Functional Conservation of the Promoter Regions of Vertebrate Tyrosinase Genes

Shigeru Sato,[1] Mika Tanaka,[2] Hirohito Miura,[3] Kazuho Ikeo,* Takashi Gojobori,* Takuji Takeuchi, and Hiroaki Yamamoto

Biological Institute, Graduate School of Science, Tohoku University, Sendai, Miyagi, Japan; *National Institute of Genetics, Mishima, Shizuoka, Japan

Tyrosinase is the key enzyme for synthesizing melanin pigments, which primarily determine mammalian skin coloration. Considering the important roles of pigments in the evolution and the adaptation of vertebrates, phylogenetic changes in the coding and flanking regulatory sequences of the *tyrosinase* gene are particularly intriguing. We have now cloned cDNA encoding tyrosinase from Japanese quail and snapping turtle. These nonmammalian cDNA are highly homologous to those of the mouse and human tyrosinases, whereas the 5′ flanking sequences are far less conserved except for a few short sequence motifs. Nevertheless, we demonstrate that the 5′ flanking sequences from the quail or turtle *tyrosinase* genes are capable of directing the expression of a fused mouse tyrosinase cDNA when introduced into cultured mouse albino melanocytes. This experimental method, which reveals the functional conservation of regulatory sequences in one cell type (the melanocyte), may be utilized to evaluate phylogenetic differences in mechanisms controlling specific gene expression in many other types of cells. We also provide evidence that the 5′ flanking sequences from these nonmammalian genes are functional *in vivo* by producing transgenic mice. Phylogenetic changes of vertebrate *tyrosinase* promoters and the possible involvement of conserved sequence motifs in melanocyte-specific expression of *tyrosinase* are discussed.

Key words: melanin/melanocytes/transcriptional regulation/transgenic mice. Journal of Investigative Dermatology Symposium Proceedings 6:10–18, 2001

Skin color has been molded by evolutionary forces to play a variety of adaptive roles in vertebrates. Melanin pigment is the major source of skin color variation, particularly in birds and mammals. Tyrosinase (E.C.1.14.18.1) is the key enzyme for melanin biosynthesis as it initiates the process by catalyzing the oxidation of tyrosine to form DOPA (L-3,4-dihydroxyphenylalanine) and further, oxidizes DOPA to DOPA-quinone (Hearing and Tsukamoto, 1991). Given the importance of melanin, and ultimately of tyrosinase, in the adaptation of vertebrates, it is clear that the genetic network-regulating enzyme function must also be, at least indirectly, scrutinized by natural selection for timely expression during the ontogeny of complex pigmentation patterns and for the effective production of melanin throughout life.

Manuscript received June 14, 2001; accepted for publication June 14, 2001.

Reprint requests to: Dr. Hiroaki Yamamoto, Biological Institute, Graduate School of Science, Tohoku University, Sendai, Miyagi 980–8578, Japan. Email: hyamamot@mail.cc.tohoku.ac.jp

Abbreviations: bHLH, basic-helix-loop-helix; Dct, dopachrome tautomerase; DOPA, L-3,4-dihydroxyphenylalanine; MITF/Mitf, microphthalmia-associated transcription factor; ORF, open reading frame; TDE, tyrosinase distal element; TE-1, tyrosinase element-1; tRNA, transfer RNA; Tyrp1, tyrosinase-related protein; UPGMA, unweighted pair-group

[1]Current address: Department of Biology, Jichi Medical School, Minamikawachi, Tochigi 329–0498, Japan.

[2]Current address: RIKEN Brain Science Institute, 2–1 Hirosawa, Wako, Saitama 351–0198, Japan.

[3]Current address: National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaragi 305–0856, Japan.

Expression of the *tyrosinase* gene is restricted to specialized cells designated as melanocytes (or melanophores). It has been demonstrated that the 5′ flanking sequences of mammalian *tyrosinase* genes determine their melanocyte-specific expression (Beermann *et al*, 1990; Tanaka *et al*, 1990; Kluppel *et al*, 1991; Shibata *et al*, 1992; Bentley *et al*, 1994; Ganss *et al*, 1994b; Porter and Meyer, 1994; Yasumoto *et al*, 1994, 1997). We hypothesized that the 5′ regulatory sequences, as well as the coding sequences, of *tyrosinase* genes have been tightly conserved by selective forces that operate during vertebrate phylogenesis. Our preliminary study showed that coding sequences, except for regions encoding putative signal peptides, in the first exon of vertebrate *tyrosinase* genes (Japanese quail, snapping turtle, mouse, and human) are highly conserved (Yamamoto *et al*, 1992). In contrast, only a few short sequence motifs are shared in the 5′ flanking regions of mammalian and nonmammalian *tyrosinase* genes (Tanaka *et al*, 1992; Yamamoto *et al*, 1992). The first such motif was an 11 bp element now termed the M-box (Lowings *et al*, 1992), previously designated as either the upstream element (Shibahara *et al*, 1991), an 11-mer motif (Jackson *et al*, 1991), or p-MSE (Yamamoto *et al*, 1992). This regulatory motif is also found in the proximal promoters of genes encoding the related melanogenic enzymes, tyrosinase-related protein-1 (Tyrp1) and tyrosinase-related protein-2 or dopachrome tautomerase (Dct) (Jackson *et al*, 1991; Shibahara *et al*, 1991; Lowings *et al*, 1992; Yamamoto *et al*, 1992; Yokoyama *et al*, 1994; Budd and Jackson, 1995; Miura *et al*, 1995; Ferguson and Kidson, 1996). Functional analyses of mammalian *tyrosinase* proximal promoters identified the M-box as a positive regulatory element for melanocyte-specific expression (Bentley *et al*, 1994; Ganss *et al*, 1994c; Yasumoto *et al*, 1997). We also found sequences homologous to the d-MSE, a 13

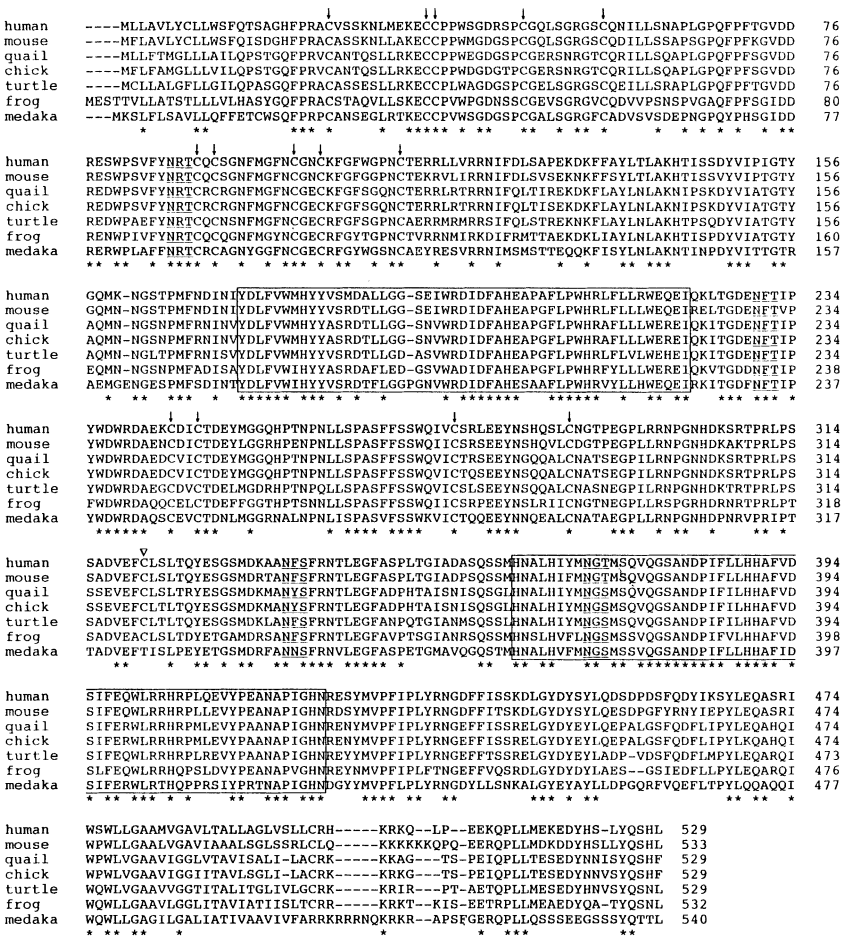

Figure 1. Alignment of deduced amino acid sequences from vertebrate tyrosinases. Amino acid sequences deduced from cDNA clones of Japanese quail (*Coturnix coturnix japonica*) and snapping turtle (*Trionix sinensis japonicus*) are aligned with previously identified sequences for tyrosinases of human (*Homo sapience*) (Kwon *et al*, 1987), mouse (*Mus musculus*) (Yamamoto *et al*, 1989), chicken (*Gallus gallus*) (Mochii *et al*, 1992), Japanese pond frog (*Rana nigromaculata*) (Takase *et al*, 1992), and medaka fish (*Oryzias latipes*) (Inagaki *et al*, 1994). Sequences were aligned using the CLUSTAL W1.7 program. Two copper-binding sites (CuA and CuB) are boxed. Cysteine residues conserved among all the proteins are indicated by *small arrows*, and one cysteine residue (conserved except for medaka fish) is indicated by an *open arrowhead*. Four conserved putative N-glycosylation sites are underlined. Gaps introduced to maximize sequence homology are represented by dashes. Asterisks indicate identical amino acids.

bp motif conserved in mouse *tyrosinase* and *Tyrp1* promoters (Yamamoto *et al*, 1992), and in the turtle flanking region. Bentley *et al* (1994) later found two other conserved sequences, a 9 bp motif (termed CR1) adjacent to the M-box and a 15 bp motif (termed CR2) about 90 bp downstream from the M-box. The CR2 contains a basic-helix-loop-helix (bHLH) factor-binding E-box motif, CATGTG, which is also the core sequence of the M-box, and a degenerate octamer element, GTGATAAT, in human, quail, chicken, and turtle genes. The authors further identified a nonconsensus SP1 motif positioned between CR1 and CR2 (Bentley *et al*, 1994).

In this study, we compared the 5′ flanking regions of vertebrate tyrosinase genes. Despite the overall sequence divergence of the 5′ flanking sequences of the quail and turtle *tyrosinase* genes, they are capable of activating transcription of a fused mouse tyrosinase cDNA when introduced into cultured mouse albino melanocytes. As a consequence, amelanotic albino melanocytes were induced to synthesize melanin. To verify those results obtained by transfection, we further demonstrate that the 5′ flanking sequences of the quail or turtle *tyrosinase* genes are able to direct expression of a fused mouse cDNA in transgenic mice, as does the flanking sequence of the human *tyrosinase* gene (Tanaka *et al*, 1992). Finally, we identify several conserved sequence motifs in the 5′ flanking regions of other vertebrate *tyrosinase* genes, including dog (Tang *et al*, 1995), Japanese pond frog (Miura *et al*, 1995), chicken (Ferguson and Kidson, 1996), and medaka fish (Inagaki *et al*, 1998).

MATERIALS AND METHODS

Cloning of quail tyrosinase cDNA Total RNA fractions were prepared from pigmented epithelium and/or whole eyes of 10-d-old Japanese quail embryos. Single-stranded cDNA were synthesized using an oligo (dT) primer flanked with *Bam*HI, *Eco*RI, and *Sma*I restriction sites, T-BES: 5′-GGATCCGAATTCCCCGGGTTTTTTTTTTTTT-TTT-3′. Subsequently, polymerase chain reaction (PCR) amplification was carried out using T-BES and a sense primer specific to quail tyrosinase cDNA. The sequence of the specific primer was 5′-GGAA-TTCGGATCCCCTGCTGCTCTGTGAGG-3′, which corresponds to bases −20 to −1 of the genomic clone Q63SH1 (Yamamoto *et al*, 1992) and is also flanked with *Eco*RI-*Bam*HI sites. In this report, position numbering is relative to the A (+1) for the first methionine unless otherwise stated. The double-stranded cDNA were digested with *Eco*RI, electrophoretically purified, and cloned into the *Eco*RI site of pUC119. Sequence data from multiple independent clones were accumulated and compared with each other, and then a consensus sequence was released because the original data were obtained by PCR.

Cloning of turtle tyrosinase cDNA Total RNA was prepared from whole eyes of 20-d-old-snapping turtle embryos. Single-stranded cDNA were prepared as described above and then the second-strand was synthesized with a specific sense primer, T-FP: 5′-GGAATTCTAG-AAAATTGCCTGCTGTTGT-3′ (bases −25 to −7) flanked with *Eco*RI and *Xba*I sites. The cDNA were digested with *Eco*RI and cloned into λgt 11 phage vector because PCR-amplification was somewhat unsatisfactory for cloning into plasmid vectors under the conditions employed. Recombinant phages were screened by PCR using T-FP and λgt 11 primers, and subcloned into the pUC119 vector. A consensus sequence was determined as described above.

Primer extension Primer extension was performed using primers end-labeled with ^{32}P (about 5×10^4 cpm by Cherenkof counting). The sequences of the primers are Q-RP: 5′-CATGGTAAACAGAAGC-ATCCTCACAGAGCAGCA-3′ (complementary to bases +18 to −15 of the quail *tyrosinase* gene) and T-RP: 5′-TCGAGGGAACTGCCCGG-ATGCAGGCTGCAG-3′ (complementary to bases +66 to +37 of the turtle *tyrosinase* gene). Total RNA prepared from pigmented epithelium or from whole eyes were annealed with primers and extension reactions were performed as previously described (McKnight and Kingsbury,

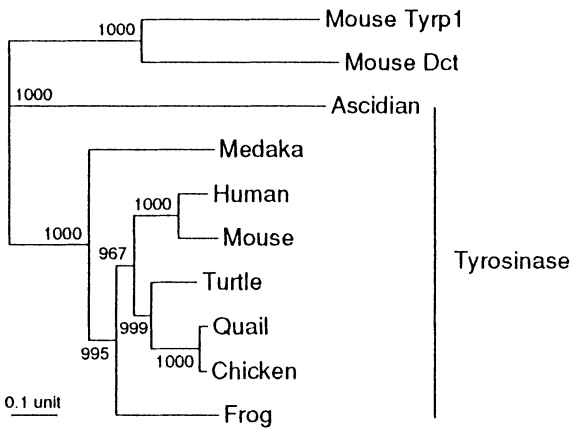

Figure 2. A representative phylogenetic tree of tyrosinase proteins. Tyrosinase sequences from seven vertebrate species (human, mouse, chicken, Japanese quail, snapping turtle, Japanese pond frog, and medaka fish) and from an ascidian (*Halocynthia roretzi*) (Sato *et al*, 1997a) were analyzed. The multiple alignment was generated by the CLUSTAL W program (version 1.7) and phylogenetic trees were constructed by the Neighbor-joining method. Branch lengths are drawn to scale indicated in units of distance as calculated in the distance matrix. Numbers above nodes are the numbers of bootstrapping trials (n = 1000) in which an identical branching pattern was produced. As an outgroup, mouse Tyrp1 (Shibahara *et al*, 1986) and Dct (Jackson *et al*, 1992) were used.

1982). In the control extension reaction, the same amount of *E. coli* tRNA was used. The products were separated by electrophoresis on 6% sequencing gels and visualized by autoradiography.

Hybrid minigenes Hybrid minigenes were constructed as follows: The quail *tyrosinase* genomic clone pQ12Xb8 (EMBL/GenBank/DDBJ Accession number: AB024279) was digested with *Fnu*4HI, and the resulting fragment (bases –13 to –1781) was blunted and ligated with the mouse tyrosinase cDNA Tyrs-J (Yamamoto *et al*, 1989), which had been digested with *Eco*RI and blunted. This quail-mouse hybrid minigene, which contained 1.8 kb of the quail *tyrosinase* promoter, was designated as qg-Tyrs-J. The snapping turtle genomic clone SB1-1 (EMBL/GenBank/DDBJ Accession number: AB024281) was digested with *Xho*I. As this clone contained an additional *Xho*I site in the far upstream region, two *Xho*I-digested fragments were simultaneously cloned into the *Xho*I site of Tyrs-J. This turtle-mouse hybrid minigene, which contained about 4.3 kb of the turtle *tyrosinase* promoter, was designated as tg-Tyrs-J. As a positive control, mg-Tyrs-J, which is a minigene composed of 2.6 kb of the mouse *tyrosinase* promoter fused to Tyrs-J, was used. This minigene (mg-Tyrs-J) has been shown to be capable of expressing tyrosinase activity in cultured albino melanocytes (Yamamoto *et al*, 1989) and in transgenic mice (Tanaka *et al*, 1990). The plasmid vector pUC118 and a construct carrying only Tyrs-J were used as negative controls.

Transfection and microinjection Cultured albino melanocytes were prepared from C57BL/6 J-Tyr^{c-2J}/Tyr^{c-2J} newborn mice according to the method of Sato *et al* (1985) and transfections were carried out as previously described (Yamamoto *et al*, 1989). In order to detect tyrosinase enzyme activity, transfected melanocytes were fixed with periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) for 30–60 min at 4°C, washed with 0.1 M sodium phosphate buffer (pH 7.4) and then incubated with 1 mg per ml DOPA in 0.1 M phosphate buffer (pH 7.4) at 37°C for 3–5 h. After completion of DOPA-reaction, cells were washed with phosphate buffer and surveyed by light microscopy for the accumulation of dark DOPA-melanin in order to localize tyrosinase activity.

Microinjection of minigenes into fertilized eggs of BALB/C albino mice was performed as described by Tanaka *et al* (1990).

Molecular phylogenetic analyzes Multiple alignments of amino acid and nucleotide sequences were generated either by the CLUSTAL W program version 1.7 (Thompson *et al*, 1994) or by the MALIGN program of the ODEN package (Ina, 1990). Amino acid substitutions were inferred using the aligned sequences and used to construct phylogenetic trees by the Neighbor-joining method (Saitou and Nei, 1987). Bootstrapping resampling was performed to create multiple (n = 1000) randomly sampled data sets from the original alignment.

RESULTS AND DISCUSSION

Cloning of quail and of turtle tyrosinase cDNA To date, vertebrate tyrosinase cDNA have been cloned from mouse (Yamamoto *et al*, 1987, 1989; Kwon *et al*, 1988; Müller *et al*, 1988; Ruppert *et al*, 1988), human (Kwon *et al*, 1987; Shibahara *et al*, 1988), chicken (Mochii *et al*, 1992), Japanese pond frog (Takase *et al*, 1992), and medaka fish (Inagaki *et al*, 1994). Partial cDNA sequences are also available for dog (Tang *et al*, 1996) and cat (van der Linde-Sipman *et al*, 1997) tyrosinases (reviewed in Shibahara *et al*, 1998).

We have isolated cDNA clones encoding tyrosinase from Japanese quail and snapping turtle. As expected from our previous analyses of the first exons of quail and turtle *tyrosinase* genes (Yamamoto *et al*, 1992), the entire nucleotide and deduced amino acid sequences were highly homologous to those of other vertebrate tyrosinases. As shown in **Fig 1**, quail tyrosinase cDNA (1894 bp) has an open reading frame (ORF) encoding 529 amino acids (*Mr* 60 333) and the turtle cDNA (1909 bp) also contains an ORF encoding 529 amino acids (*Mr* 60 167); these ORF are similar in length to those of tyrosinase cDNA isolated from other vertebrates. In addition, the sizes of the major transcripts of quail and turtle tyrosinases were determined to be approximately 2.1 kb by northern hybridization (data not shown).

In each of the seven vertebrate tyrosinases aligned in **Fig 1**, a signal peptide (von Heijne, 1986) and a transmembrane domain (Klein *et al*, 1985) were identified at the N-terminus and near the C-terminus, respectively. These two regions, together with a cytoplasmic tail at the C-terminal end, show relatively low similarity among aligned sequences compared with the overall high homology of other regions. As expected, two putative copper-binding sites (CuA and CuB) are highly conserved in all vertebrate tyrosinases. In these regions, which are also found in plant and fungal tyrosinases, six conserved histidine residues that are symmetrically arranged are believed to form a binuclear copper center and are crucial for specific copper-binding and/or enzymatic activity (Hearing and Tsukamoto, 1991; Oetting and King, 1994a; van Gelder *et al*, 1997). There are 14 cysteine residues at conserved positions distributed unevenly in the sequences (**Fig 1**). The first 10 cysteine residues cluster near the N-termini and the other five (four in medaka fish tyrosinase) residues reside between two copper-binding sites. Amino acid substitutions within the N-terminal cysteine rich region are found in BALB/C albino (Tyr^{c}/Tyr^{c}) mouse (C103S) (Shibahara *et al*, 1990; Yokoyama *et al*, 1990) and among patients diagnosed with OCA1 oculocutaneous albinism (C55Y and C89R) (Oetting and King, 1994b). The N-terminal region has been proposed to play a role in the formation of multienzyme complexes by Jackson *et al* (1992) based on the characteristic sequence pattern known as an "EGF-motif". Also, the observation that vertebrate tyrosinases become very fragile under reducing conditions (Nishioka, 1978) suggests that some cysteine residues in these regions are responsible for developing the correct molecular conformation required for catalytic activity. Comparison of these deduced amino acid sequences with those deposited in the albinism database (http://www.cbc.umn.edu/tad) showed no matching except for the H19S mutation that may cause albinism in humans. It is expected that this substitution results in aberrant cleavage of the signal peptide sequence (Oetting *et al*, 1998). An alignment of amino acid sequences (**Fig 1**) shows that quail, chick, turtle, frog, and medaka all have this substitution. It is intriguing to see whether this substitution would affect melanization in pigment cells.

We found nine putative N–glycosylation sites of the form NXS or NXT in the quail tyrosinase sequence and eight such sites in the turtle sequence. Although there are many putative N-glycosylation sites in each vertebrate tyrosinase, only four sites are shared in common (**Fig 1**). This is in agreement with an earlier report that

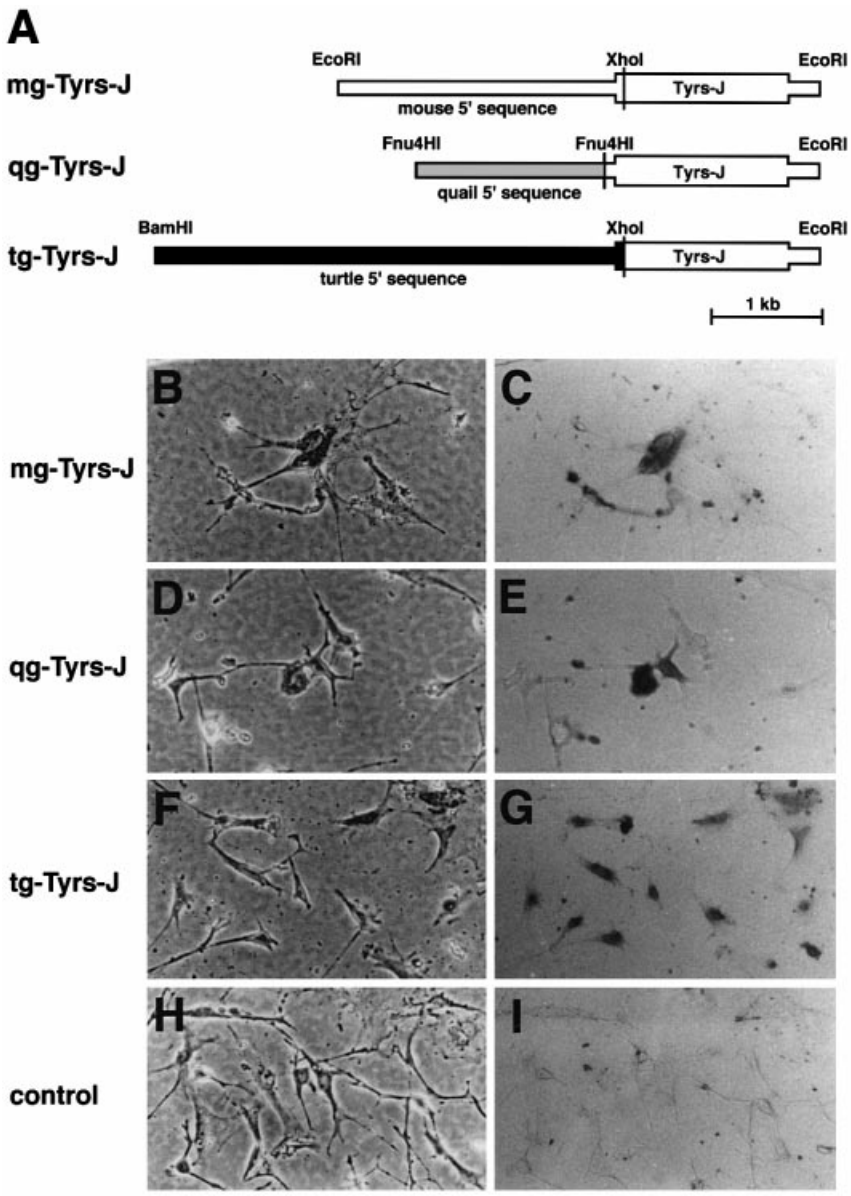

Figure 3. Expression of hybrid minigenes in mouse albino melanocytes. (*A*) Hybrid minigenes. A mouse tyrosinase cDNA (Tyrs-J) was fused to the 5′ flanking sequences of a 2.6 kb mouse (mg-Tyrs-J), a 1.8 kb Japanese quail (qg-Tyrs-J), and a 4.3 kb snapping turtle (tg-Tyrs-J) *tyrosinase*, respectively. (*B–I*) Cultured mouse albino melanocytes transfected with mg-Tyrs-J (*B*, *C*), qg-Tyrs-J (*D*, *E*), tg-Tyrs-J (*F*, *G*), or control pUC118 (*H*, *I*). Tyrosinase activity localized by the accumulation of dark DOPA-melanin was detected in cells transfected with the minigenes (*C*, *E*, *G*), but not with the control plasmid (*I*). Photographs of the same field were taken under phase-contrast (*B*, *D*, *F*, *H*) and without phase-contrast (*C*, *E*, *G*, *I*).

showed the presence of four glycosylated sugar chains per tyrosinase molecule purified from hamster melanoma (Ohkura *et al*, 1984). Thus, if N-glycosylation is essential to express tyrosinase activity and is necessary for tyrosinase to be correctly processed and localized in melanosomes, the reason for the conservation of these four sites is evident.

To infer evolutionary relationships between vertebrate tyrosinase proteins, molecular phylogenetic trees were constructed based on entire amino acid sequences. A representative tree obtained by the neighbor-joining method (Saitou and Nei, 1987) is shown in **Fig 2**. Nodes (branch points) of the tree correlate well with the divergence of species (Nei, 1987). In addition, the unweighted pair-group method with arithmetic mean (UPGMA) (Sokal and Michener, 1985), another distance matrix method, and the maximum parsimony method (Felsenstein, 1982) gave the same tree topology.

Function of nonmammalian 5′ flanking sequences in mouse melanocytes Human and mouse *tyrosinase* genes share homologous sequences scattered in their 5′ flanking regions like an archipelago, although these vertebrate sequences share little similarity with the 5′ flanking sequences of quail and turtle genes (Tanaka *et al*, 1992; Yamamoto *et al*, 1992). We used transfection to examine whether the highly diverged 5′ flanking sequences of nonmammalian tyrosinase genes are capable of activating transcription in cultured mouse albino melanocytes. One of the transfected hybrid minigenes used, qg-Tyrs-J, consisted of an 1.8 kb quail *tyrosinase* 5′ flanking sequence fused to a mouse tyrosinase cDNA (Tyrs-J), while the other, tg-Tyrs-J, consisted of a 4.3 kb turtle *tyrosinase* 5′ flanking sequence fused to Tyrs-J. As shown in **Fig 3**, melanization occurred in mouse melanocytes transfected with qg-Tyrs-J or tg-Tyrs-J, but not with control constructs. Intense melanization of the culture media was also observed (data not shown).

To verify these results, we produced transgenic mice by microinjection of the minigenes into fertilized eggs of BALB/C albino mice (**Fig 4**). These transgenic founder mice have pigmented eyes and agouti-patterned brown hair as expected from the genetic background of BALB/C (*A/A*, $Tyrp1^b/Tyrp1^b$). Their phenotypes resemble mice carrying mg-Tyrs-J (Tanaka *et al*, 1990) or hg-Tyrs-J (Tanaka *et al*, 1992), the latter being a minigene composed of 1.2 kb human *tyrosinase* 5′ flanking sequence fused to Tyrs-J. Our results suggest that despite the overall sequence divergence, 5′ regulatory sequences from nonmammalian sources can interact with mouse *trans*-acting factors responsible for intrinsic

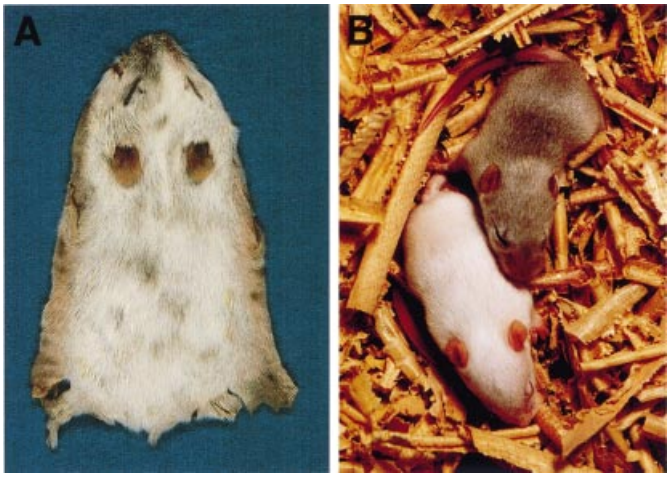

Figure 4. Expression of hybrid minigenes in transgenic mice. (*A*) The entire skin taken from a transgenic founder mouse carrying qg-Tyrs-J. Patches of brown colored-hair, which indicate chimeric distribution of cells with the integrated transgene, are evident. The photograph was taken after the founder mouse unfortunately died in an accident. (*B*) A transgenic founder mouse carrying tg-Tyrs-J (*left*) and a nontransgenic litter mate (*right*). The transgenic mouse has black eyes and agouti hair indicating early integration of the transgene.

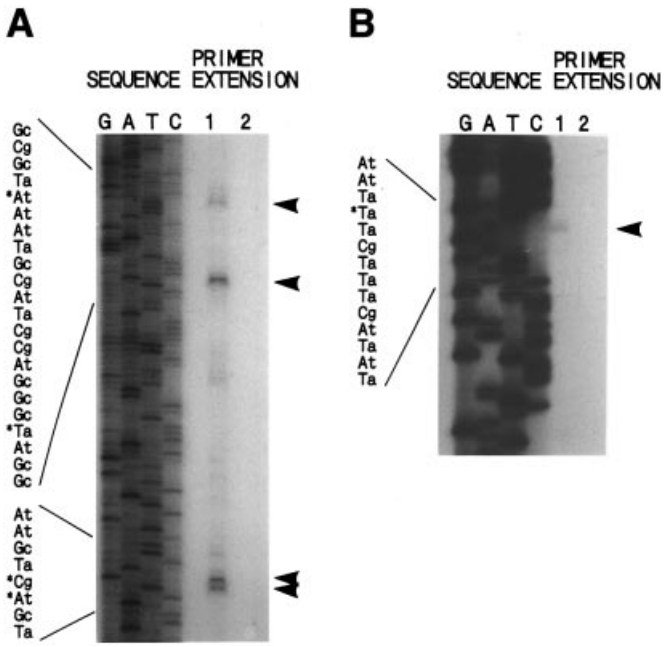

Figure 5. Mapping of transcription initiation sites. (*A*) Transcription initiation sites of the Japanese quail *tyrosinase* gene. The primer extension products obtained with 50 μg of RNA prepared from pigmented epithelium (*lane 1*) or control tRNA (*lane 2*) were separated on a sequencing gel. The products were run next to a corresponding sequencing ladder obtained using the genomic clone pQ12Xb8 and the same primer. (*B*) Transcription initiation sites of the snapping turtle *tyrosinase* gene. The extension products obtained with 20 μg of RNA prepared from whole eyes (*lane 1*) or control tRNA (*lane 2*) were run next to a sequencing ladder obtained using the genomic clone SB1-1 and the same primer. The sequence in lower case letters is determined directly from the sequencing gels, while the upper case letters represent the complementary sequence (sense strand). *Asterisks* and *arrowheads* correspond to positions of the extended products.

mouse *tyrosinase* gene expression. Specific transcription activation by the mouse or quail *tyrosinase* promoters was previously noted in cultured chicken albino melanocytes infected with avian retrovirus vectors carrying Tyrs-J driven by these promoters (Akiyama *et al*, 1994). Therefore, the presence of evolutionarily conserved regulatory sequence motifs was anticipated in these vertebrate *tyrosinase* promoters.

Conserved sequences near the initiation sites We determined the transcription start sites of the quail and turtle *tyrosinase* genes by primer extension experiments to compare precisely the sequences immediately 5′ to the potential *cis*-elements involved in the transcription initiation. The transcription start sites of the quail *tyrosinase* gene were mapped to positions –180, –166, –113, and –112 (**Fig 5*A***) and of the turtle gene to the T at –86 (**Fig 5*B***), while those of the mouse and human genes have been assigned to positions –79 or –80 (Kikuchi *et al*, 1989; Ruppert *et al*, 1988; Takeda *et al*, 1989; Yamamoto *et al*, 1989; Giebel *et al* 1991). Interestingly, there is a TATA-like sequence adjacent to CR2 in the human and mouse promoters, and transcription was shown to start within the CR2 (**Fig 6**). Bentley *et al* (1994) discussed the possibility that a combination of factors recognizing the TATA-like sequence and CR2 region plays an important role in *tyrosinase* expression. Two TATA-like sequences are also found at positions flanking the CR2 region in the frog promoter (Miura *et al*, 1995), so that similar factors may be operating in frog melanophores. In the quail and turtle promoters, however, there are no TATA-like sequences at the corresponding positions or around start points further downstream from the CR2 (**Figs 5, 6**). Studies of the chicken *tyrosinase* promoter revealed a similar situation (Ferguson and Kidson, 1996). It may be possible that in the case of quail and chicken *tyrosinase* genes, transcription initiation is regulated via the conserved SP1 motif and CR2 (Bentley *et al*, 1994; Ferguson and Kidson, 1996). If so, another scenario may be required to explain the expression of the turtle and medaka fish *tyrosinase* genes (Inagaki *et al*, 1998), both of which lack both the TATA-like sequences and the conserved SP1 motifs adjacent to CR2.

Other conserved sequences in the 5′ flanking regions Previously, we (Yamamoto *et al*, 1992) and Bentley *et al* (1994) found highly conserved short sequence motifs in the quail and turtle *tyrosinase* promoters. Besides the CR2 and SP1 motifs, there is an M-box/CR1 and two additional E-box motifs (not CATGTG) at similar positions between the two promoters (**Fig 6**), although one of the non-CATGTG E-box motifs is located 3′ to the transcription start sites in the turtle gene (**Fig 5**). Two non-CATGTG E-box motifs are found at similar positions in the chicken *tyrosinase* promoter as well.

Shibata *et al* (1992) identified a melanocyte-specific enhancer element, termed the tyrosinase distal element (TDE) (Yasumoto *et al*, 1994), located 1.85 kb upstream from the transcription start site in the human *tyrosinase* promoter. This 39 bp element contains at its center a bHLH factor-binding E-box motif, CATGTG, which also appears in the M-box and in the CR2 (Inr-E). Homologous motifs, which also have the CATGTG-type E-box sequences, were found unexpectedly 0.9 kb downstream compared with human TDE in each of the mouse, quail, chicken, and turtle *tyrosinase* promoters. As shown in **Fig 6**, conservation (28/46 identical) of those sequences is even more striking than in regions around the CR2 (15/26). Note that the homologous region extends toward 3′ from the E-box. Although some similarity was found between the human TDE sequence and the mouse tyrosinase element-1 (TE-1), which has been described as a putative melanocyte-specific enhancer element (Ponnazhagan and Kwon, 1992), the TE-1 sequence has no effect on the formation of melanoma nuclear protein-TDE complexes in band shift assays (Yasumoto *et al*, 1994), indicating that factor(s) interacting with the TE-1 cannot recognize the conserved TDE sequence. A database search failed to detect any consensus sequence motifs for known transcription factors in other parts of the TDE sequences. Thus, apart from the CATGTG motifs essential for TDE enhancer activity (Yasumoto *et al*, 1994), the

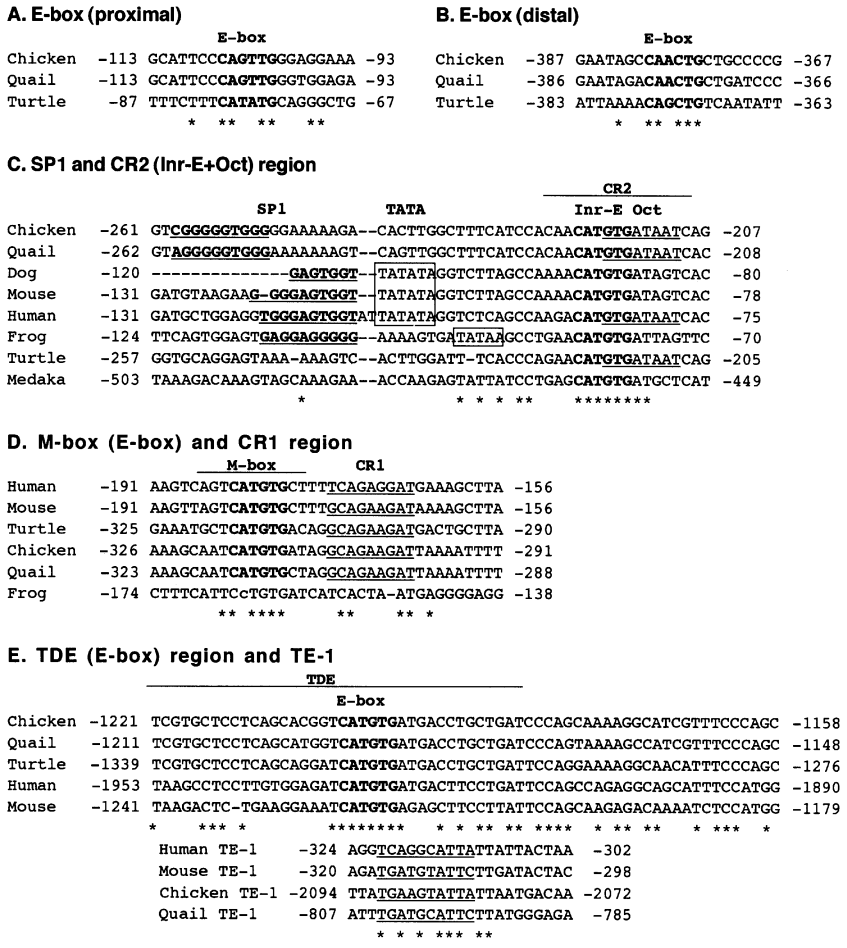

Figure 6. Conserved sequence motifs in the *tyrosinase* 5′ flanking regions. Nucleotide sequences of the Japanese quail and snapping turtle promoters were determined in this study and aligned with published sequences from human (Yasumoto *et al*, 1994), mouse (Yamamoto *et al*, 1989), chicken (Ferguson and Kidson, 1996), Japanese pond frog (Miura *et al*, 1995), and dog (Canis familiaris) (Tang *et al*, 1996). (*A*) E-box (proximal). These non-CATGTG type E-boxes are in the first exons except in the chicken promoter (Ferguson and Kidson, 1996). (*B*) E-box (distal). The E-box motif at this position may be responsible for determining the melanocyte specificity of the quail promoter (Akiyama *et al*, 1994). (*C*) SP1 and CR2 region. Nonconsensus SP1-binding motifs (underlined boldface) are conserved except in the snapping turtle promoter. TATA-like sequences are boxed. In the CR2 (overlined), CATGTG E-box motifs (Inr-E) are in boldface and degenerate octamer motifs are underlined. Up to the 5′ end of the published dog sequence is shown. (*D*) M-box and CR1 region. The CATGTG E-box motifs in the M-box (overlined) are shown in boldface while a mismatch found in the frog promoter is in lower case. The CR1 (underlined) is not found in the frog sequence. (*E*) TDE region and TE-1. The CATGTG E-box motifs in the TDE (overlined) are shown in boldface. Similarity was found between a part of the TDE sequence and TE-1 (underlined), which are located at similar positions in the mouse and human proximal promoters. Although the functional significance of the human and mouse TE-1 has been controversial (Bentley *et al* 1994; Ganss *et al*, 1994c), sequence motifs homologous to mammalian TE-1 are also found in the quail and chicken promoters. The sequences covering TE-1 are placed to maximize similarity with TDE sequences. Note that the position numbering is relative to the A (+1) for the first methionine because transcription starts from heterologous positions in the chicken and quail genes and the start site of the frog gene is not known. In each aligned sequence, *asterisks* indicate identical bases.

significance of the surrounding long conserved sequences is not known at this moment.

We also searched for additional sequences homologous to mouse *tyrosinase* regulatory elements in nonmammalian promoters. In the quail and chicken *tyrosinase* promoters, there is a sequence homologous (9/10 identity) to the TE-1 described above at bases –803 to –794 and at –2090 to –2081, respectively. In the quail promoter we found a sequence homologous (18/27) to hs–1 (Ganss *et al*, 1994b), a melanocyte specific enhancer located –12 kb upstream of the mouse *tyrosinase* gene, at bases –727 to –740.

Roles of conserved CATGTG motifs As described above, CATGTG motifs located at three different positions (**Fig 6**) in the mammalian *tyrosinase* promoters play important roles in the activation of melanocyte-specific transcription (Bentley *et al*, 1994; Ganss *et al*, 1994a; Yasumoto *et al*, 1994, 1997). Those studies also revealed that the bHLH-Leucine-Zipper (bHLH-LZ) microphthalmia-associated transcription factor (human: MITF, mouse: Mitf) can activate *tyrosinase* expression through these motifs in transient transfection assays (Hodgkinson *et al*, 1993; Hemesath *et al*, 1994; Steingrimsson *et al*, 1994; Yasumoto *et al*, 1994, 1995, 1997; Moore, 1995; Sato *et al*, 1997b). Although ubiquitous bHLH-LZ factors, such as USF and/or related proteins, also bind the same sites and can efficiently transactivate their expression in cultured cells, those proteins do not always dictate cell-type specificity as does MITF/Mitf (Bentley *et al*, 1994; Yasumoto *et al*, 1994; Aksan and Goding, 1998). Therefore, given that MITF/Mitf is the only bHLH-LZ factor preferentially expressed in melanocytes and that mice lacking functional Mitf do not produce any melanocytes or melanin, MITF/Mitf homologs and conserved CATGTG motifs in the *tyrosinase* promoters are most likely involved in the expression of the gene in avians

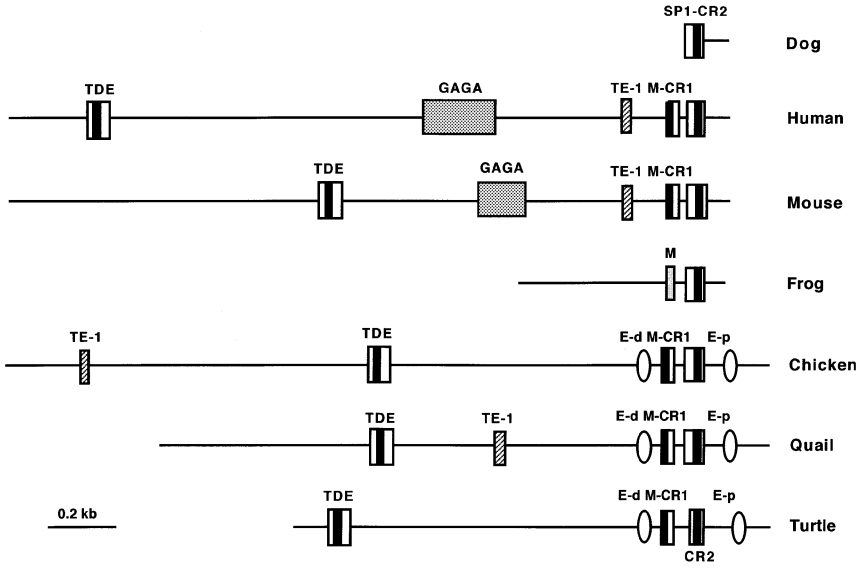

Figure 7. Localization of conserved sequence motifs in *tyrosinase* 5′ flanking regions. Schematic illustrations of 5′ flanking regions and parts of the first exons of *tyrosinase* genes from human, dog, mouse, chicken, quail, and snapping turtle are shown. The conserved sequence motifs, including CATGTG-type E-box motifs (closed box), are depicted in this figure. In the turtle promoter, the nonconsensus SP1-binding motif is not conserved. E-p, E-box (proximal); SP1/CR^2, SP1 and CR2 region; M/CR1, M-box and CR1 region; E-d, E-box (distal); GAGA, GA dinucleotide repeat; TDE, TDE region.

(Mochii *et al*, 1998a, b) and in reptiles. Our results from transfection and transgenic mouse experiments suggest that one transcription factor responsible for the activation of qg-Tyrs-J and tg-Tyrs-J minigenes is mouse Mitf and that the activation was mediated via multiple CATGTG motifs (**Figs 3**, **4**, **6**).

Observations that the relative roles of three CATGTG motifs differ in the context of the mouse and human *tyrosinase* promoters (Bentley *et al*, 1994; Yasumoto *et al*, 1995, 1997) suggest that sequences adjacent to those motifs have significant effects on the affinity and/or behavior of binding factors such as MITF/Mitf. Eisen *et al* (1995) showed that repression of the human *tyrosinase* promoter activity by Brn-2, a POU domain transcription factor, correlates with Brn-2 binding to the initiation site (CR1). The Inr-E overlaps with the Brn-2 binding octamer element, GTGATAAT (**Fig 6**), in CR1 in a mutually exclusive fashion with USF *in vitro* and with Brn-2 expression preventing activation of the promoter by Mitf *in vivo*. It must be noted that this putative Brn-2 binding octamer sequence is not conserved in the frog and medaka fish or even in mouse and dog *tyrosinase* promoters.

Although promoter mutagenesis experiments have shown that the M-box is necessary for full activation of mammalian *tyrosinase* promoters (Bentley *et al*, 1994; Ganss *et al*, 1994c), it does not seem to be an essential *cis*-element necessary to drive expression in melanin-producing pigment cells. For example, the mouse *tyrosinase* promoter fragment containing the Inr-E but not the M-box confers melanocyte specificity in cultured cells (Bentley *et al*, 1994; Yasumoto *et al*, 1995), and transgenes driven by the mouse *tyrosinase* promoter (either 2.2 or 0.27 kb of the 5′ flanking region) with a mutated M-box are still capable of inducing pigmentation in eyes and hairs in transgenic mice (Ganss *et al*, 1994c). Yasumoto *et al* (1997) demonstrated that the affinity of MITF to the human *tyrosinase* M-box is lower than to the Inr-E and TDE, and that each affinity *in vitro* correlates with the promoter activity *in vivo*. More precisely, Aksan and Goding (1998) showed, based on evidence obtained by *in vitro* binding and yeast one hybrid assays, that a CATGTG motif should be flanked by a T residue for the efficient Mitf binding (Aksan and Goding, 1998). Mitf binds either TCATGTG, CATGTGA, or preferably TCATGTGA, which is found in the M-boxes of turtle and chicken *tyrosinase* promoters and in all known TDE (**Fig 6**). When we compared the sequences corresponding to the M-box region in *tyrosinase* genes from other vertebrates (Amphibia and Pisces), the core sequence of the M-box, CATGTG, is replaced by CCTGTG in the frog gene (Miura *et al*, 1995), a sequence that should not bind bHLH proteins. This is surprising since, on the whole, the frog M-box region is highly conserved: the sequence is identical in nine of 11 nucleotides to the chicken M-box (**Fig 6**). Furthermore, no intact M-box has been found in the medaka fish *tyrosinase* promoter (Inagaki *et al*, 1998). At present, *cis*-elements required for the pigment cell-specific expression of the frog and fish *tyrosinase* genes have not been examined in detail; however, considering that the upstream sequences of 748 bp for the frog promoter and 3.0 kb for the medaka fish promoter regions are sufficient for *tyrosinase* expression (Miura *et al*, 1995; Inagaki *et al*, 1998), and that Inr-E, which has a 5′ flanking T residue essential for the ability of Mitf to bind DNA (Aksan and Goding, 1998), appears to be the single evolutionarily conserved sequence, we expect that this element also plays an important role for regulating *tyrosinase* expression in nonmammalian vertebratesn.

Evolution of tyrosinase promoters of vertebrates **Figure 7** depicts the positions of conserved sequence motifs in the proximal promoters of *tyrosinase* genes from amniotes. These promoters are characterized by the presence of three highly conserved regions (TDE, M-box/CR1, and CR2) each containing a CATGTG E-box. The distance between the M-box and the CR2 is constant (82–96 bp), while the position of the TDE relative to the CR2 differs significantly between promoters. Yasumoto *et al* (1994) have shown that the TDE can exert its enhancer activity from 1.8 kb upstream in the human promoter, and that the orientation of the TDE does not affect its activity in transient transfection assays. Therefore, it is possible that, except for the position of the CR2, which may be utilized for transcription initiation, the precise order and location of other conserved motifs has little functional significance and reflects the organization of an ancestral *tyrosinase* promoter. If this is the case, the distance between the CR2 and the first methionine seems to have widened in the lineage giving rise to reptiles and birds, and the long GA repeat, which is characteristic of mammalian promoters, has been inserted (Kominami *et al*, 1983). Alternatively, the multiplication of the GA dinucleotide occurred after the divergence of the mammalian lineage from the clade comprising birds and reptiles.

While the core promoter (CR2) is conserved in hitherto cloned vertebrate *tyrosinase* genes, the TDE and M-box are not found in the reported promoter sequences of medaka fish and frogs. This raises the possibility that the M-box and the TDE were acquired or evolved later in the lineage leading to present-day amniotes after the divergence with amphibians. We note the presence of a M-box-like sequence in the frog gene, however, so that it is also possible that the origin of the M-box is in fact much older. Furthermore, because the M-box is found in the proximal promoters of other members of mammalian tyrosinase family genes (*Tyrp*1 and *Dct*) and of other vertebrate pigment cell-specific

genes, such as *QNR*-71 (Turque *et al*, 1996) and *melastatin* (Hunter *et al*, 1998), we should bear in mind that it is relatively easy to gain a promoter element of about 10 bp. Further investigation into the structures of *tyrosinase* genes from various vertebrates, particularly from recently isolated fishes and closely related ancestral chordates (Sato *et al*, 1997a), is clearly needed to understand how vertebrate *tyrosinase* promoters were molded during the evolution of vertebrates. In addition, characterization of MITF/Mitf homologs isolated from avians (Mochii *et al*, 1998a, b) and other nonmammalian vertebrates (our unpublished data) will provide information needed to determine the importance of evolutionarily conserved CATGTG motifs in the process.

In summary, our data indicate that, despite overall sequence divergence, 5′ flanking sequences of the quail and turtle *tyrosinase* genes are capable of activating transcription of a fused mouse tyrosinase cDNA in cultured mouse albino melanocytes. This transgenic procedure using a cultured differentiated type of cell, such as a melanocyte, that is common to all vertebrates, as the host for a cDNA from one class of vertebrates (e.g., mammals: mouse) and regulatory sequences from another species (e.g., reptiles: turtle; birds: quail) provides a convenient setting to determine phylogenetic, structural, and functional changes among vertebrate melanocytes at the genetic and metabolic levels. The procedure may be utilized to evaluate comparable phylogenetic changes in many other types of specialized cells in vertebrates. To verify the results obtained in cultured cells, we showed that the 5′ flanking sequences from quail or turtle genes can direct expression of the fused mouse cDNA in transgenic mice. Finally, by comparing 5′ flanking sequences we identified several potential regulatory sequence motifs conserved in vertebrate *tyrosinase* promoters.

This study was started when H.M., M.T., S.S., and H.Y. were in Dr. T. Takeuchi's laboratory at Tohoku University. The authors wish to dedicate this paper to the late Dr. Takeuchi. We thank Dr. Colin Goding for helpful discussions, Dr. Walter Quevedo Jr. for reading an earlier version of the manuscript and for his encouragement, and Dr. Hiromichi Yonekawa for his kind supply of C57BL/6 J-Tyr^{c-2J}/Tyr^{c-2J} mice. We also thank Kosuke Nasu who has contributed to an early phase of this project. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan and the NIG Cooperative Research Programs to H.Y. (32-′92 and 52-′93), and supported in part by a Research Fund from the Naito Foundation to H.Y.

The nucleotide sequences of quail and turtle tyrosinase cDNAs have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases as follows: Quail tyrosinase cDNA, Accession: AB024278; Turtle tyrosinase cDNA, Accession: AB024280

REFERENCES

Akiyama T, Whitaker B, Federspiel M, Hughes SH, Yamamoto H, Takeuchi T, Brumbaugh J: Tissue-specific expression of mouse tyrosinase gene in cultured chicken cells. *Exp Cell Res* 214:154–162, 1994

Aksan I, Goding CR: Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-Box elements In vitro and In vivo. *Mol Cell Biol* 18:6930–6938, 1998

Beermann F, Ruppert S, Hummler E, Bosch FX, Muller G, Ruther U, Schutz G: Rescue of the albino phenotype by introduction of a functional tyrosinase gene into mice. *EMBO J* 9:2819–2826, 1990

Bentley NJ, Eisen T, Goding CR: Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol Cell Biol* 14:7996–8006, 1994

Budd PS, Jackson IJ: Structure of the mouse tyrosinase-related protein-2/dopachrome tautomerase (Tyrp2/Dct) gene and sequence of two novel slaty alleles. *Genomics* 29:35–43, 1995

Eisen T, Easty DJ, Bennett DC, Goding CR: The POU domain transcription factor Brn-2. elevated expression in malignant melanoma and regulation of melanocyte-specific gene expression. *Oncogene* 11:2157–2164, 1995

Felsenstein J: Numerical methods for inferring evolutionary trees. *Q Rev Biol* 57:379–404, 1982

Ferguson CA, Kidson SH: Characteristic sequences in the promoter region of the chicken tyrosinase-encoding gene. *Gene* 169:191–195, 1996

Ganss R, Schutz G, Beermann F: The mouse tyrosinase gene. Promoter modulation by positive and negative regulatory elements. *J Biol Chem* 269:29808–29816, 1994a

Ganss R, Montoliu L, Monaghan AP, Schütz G: A cell-specific enhancer far upstream of the mouse tyrosinase gene confers high level and copy number-related expression in transgenic mice. *EMBO J* 13:3083–3093, 1994b

Ganss R, Schmidt A, Schutz G, Beermann F: Analysis of the mouse tyrosinase promoter in vitro and in vivo. *Pigment Cell Res* 7:275–278, 1994c

van Gelder CW, Flurkey WH, Wichers HJ: Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* 45:1309–1323, 1997

Giebel LB, Strunk KM, Spritz RA: Organization and nucleotide sequences of the human tyrosinase gene and a truncated tyrosine-related segment. *Genomics* 9:435–445, 1991

Hearing VJ, Tsukamoto K: Enzymatic control of pigmentation in mammals. *FASEB J* 5:2902–2909, 1991

von Heijne G: A new method for predicting signal sequence cleavage sites. *Nucl Acids Res* 14:4683–4690, 1986

Hemesath TJ, Steingrimsson E, McGill G, *et al*: Microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. *Genes Dev* 8:2770–2780, 1994

Hodgkinson CA, Moore KJ, Nakayama A, Stingrimsson E, Copeland NG, Jenkins NA, Arnheiter H: Mutation at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix zipper protein. *Cell* 74:395–404, 1993

Hunter JJ, Shao J, Smutko JS, *et al*: Chromosomal localization and genomic characterization of the mouse melastatin gene (Mlsn1). *Genomics* 54:116–123, 1998

Ina Y: *Molecular Evolutionary Analysis of Hepatitis B Viruses and its Application to the Design of the Anti-Virus Drugs*. Master thesis, Shizuoka University, Shizuoka, Japan, 1990

Inagaki H, Bessho Y, Koga A, Hori H: Expression of the tyrosinase-encoding gene in a colorless melanophore mutant of the medaka fish, *Oryzias latipes*. *Gene* 150:319–324, 1994

Inagaki H, Koga A, Bessho Y, Hori H: The tyrosinase gene from medakafish: transgenic expression rescues albino mutation. *Pigment Cell Res* 11:283–290, 1998

Jackson IJ, Chambers DM, Budd PS, Johnson R: The tyrosinase-related protein-1 gene has a structure and promoter sequence very different from tyrosinase. *Nucl Acids Res* 19:3799–3804, 1991

Jackson IJ, Chambers DM, Tsukamoto K, Copeland NG, Gilbert DJ, Jenkins NA, Hearing V: A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse slaty locus. *EMBO J* 11:527–535, 1992

Kikuchi H, Miura H, Yamamoto H, Takeuchi T, Dei T, Watanabe M: Characteristic sequences in the upstream region of the human tyrosinase gene. *Biochim Biophys Acta* 1009:283–286, 1989

Klein P, Kanehisa M, DeLisi C: The detection and classification of membrane-spanning proteins. *Biochim Biophys Acta* 815:468–476, 1985

Kluppel M, Beermann F, Ruppert S, Schmid E, Hummler E, Schutz G: The mouse tyrosinase promoter is sufficient for expression in melanocytes and in the pigmented epithelium of the retina. *Proc Natl Acad Sci USA* 88:3777–3781, 1991

Kominami R, Muramatsu M, Moriwaki K: A mouse type 2 Alu sequence (M2) is mobile in the genome. *Nature* 301:87–89, 1983

Kwon BS, Haq AK, Pomerantz SH, Halaban R: Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc Natl Acad Sci USA* 84:7473–7477, 1987

Kwon BS, Wakulchik M, Haq AK, Halaban R, Kestler D: Sequence analysis of mouse tyrosinase cDNA and the effect of melanotropin on its gene expression. *Biochem Biophys Res Commun* 153:1301–1309, 1988

van der Linde-Sipman JS, de Wit MM, van Garderen E, Molenbeek RF, van der Velde-Zimmermann D, de Weger RA: Cutaneous malignant melanomas in 57 cats. identification of (amelanotic) signet-ring and balloon cell types and verification of their origin by immunohistochemistry, electron microscopy, and in situ hybridization. *Vet Pathol* 34:31–38, 1997

Lowings P, Yavuzer U, Goding CR: Positive and negative elements regulate a melanocyte-specific promoter. *Mol Cell Biol* 12:3653–3662, 1992

McKnight SL, Kingsbury R: Transcriptional control signals of a eukaryotic protein-coding gene. *Science* 217:316–324, 1982

McLean IW, Nakane PK: Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J Histochem Cytochem* 22:1077–1083, 1974

Miura I, Okumoto H, Makino K, Nakata A, Nishioka M: Analysis of the tyrosinase gene of the Japanese pond frog, Rana nigromaculata: cloning and nucleotide sequence of the genomic DNA containing the tyrosinase gene and its flanking regions. *Jpn J Genet* 70:79–92, 1995

Mochii M, Iio A, Yamamoto H, Takeuchi T, Eguchi G: Isolation and characterization of a chicken tyrosinase cDNA. *Pigment Cell Res* 5:162–167, 1992

Mochii M, Ono T, Matsubara Y, Eguchi G: Spontaneous transdifferentiation of quail pigmented epithelial cell is accompanied by a mutation in the Mitf gene. *Dev Biol* 196:145–159, 1998a

Mochii M, Mazaki Y, Mizuno N, Hayashi H, Eguchi G: Role of Mitf in differentiation and transdifferentiation of chicken pigmented epithelial cell. *Dev Biol* 193:47–62, 1998b

Moore KJ: Insight into the microphthalmia gene. *Trends Genet* 11:442–448, 1995

Müller G, Ruppert S, Schmid E, Schütz G: Functional analysis of alternatively spliced tyrosinase gene transcripts. *EMBO J* 7:2723–2730, 1988

Nei M: *Molecular Evolutionary Genetics*. New York: Columbia University Press, 1987

Nishioka K: Particulate tyrosinase of human malignant melanoma. Solubilization, purification following trypsin treatment, and characterization. *Eur J Biochem* 85:137–146, 1978

Oetting WS, King RA: Molecular basis of oculocutaneous albinism. *J Invest Dermatol* 103:131S–136S, 1994a

Oetting WS, King RA: Analysis of tyrosinase mutations associated with tyrosinase-related oculocutaneous albinism (OCA1). *Pigment Cell Res* 7:285–290, 1994b

Oetting WS, Fryer JP, King RA: Mutations of the human tyrosinase gene associated with tyrosinase related oculocutaneous albinism (OCA1). Online. *Hum Mutat* 12:433–434, 1998

Ohkura T, Yamashita K, Mishima Y, Kobata A: Purification of hamster melanoma tyrosinases and structural studies of their asparagine-linked sugar chains. *Arch Biochem Biophys* 235:63–77, 1984

Ponnazhagan S, Kwon BS: A cis-acting element involved in mouse tyrosinase gene expression and partial purification of its binding protein. *Pigment Cell Res* 5:155–161, 1992

Porter SD, Meyer CJ: A distal tyrosinase upstream element stimulates gene expression in neural-crest-derived melanocytes of transgenic mice: position-independent and mosaic expression. *Development* 120:2103–2111, 1994

Ruppert S, Müller G, Kwon B, Schütz G: Multiple transcripts of the mouse tyrosinasegene are generated by alternative splicing. *EMBO J* 7:2715–2722, 1988

Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425, 1987

Sato C, Ito S, Takeuchi T: Establishment of a mouse melanocyte clone which synthesizes both eumelanin and pheomelanin. *Cell Struct Funct* 10:421–425, 1985

Sato S, Masuya H, Numakunai T, *et al*: An ascidian tyrosinase gene. its unique structure and expression in the developing brain. *Dev Dynamics* 208:363–374, 1997a

Sato S, Roberts K, Gambino G, Cook A, Kouzarides T, Goding CR: CBP/p300 as a co-factor for the Microphthalmia transcription factor. *Oncogene* 14:3083–3092, 1997b

Shibahara S, Okinaga S, Tomita Y, Takeda A, Yamamoto H, Sato M, Takeuchi T: A point mutation in the tyrosinase gene of BALB/c albino mouse causing the cysteine to serine substitution at position 85. *Eur J Biochem* 189:455–461, 1990

Shibahara S, Taguchi H, Muller RM, Shibata K, Cohen T, Tomita Y, Tagami H: Structural organization of the pigment cell-specific gene located at the brown locus in mouse. Its promoter activity and alternatively spliced transcript. *J Biol Chem* 266:15895–15901, 1991

Shibahara S, Tomita Y, Sakakura T, Nager C, Chaudhuri B, Muller R: Cloning and expression of cDNA encoding mouse tyrosinase. *Nucl Acids Res* 14: 2413–2427, 1986

Shibahara S, Yasumoto K, Takahashi K: Genetic regulation of the pigment cell. In: *The Pigmentary System. Physiology and Pathophysiology* (JJ Norlund, RE Boissy, VJ Hearing, RA King, J-P Ortone, eds). New York: Oxford University Press, 1998, pp. 251–273

Shibahara S, Tomita Y, Tagami H, Muller R, Cohen T: Molecular basis for the heterogeneity of human tyrosinase. *Tohoku J Exp Med* 156:403–414, 1988

Shibata K, Muraosa Y, Tomita Y, Tagami H, Shibahara S: Identification of a cis-acting element that enhances the pigment cell- specific expression of the human tyrosinase gene. *J Biol Chem* 267:20584–20588, 1992

Sokal RR, Michener CD: A statistical method for evaluating systematic relationships. *University Kansas Sci Bull* 28:1409–1438, 1985

Steingrimsson E, Moore KJ, Lamoreux ML, *et al*: Molecular basis of mouse microphthalmia (mi) mutations helps explain their developmental and phenotypic consequences. *Nat Genet* 8:256–263, 1994

Takase M, Miura I, Nakata A, Takeuchi T, Nishioka M: Cloning and sequencing of the cDNA encoding tyrosinase of the Japanese pond frog, Rana nigromaculata. *Gene* 121:359–363, 1992

Takeda A, Tomita Y, Okinaga S, Tagami H, Shibahara S: Functional analysis of the cDNA encoding human tyrosinase precursor. *Biochem Biophys Res Commun* 162:984–990, 1989

Tanaka S, Yamamoto H, Takeuchi S, Takeuchi T: Melanization in albino mice transformed by introducing cloned mouse tyrosinase gene. *Development* 108:223–227, 1990

Tanaka M, Tanaka S, Miura H, Yamamoto H, Kikuchi H, Takeuchi T: Conserved regulatory mechanisms of tyrosinase genes in mice and humans. *Pigment Cell Res* 5:304–311, 1992

Tang Q, Williams RW, Hogan D, Valentine V, Goldowitz D: Canis familiaris tyrosinase (tyr) gene, exon 1, partial cds. DDBJ/EMBL/GenBank nucleotide sequence databases (accession number U42219), 1996

Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res* 22:4673–4680, 1994

Turque N, Denhez F, Martin P, *et al*: Characterization of a new melanocyte-specific gene (QNR-71) expressed in v-myc-transformed quail neuroretina. *EMBO J* 15:3338–3350, 1996

Yamamoto H, Takeuchi S, Kudo T, Makino K, Nakata A, Shinoda T, Takeuchi T: Cloning and sequencing of mouse tyrosinase cDNA. *Jpn J Genet* 62:271–274, 1987

Yamamoto H, Kudo T, Masuko N, *et al*: Phylogeny of regulatory regions of vertebrate tyrosinase genes. *Pigment Cell Res* 5:284–294, 1992

Yamamoto H, Takeuchi S, Kudo T, Sato C, Takeuchi T: Melanin production in cultured albino melanocytes transfected with mouse tyrosinase cDNA. *Jpn J Genet* 66:121–135, 1989

Yasumoto K, Yokoyama K, Shibata K, Tomita Y, Shibahara S: Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol Cell Biol* 14:8058–8070, 1994

Yasumoto K, Mahalingam H, Suzuki H, Yoshizawa M, Yokoyama K, Shibahara S: Transcriptional activation of the melanocyte-specific genes by the human homolog of the mouse Microphthalmia protein. *J Biochem* 118:874–881, 1995

Yasumoto K, Yokoyama K, Takahashi K, Tomita Y, Shibahara S: Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. *J Biol Chem* 272:503–509, 1997

Yokoyama T, Silversides DW, Waymire KG, Kwon BS, Takeuchi T, Overbeek PA: Conserved cysteine to serine mutation in tyrosinase is responsible for the classical albino mutation in laboratory mice. *Nucl Acids Res* 18:7293–7298, 1990

Yokoyama K, Yasumoto K, Suzuki H, Shibahara S: Cloning of the human DOPAchrome tautomerase/tyrosinase-related protein 2 gene and identification of two regulatory regions required for its pigment cell-specific expression. *J Biol Chem* 269:27080–27087, 1994