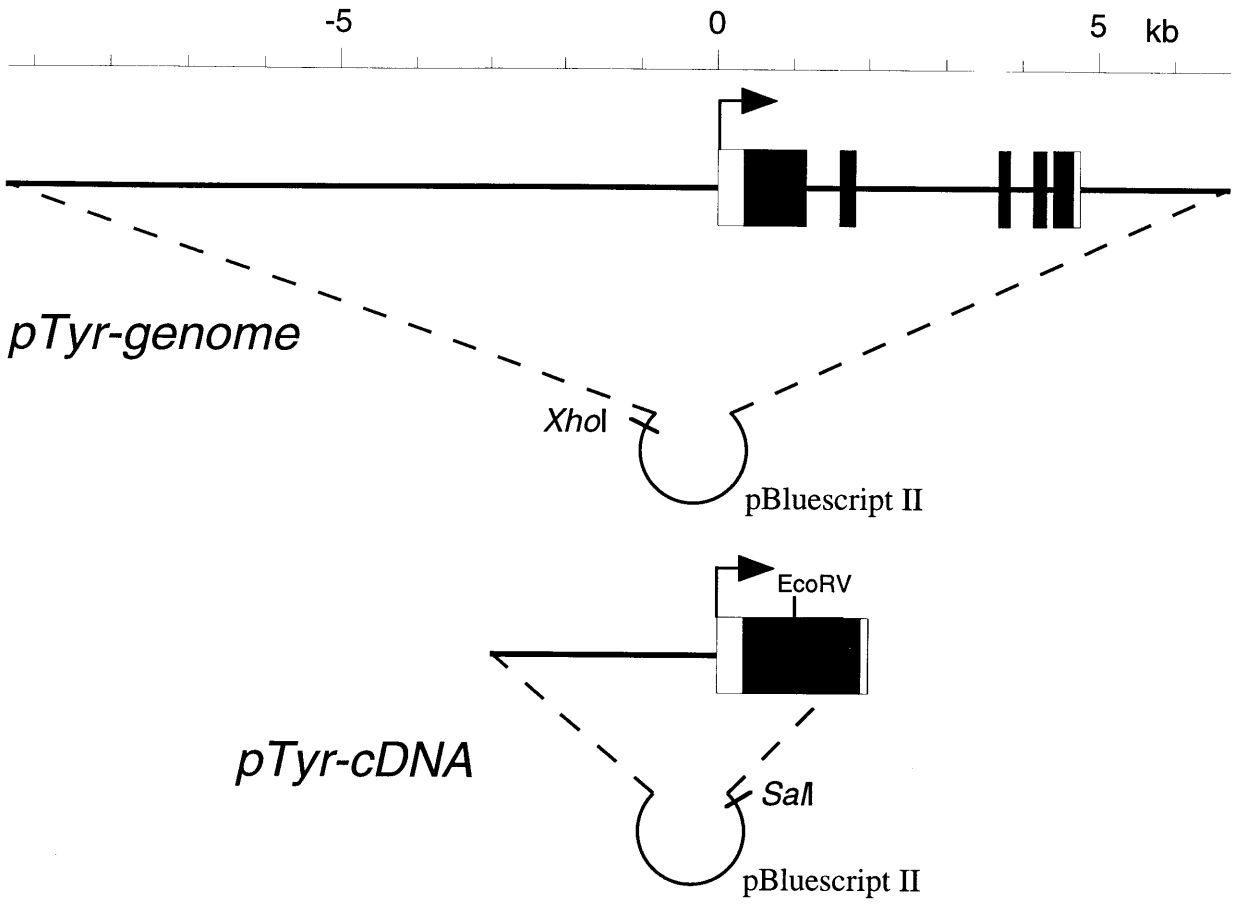

Figure 7. The tyrosinase gene expression vector construct.
pTyr-genome and *pTyr-cDNA* plasmids (see text) were used. Linear plasmids with *Xho*I digest for *pTyr-genome* and *Sal*I digest for *pTyr-cDNA* or circular plasmids without digestion were microinjected into albino-i^1 eggs.

Table 1. Survival of embryos and number of pigmented F_0 embryos after *pTyr-genome* and *pTyr-cDNA* injection into albino-i^1 eggs

	pTyr-genome linear	pTyr-genome circular	pTyr-cDNA linear	pBluescript linear
	No. (%)	No. (%)	No. (%)	No. (%)
a. No. of fertilized eggs injected	114 (100) (a/a)	51 (100)	118 (100)	138 (100)
b. No. of embryos surviving to 5th day	53 (46) (b/a)	21 (41)	45 (38)	60 (43)
c. No. of embryos pigmented	15 (28) (c/b)	1 (5)	10 (22)	0 (0)

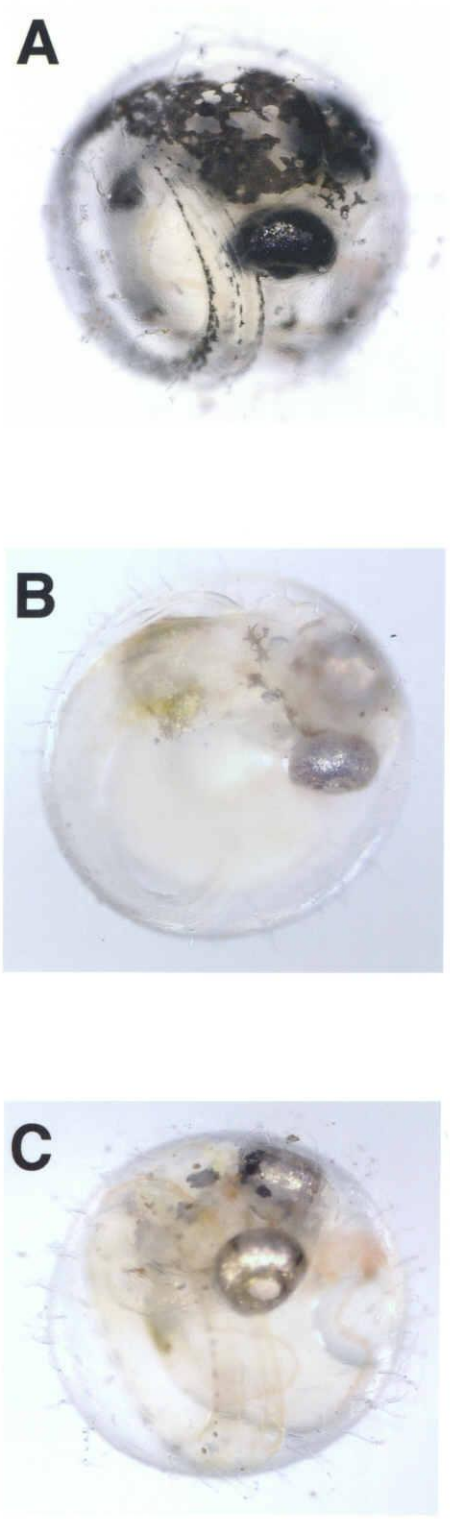

Figure 8. Transgenic albino medaka embryos exhibiting mosaic melanin pigmentation.
(A) Control wild-type embryo, (B) albino i^{l} embryo, and (C) injected (F0) embryo, each at the hatching stage, 8 days after spawning. The injected embryo exhibits scattered heavily melanized melanophores in the dorsal head skin and mosaic melanization over the eye caps.

DISCUSSION

Transcription Initiation Sites

The present study demonstrated that the major site of transcription, at +1 of the medaka sequence in Fig. 6, starts with a longer untranslated leader sequence (340 nucleotides long) from the AUG start codon than mammals (80 nt in human). In a previous study (Inagaki et al., 1994), I performed Northern analysis of medaka tyrosinase mRNA, and showed expression of an approximately 2.2 kb mRNA in eye and skin of wild-type medaka. This includes the 1.6 kb of the coding region, 0.1 kb of 3' UTR+polyA signal and 340 bases of the 5' leader sequence, indicating a long 5' region. Thus, it is clear that the transcription initiation site in medaka is positioned at +1 site in the medaka sequence and is apparently different from the mammalian whose initiation site is close to the motif closely associated with the gene expression.

Several experiments in mice have shown that 2 to 12 kb in the 5' upstream region of the tyrosinase gene confer tissue specificity in expression, but not the wild-type level of melanin production (Shibata et al., 1992; Ganss et al., 1994). A YAC vector which contains 250 kb region of the mouse tyrosinase gene generates full melanization in transgenic mice (Schedl et al., 1993). These data suggest that the mouse and/or human tyrosinase genes must

have other important regulatory elements in the far upstream/downstream regions. Whether or not this is the case for the medaka tyrosinase gene, the transgenic experiment in the present study clearly showed that as little as 3 kb of the 5' upstream region is sufficient for tissue specific expression.

The Medaka *i* Locus is the Tyrosinase Gene

In the present study, I used the albino-i^1 mutant to perform an albino rescue experiment, and confirmed melanin pigmentation in transgenic eggs. Since the i^1 mutant is due to transposon insertion in the tyrosinase gene (see Koga and Hori, 1997), there is a possibility that the albino phenotype could revert to the wild-type phenotype due to precise transposon excision (Koga et al., 1996). To test this point, a thousand i^1 eggs were examined but none gave rise to embryos with melanin production. Control injection of the plasmid without the tyrosinase gene also revealed no recovery (Table 1). Hyodo-Taguchi et al. (1997) also analyzed approximately 2,000 albino i^1 embryos, and observed that not a single one showed a pigmented phenotype.

Albino Rescue Experiments

I have described here that introduction of the tyrosinase gene into albino-i^1 fish results in melanin pigmentation in the eye and skin. No ectopic expression was observed. The mosaic pattern of pigmentation has also been

seen in all other stable transgenic experiments performed with medaka so far (Ozato et al., 1989; Kinoshita et al., 1996). Recently, Hyodo-Taguchi et al. (1997) performed a similar transgenic experiment employing a mock gene, *i.e.*, mouse tyrosinase cDNA plasmid *pmTyr4*, and albino-i^{1} as recipient. Transient phenotypic rescue, with mosaic expression just like in the present case, was shown.

Matsumoto et al. (1992) introduced another mouse tyrosinase cDNA plasmid, *mg-Tyr-J*, into the eggs of the orange-red mutant (genotype *b/b*; *i+/i+*), and described rescue from the orange-colored phenotype, with recognizable melanin deposition in the skin. Furthermore, Ono et al. (1997) recently confirmed previous results using an electroporation method in order to produce large numbers of transgenic fish and a specific mouse tyrosinase antibody to assess protein expression.

In a comparable attempt to rescue the *b/b* mutant phenotype, Hyodo-Taguchi et al. (1997) carried out an experiment, using *pmTyr4* and an orange-mutant of medaka inbred strain, HO4C (genotype *b/b, i+/i+*). Contrary to the results of Matsumoto et al. (1992) and Ono et al. (1997), they did not observe any phenotypic effects. This might have been expected, however, as the orange-red variety of medaka is due to a mutation that does not affect tyrosinase mRNA (Inagaki et al., 1994) or protein levels (Hishida et al., 1961; Hirose and Matsumoto, 1993). The *i* locus has been established to be nonallelic

with the *b* series alleles (Yamamoto, 1969) and recent linkage map analysis indicated positions in different linkage groups, *i.e.*, linkage group 2 for *b* (Wada et al., 1995) and 18 for i^{l} (Naruse et al., personal communication).

The contradictory results of Hyodo-Taguchi et al. (1997) and Matsumoto et al. (1992) might be explained by differences in the sizes of the 5' upstream regions of the two mouse minigene plasmids *pmTyr4* and *mg-Tyr-J*. Thus the transcript from *mg-Tyr-J* might complement a *b* locus mutation not covered by *pmTyr4*. It can be expected that a full and stable rescue of the *b/b* mutation in medaka would be achieved if the authentic medaka tyrosinase sequences such as the *pTyr-genome* construct used in this study are used for gene transfer. It is probable that cloning and sequencing of the medaka *b* locus gene will resolve this question.

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