Cloning and expression of cDNA encoding mouse tyrosinase

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ABSTRACT

We have isolated a pigment cell-specific cDNA clone from a B16 mouse melanoma cDNA library by differential hybridization. The mRNA of isolated cDNA is highly expressed in B16 melanoma cells and in black mouse (C57BL/6) skin, but is not detectable in mouse neuroblastoma cells nor in K1735 mouse amelanotic melanoma cells. The protein sequence deduced from the nucleotide sequence of the cloned cDNA shows significant similarity to the entire region of *Neurospora* tyrosinase. To know the identity of cDNA, we transfected K1735 amelanotic melanoma and COS-7 cells with the cDNA carried in a simian virus 40 vector (pKCRH2). We confirmed that the isolated cDNA encodes mouse tyrosinase by immunofluorescence staining of transfected cells using two different anti-T₄-tyrosinase monoclonal antibodies. Tyrosinase is composed of 513 amino acids with a molecular weight of 57,872 excluding a hydrophobic signal peptide of 24 amino acids.

INTRODUCTION

Pigmentation is one of most demonstrable phenotypes in animal kingdom. Melanins are principal pigments found in all living organisms including bacteria, fungi, plants, and animals. Mammalian tyrosinase (EC 1.14.18.1) is a copper-containing enzyme (1,2) that is essential for melanin biosynthesis in pigment cells. The reaction pathway of melanin biosynthesis was determined about sixty years ago (reviewed in ref. 3) and tyrosinase catalyzes the first two reactions of this pathway: the conversion of tyrosine to dihydroxyphenylalanine (dopa), and dopa to dopaquinone (reviewed in ref. 4). Dopaquinone is subsequently converted to dopachrome by a rapid autooxidation. Until recently, melanin was believed to be formed spontaneously from dopaquinone. However, recent studies indicated that the subsequent reaction from dopachrome to 5,6-dihydroxyindole is accelerated by the dopachrome conversion factor (5,6) found in both pigmented and

amelanotic melanoma cells, and tyrosinase also catalyzes the conversion of 5,6-dihydroxyindole to melanochrome (7). This last reaction is also regulated negatively by the indole blocking factor (6), whose inhibitory effect can be removed by treatment with melanocyte stimulating hormone. Thus, the regulation of melanin biosynthesis is more complicated than previously believed.

We are interested in the cDNA clone for mouse tyrosinase as a probe to study the regulation of melanin biosynthesis for the following reasons. Melanin is specifically produced by melanocytes, which are derived from neural crest cells except for retinal melanocytes (reviewed in ref. 8). All pigment found in mouse is produced by melanocytes and various mutants affecting coat and eye colour are known (9). As the melanoblasts (prospective melanocytes) do not contain pigment (10), tyrosinase cDNA clone will be a suitable molecular probe to study the differentiation of melanoblasts in various mouse strains during fetal development. The regulatory mechanisms of melanin biosynthesis are clinically significant, because the intermediate substances in melanin formation are toxic to pigment cells (11) and sometimes destroy the cultured malignant melanoma cells (12,13). It may be possible to develop the unique chemotherapy against malignant melanoma (reviewed in ref. 14). To establish the rationale for chemotherapy, we may need to clarify the structure of tyrosinase and other factors involved in melanin biosynthesis. Furthermore, tyrosinase cDNA clone will be a useful tool to analyze the molecular basis of albinism.

Accordingly, as the first step we isolated the cDNA clone for mouse tyrosinase, a key enzyme for melanin biosynthesis and deduced the primary structure of tyrosinase.

MATERIALS AND METHODS

Preparation of RNA

Total RNA was prepared (15) from B16 mouse melanoma cells, mouse neuroblastoma cells (NB₂A), K1735 mouse amelanotic melanoma cells, and black mouse (C57BL/6) skin of 5-10 days after birth. Poly(A)⁺RNA was isolated by oligo(dT)-cellulose chromatography (16).

Isolation of Pigment Cell-Specific cDNA Clones

A cDNA library was constructed by the method of Okayama and Berg (17) using 2 μ g of B16 melanoma poly(A)⁺RNA. *E. coli* HB101 was used for transformation. To prevent the fusion of each colonies, we spread about 500 colonies per nitrocellulose filter (82 mm diameter). Two sets of replica were made and screened with ³²P-labelled cDNA transcribed from B16 melanoma poly(A)⁺RNA or from neuroblastoma poly(A)⁺RNA by random priming method (18). The hybridization mixture contained 10⁶ cpm/ml of cDNA and was incubated at 60°C for 24 hr. We chose all candidate clones which hybridized with B16 melanoma cDNA probe, but which did not hybridize with neuroblastoma cDNA probe, and then inoculated onto three sets of nitrocellulose filters. Two sets of filters were hybridized again with B16 melanoma cDNA or with neuroblastoma cDNA to confirm the above criteria. The third set of filters was hybridized with black skin cDNA prepared as above. In this way, we screened about 10,000 transformants and isolated four independent cDNA clones which hybridized with B16 melanoma cDNA, but which did not hybridize with neuroblastoma cDNA. Two of them also hybridized with black skin cDNA. Plasmid DNA was isolated and used for further analysis. The clone, pMT5, is one of two clones thus obtained. Using ³²P-labelled DdeI-DdeI fragment (nucleotide residues 107-275, see Fig. 4) derived from pMT5, we screened the original B16 melanoma cDNA library (about 10,000 transformants) and obtained the full-length cDNA clone, pMT4. The nucleotide numbers shown within parenthesis indicate the 5'-terminal nucleotide generated by cleavage. All cloned DNA probes used in this study were labelled with [α -³²P]-dCTP by the method of Feinberg and Vogelstein (19).

RNA Blot Analysis

RNA was subjected to gel electrophoresis (20) using a minigel, transferred to a nitrocellulose filter, and hybridized with ³²P-labelled PvuII-PvuII fragment (nucleotide residues 947-1673, see also Fig. 4) derived from pMT5. The size markers were mouse rRNA and *E. coli* rRNA (21).

Construction of Expression Plasmids Carrying Tyrosinase cDNA

The expression vector, pKCRH2 (22), was linearized and both single stranded ends were filled in as described (20). The

resulting blunt-ends were used for ligation with two fragments prepared from pMT4, PvuII-AvaI fragment (nucleotide residues -164 to 602) and AvaI-EcoRV fragment (nucleotide residues 602 to 1831), which cover the entire protein-coding region (see Figure 3). Recombinant plasmids were cloned in *E. coli* HB101 and plasmids carrying both fragments in correct order with two different orientation were isolated. One plasmid, pKCRH2MT thus obtained, carries insert in the same orientation with respect to simian virus 40(SV40) early gene transcription. The similar structures of pKCRH2 carrying other cDNAs were shown elsewhere (20,22).

Transfection of K1735 Amelanotic Melanoma and COS-7 Cells and Indirect Immunofluorescence Staining of Transfected Cells

K1735 amelanotic melanoma cells (23,24) were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum, glutamine, nonessential amino acids and antibiotics. COS-7 cells (25) were maintained as described (20). About 70% confluent cells, seeded in Lab-Tek Tissue Culture Chamber/Slides (the size of chamber, 9x20 mm), were fed with fresh medium 4h before addition of expression plasmid DNA. Plasmid DNA (1 μ g/chamber) was used to transfect cells by the calcium phosphate method (26,27). After 45h incubation, the cells were fixed and then examined for expression of tyrosinase by indirect immunofluorescence staining using two specific anti-T₄-tyrosinase monoclonal antibodies, TMH-1 and TMH-2 (28,29) according to the method described (29). These two monoclonal antibodies recognize different epitopes of T₄-tyrosinase (29). We transfected cells with one plasmid at least in four chambers and two of them were used as a control for background fluorescence. We examined five areas of each chamber (about 1,000 cells per area) and counted the fluorescence-positive cells. Therefore, we routinely examined at least 10,000 cells transfected with pKCRH2MT to calculate the percentage of immunofluorescence-positive cells.

DNA Sequence Determination

DNA fragments were 5'-end labelled with [γ -³²P]-ATP and polynucleotide kinase, and sequenced by the method of Maxam and Gilbert (30).

Computer Analysis

Optimal alignment of *Neurospora* and mouse tyrosinase was made and its statistical significance was evaluated as described (31). Each sequence was randomized and then aligned to each other. This procedure was repeated 100 times and hence the mean ($\bar{x}$) and standard deviation (s.d.) were obtained. The alignment score (A), defined as $A = (s - \bar{x})/s.d.$, was then calculated, where s indicates the actual score.

RESULTS AND DISCUSSION

Isolation of Pigment Cell-Specific cDNA Clones

As no specific probe was available to isolate a cDNA clone for mammalian tyrosinase, we used the differential hybridization. Our cloning strategy (Fig. 1A) was based on the following assumptions : 1) B16 mouse melanoma cells are abundant in tyrosinase mRNA; 2) mouse neuroblastoma cells (NB₂A), also derived from the neural crest, contain no or only a few copies of tyrosinase mRNA per cell; 3) black mouse (C57BL/6) skin is also abundant in tyrosinase mRNA. Accordingly we constructed a cDNA library in the Okayama-Berg vector (17) using poly(A)⁺RNA prepared from B16 melanoma cells. By comparing the patterns of hybridization in sets of replica filters, we identified four clones which hybridized with B16 melanoma cDNA, but which did not hybridize with neuroblastoma cDNA. Then, we hybridized these four clones with black skin ³²P-cDNA and obtained two independent positive clones. We confirmed by Northern blot analysis that these two clones are consistent with our assumptions. One clone, designated pMT5, had an insert of about 1800 nucleotides including poly(dG) tail and poly(dA) tail and hybridized with mRNA of about 3000 nucleotides long (Fig. 1B). In this Northern blot analysis, we used 100 ng of B16 melanoma RNA, 3 µg of black skin RNA and 3 µg of neuroblastoma poly(A)⁺RNA. Therefore, it is apparent that B16 melanoma cells are highly abundant in mRNA for pMT5, and neuroblastoma cells contain no detectable levels of this mRNA. Furthermore, K1735 amelanotic melanoma cells (23,24,29), a nonpigmented variant of mouse melanoma, contain no detectable levels of hybridizable mRNA (Fig. 1C), suggesting that pMT5 encodes the protein related to pigmentation. As the mRNA for

Fig. 1. Isolation of pigment cell-specific cDNA

A. cDNA cloning strategy. **B.** and **C.** Northern blot analysis. Lanes in **B** indicated contain 100 ng of B16 melanoma RNA, 3 µg of black skin RNA and 3 µg of neuroblastoma poly(A)⁺RNA. Lanes in **C** contain 3 µg of black skin RNA and 3 µg of K1735 amelanotic melanoma poly(A)⁺RNA. The size markers were mouse rRNA and *E. coli* rRNA.

pMT5 is long enough to encode tyrosinase, whose reported molecular weight is within the range of about 57,000 - 70,000 (2,32-34), we characterized pMT5. In order to isolate full-length cDNA clone, we screened the original B16 melanoma cDNA library with Dde I-Dde I fragment (nucleotide residues 107-275, see Fig. 4) derived from pMT5. From 10,000 transformants, we obtained the full-length cDNA clone, pMT4.

Transient Expression of Tyrosinase cDNA in K1735 Amelanotic Melanoma Cells and COS-7 Cells

As the amino acid sequence of tyrosinase is not known, we wanted to confirm that the isolated cDNA actually encodes tyrosinase. Consequently, we transfected K1735 amelanotic melanoma cells and COS-7 cells (monkey kidney cells) with the expression vector pKCRH2 (22) containing entire protein-coding region of cloned cDNA and examined the transient expression of tyrosinase in transfected cells by using two different anti-T₄-tyrosinase monoclonal antibodies, TMH-1 and TMH-2 (28,29). The

Fig. 2. Transient expression of tyrosinase cDNA in K1735 amelanotic melanoma cells and COS-7 cells

Immunofluorescence-positive K1735 amelanotic melanoma cell (A and B) and COS-7 cell (E and F) are shown under phase-contrast (A and E) and fluorescence (B and F) microscope. The K1735 amelanotic melanoma cells transfected with pKCRH2 are also shown (C and D). The examples shown were stained by using a monoclonal antibody, TMH-1.

expression vector, pKCRH2, contained the SV40 early gene promoter, donor and acceptor splice sites derived from the rabbit β -globin gene, and polyadenylation sites derived from the rabbit β -globin gene and the SV40 early gene (22). K1735 amelanotic melanoma cells were originally produced by ultraviolet exposure (23) and they subsequently lost the ability of pigmentation (28,29). We mainly used K1735 cells for transfection because of the following reasons: 1) the presence of melanosomes, pigment granules, is probably essential for the complete posttranslational modifications of tyrosinase, as tyrosinase is closely associated with melanosomes during maturation of tyrosinase (35); 2) specific monoclonal antibodies, TMH-1 and TMH-2, can recognize only mature form of tyrosinase (so-called T_4 tyrosinase) (28,29); 3) these monoclonal antibodies did not react

Fig. 3. Restriction map and sequencing strategy for tyrosinase cDNA

The restriction map shows relevant restriction endonuclease sites. The poly(dA) tail and poly(dG) tail are not included. The protein-coding region is indicated by an open box and a putative signal peptide is indicated by a closed box. The direction and extent of sequence determinations are shown by horizontal arrows under solid lines indicating each clone used. The sites of 5'-end labelling are indicated by short vertical lines at the end of arrows and the slash marks at the end of arrows indicate the *Pst*I sites of 5'-end labelling located on the vector DNA (17). The scale at the top indicates the nucleotide number (see Figure 4) and the numbers for 5'- and 3'-ends of cloned cDNA were also shown. The sites of *Pvu*II, *Ava*I, and *Eco*RV used for construction of expression plasmid are indicated by asterisks.

with K1735 cells (28,29); 4) K1735 cells do not contain detectable levels of hybridizable mRNA with our cDNA probe (see Fig. 1C); and 5) we confirmed that SV40 promoter works properly in these cells using a recombinant plasmid pSV2-cat (36), in which chloramphenicol acetyltransferase coding sequences were placed under SV40 early region promoter (data not shown).

In three independent transfection experiments, we found about 0.5, 0.9 and 1.8% of K1735 cells transfected with pKCRH2MT are immunofluorescence-positive (Fig. 2 A and B). As both monoclonal antibodies gave the similar results, we showed the examples stained by using TMH-1. The number of positive cells varied among experiments but we always found the positive cells only in the K1735 cells transfected with pKCRH2MT. No immunofluorescence-positive cells were found in K1735 cells transfected with pKCRH2 alone (Fig. 2 C and D) or with pKCRH2 carrying the same insert as pKCRH2MT but in the opposite

orientation. Furthermore, we have never found fluorescence-positive cells in the transfected cells stained for background fluorescence. Subsequently, we also used COS-7 cells for transfection and obtained the similar results. About 0.8% of COS-7 cells transfected with pKCRH2MT reacted with monoclonal antibodies (Fig. 2 E and F). No immunofluorescence-positive cells were found in control systems. These results clearly indicate that the isolated cDNA actually encodes tyrosinase.

Nucleotide Sequence

We determined the entire nucleotide sequence of cloned cDNA according to the strategy shown in Fig. 3. The nucleotide sequence of tyrosinase cDNA revealed a single long open reading frame, beginning with the ATG codon at nucleotide residues 1-3 (Fig. 4). This reading frame codes for a polypeptide of 537 amino acids with a molecular weight of 60,819. The assigned ATG is preceded by an in-frame termination codon TGA (nucleotide residues -21 to -19) and the termination codons are also found in two other reading frames in the upstream region of the assigned ATG (nucleotide residues -170 to -172 and -37 to -35). Therefore, there is no other open reading frame that can encode a long polypeptide (>100 amino acid residues). The sequence flanking the assigned ATG (nucleotide residues -5 to 4) is not a typical favored sequence for eucaryotic initiation sites described by Kozak (37), but the same sequence is found in her list of 211 mRNAs examined (37). The codon specifying carboxy-terminal valine (amino acid residue 513) is followed by the translational termination codon TGA (nucleotide residues 1612-1614).

No nucleotide differences were observed between the clones pMT4 and pMT5, except that both clones differ in the length (see also Fig. 3). The clone pMT4 contains 838 nucleotides of 3'-untranslated region excluding about 160 nucleotides of poly(dA) tail and contains three polyadenylation signals, one copy of ATTAAA (38) (nucleotide residues 1732-1737) and two copies of AATAAA (39) (nucleotide residues 2418-2423 and 2427-2432). Two copies of AATAAA were found 32 and 23 nucleotides upstream from poly(dA) tail of pMT4. The clone pMT5 has the insert of 1724 nucleotides (nucleotide residues 31-1754) and contains 140 nucleotides of 3'-untranslated region excluding about 30

CAATTAACAGCTGGCATCAGGGGAAAAGCAGACATCCAACAACACTAGCTCTGAAGGAGA	-115
TCAGCAGAGAAACCTTCACGGGATTCATGCTGTATTTTCATCTAGCACCCCTGCTCTCT	- 55
CCATGCAAGAGCAGCATAGGAGACCTGTGTTCTGAACTCTTGCTTCGAGAAGAAATGAAA	6
TCTTACAACGCTCCTCCCCCTAGCCTATATCTCCCTTTTCTCGATGCTGTTTTATCAGGTT	66
SerTyrAsnValLeuProLeuAlaTyrIleSerLeuPheLeuMetLeuPheTyrGlnVal	
-20	-10
TGGGCTCAGTTTCCACGAGAGTGTGCCAATATTGAGGCTCTGAGACGTGGGTGTGTGC	126
TrpAlaGlnPheProArgGluCysAlaAsnIleGluAlaLeuArgArgGlyValCysCys	
-1 1	10
CCAGACCTGCTCCCTTCTCTGGACCGGGGACTGACCCTTGTGGCTCATCATCAGGAAGA	186
ProAspLeuLeuProSerSerGlyProGlyThrAspProCysGlySerSerSerGlyArg	
20	30
GGCAGGTGTGTGGCTGTGATTGCAGACTCCCGACCCACAGCCGCATTATCCCACGAT	246
GlyArgCysValAlaValIleAlaAspSerArgProHisSerArgHisTyrProHisAsp	
40	50
GGTAAAGATGACCGAGAAGCCTGGCCTCTGAGGTTCTTTAATAGAACATGTCAGTGAAT	306
GlyLysAspAspArgGluAlaTrpProLeuArgPhePheAsnArgThrCysGlnCysAsn	
60	70
GATAATTTCTCAGGACACAACCTGTGGGACTTGCCGCTCTGGGTGAGAGGAGCTGCATGC	366
AspAsnPheSerGlyHisAsnCysGlyThrCysArgProGlyTrpArgGlyAlaAlaCys	
80	90
AACCGAAAAATTCACAGTCAGGAGAAATCTTCTAGACTTAAGTCAGAGAAGAAAGAGC	426
AsnGlnLysIleLeuThrValArgArgAsnLeuLeuAspLeuSerProGluGluLysSer	
100	110
CACCTTGTGTCAGGGCCTTGGATATGGCGAAGCGCACAACTACCCCTCAATTTGTCTATGCC	486
HisPheValArgAlaLeuAspMetAlaLysArgThrThrHisProGlnPheValIleAla	
120	130
ACAAGGAGGTAGAAACATATCGGACCGAGTGGCAACACACCACAATTTGAGAACATT	546
ThrArgArgLeuGluAspIleLeuGlyProAspGlyAsnThrProGlnPheGluAsnIle	
140	150
TCCGTTTATACTACTTTGTTGGACACACTATTATTAGTCAAAAAACCTTCTCGGG	606
SerValTyrAsnTyrPheValTrpThrHisTyrTyrSerValLysLysThrPheLeuGly	
160	170
ACAGGACAGGAAAGCTTTGGGGATGTGGATTCTCTCAAGGACCCGCTTTCTCACA	666
ThrGlyGlnGluSerPheGlyAspValAspPheSerHisGluGlyProAlaPheLeuThr	
180	190
TGGCAGGTACCATCTGCTGCAGCTGGAGAGAGACATGCAGGAGATGCTGCAGGAGCCT	726
TrpHisArgTyrHisLeuLeuGlnLeuGluArgAspMetGlnGluMetLeuGluAlaPro	
200	210
TCTTTCTCCCTCTCTTACTGGAATTTTGCAACTGGGAAAAACGTCTGCGATGTCTGCACT	786
SerPheSerLeuProTyrTrpAsnPheAlaThrGlyLysAsnValCysAspValCysThr	
220	230
GATGACTTGATGGGATCCAGAAGCAACTTCGATTCTACTCTTATAAGCCCCAACTCTGCT	846
AspAspLeuMetGlySerArgSerAsnPheAspSerThrLeuIleSerProAsnSerVal	
240	250
TTTTCTCAATGGAGAGTGTCTGTGAATCCTTGGAGAGTACGATACCTTGGGAACACTT	906
PheSerGlnTrpArgValValCysGluSerLeuGluGluTyrAspThrLeuGlyThrLeu	
260	270
TGTAACAGCACTGAGGTGGACCAATCAGGAGAAACCCAGCTGGAATGTAGGAGACCA	966
CysAsnSerThrGluGlyGlyProIleArgArgAsnProAlaGlyAsnValGlyArgPro	
280	290
GCAGTGCAGCGTCTCTCTGAGCCACAGGATGTCAGTCTGAGGAGTCCGTGTATTT	1026
AlaValGlnArgLeuProGluProGlnAspValThrGlnCysLeuGluValArgValPhe	
300	310
GACACACCTCTCTTTTATTCCAATTCTACAGACAGTTTTCGAAATACAGTGAAGGTTAC	1086
AspThrProProPheTyrSerAsnSerThrAspSerPheArgAsnThrValGluGlyTyr	
320	330
AGTGTCTCCACGGGAAAAATAGACCCTGCTGTTTCGAAGCCTTCACAACCTGGCCACCTC	1146
SerAlaProThrGlyLysTyrAspProAlaValArgSerLeuHisAsnLeuAlaHisLeu	
340	350
TTCTGTAATGGAACGGGAGGACAAACCCATTGTCTCCCAATGATCTATTTTTGCTCTC	1206
PheLeuAsnGlyThrGlyGlyGlnThrHisLeuSerProAsnAspProIlePheValLeu	
360	370
CTGCACACTTTCAGTGTGCGGTCTTTGACGAATGGCTAAGGAGGTATTAACGCCGATATT	1266
LeuHisThrPheThrAspAlaValPheAspGluTrpLeuArgArgTyrAsnAlaAspIle	
380	390
CTACCTTCCCGTTGGAAAAACGCACCTATTGGACATAACAGGCAATACAACATGGTGCCA	1326
SerThrProLeuGluAsnAlaProIleGlyHisAsnArgGlnTyrAsnMetValPro	
400	410
TTCTGGCCTCCAGTTACCAACACAGAAATGTTGTACTGCTCCAGACAATCTGGGATAT	1386
PheTrpProProValThrAsnThrGluMetPheValThrAlaProAspAsnLeuGlyTyr	
420	430
GCTTATGAAGTTCAATGGCCAGGTGAGGAGTTACTGTATCTGGAATCATTACCATTTGCT	1446
AlaTyrGluValGlnTrpProGlyGlnGluPheThrValSerGluIleIleThrIleAla	
440	450
GTAGTGGCTGCGTTGTACTTGTAGCTGCCATTTTCGGGGTGTCTTCTGTCTGATCCGT	1506
ValValAlaAlaLeuLeuLeuValAlaAlaIlePheGlyValAlaSerCysLeuIleArg	
460	470

TCTAGAAGCACCAAGAATGAAGCCAACCGCTCTCCTCACTGATCACTATCAACGCTAT	1566
SerArgSerThrLysAsnGluAlaAsnGlnProLeuLeuThrAspHisTyrGlnArgTyr	
480	490
GCTGAGGACTATGAGGAGCTCCCGAATCCTAACCACTCCATGGTCTGATAGCGCGCTGCC	1626
AlaGluAspTyrGluGluLeuProAsnProAsnHisSerMetValEnd	
500	510
CATCCCGCACGCAGCAGGTGGGAAGCAACCTGATTGAAACACAGCTGTCAACCGTATT	1686
TCCTTGGGCTTCTCTCTTTCTTCCCAAGATAGATGTGGTTTGGAAATTAAGCATTTGCTC	1746
TCCAGTATGCACAGTTTAGACTCCTTTAATGGGTTTAAATAATTACTTATTGAAGAT	1806
GGCAATTATACACAGTTGTGTGATATCTGAGAGGATAATTGGATGAACTTGGTGGTATTC	1866
TAAAGATGGCCTGTATGCTGTGAAAAAGCAATGCTTTGTTACACAGGAAAAATAAGTTT	1926
GATGTAATAATCTCTATTTCAAATATATGAGTGCCCATCTTATGGAGTTAACATGTGAAT	1986
GCATTGTTATTGTTGGTACTAATCAAAAGTTATAAAAGCAGCCATGTTGATTAGTTACAA	2046
CAAAATCACATGACATTATAAACAAATAGAAATCTGATTATCTTTCTGAACTGCTCTCAT	2106
TTTGCTTTCAGCGTATTATTGGCCCTCAGTGCAAGGCCACCACAAAGTCACAGTCTTG	2166
CCACACATGGTAAAGACGAAAGCTTCACTTGCTGGAACACATCTGCTCTTGATATTTGC	2226
CAATGTGTTCCCTAGCTCAGTTCTCTGGACATGAAACTGCCTGGGCCACAGTTCACCTTG	2286
AATTCTGATCTTTAGAGGCTGCGAGGAAGGTATCAGTCAGAGGACACAATGTTCTGTG	2346
CATCTTATCAAAATGTTGATTTCATTCAACAGCATTGTTACAGCACTTATCAGCCTTCT	2406
GTGTATTTCAAAATAAATAATAAAATCTTCTTCATTGGTT	2449

Fig. 4. Nucleotide sequence of cloned cDNA encoding mouse tyrosinase and its deduced amino acid sequence

The nucleotide sequence of the message strand is shown. Nucleotides are numbered in the 5' to 3' direction and numbers are shown on the right side of sequence. Nucleotide residue 1 is the A of the initiating methionine codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The predicted amino acid sequence is shown below the nucleotide sequence and numbered beginning with the amino-terminal residue of the mature tyrosinase. The preceding residues of a putative signal peptide are indicated by negative numbers. Potential glycosylation signals (45,46) and potential polyadenylation signals (38,39) are underlined. The sites of poly(dA) tail addition are indicated by small vertical arrows.

nucleotides of poly(dA) tail. One polyadenylation signal, ATTAAA (nucleotide residues 1732-1737) was found 23 nucleotides upstream from poly(dA) tail of pMT5. Therefore, tyrosinase mRNA is polyadenylated at least at two sites (Fig. 4). Apparently, the pMT5 is transcribed from a minor species of mRNA of about 2,300 nucleotides, which was detectable in B16 melanoma RNA (Fig. 1B) and in skin RNA only after longer exposure (data not shown).

Amino acid sequence

The sequence of 24 amino acid residues starting with the assigned ATG codon exhibits a feature characteristic of the signal peptide of secretory and membrane-associated proteins (40), which mainly contains hydrophobic amino acids and terminates with an amino acid having a small side chain such as alanine, glycine, or serine (41). Therefore, a possible site for cleavage of the signal peptide of tyrosinase precursor is after the alanine residue at position -1 (Fig. 4). This prepeptide is probably involved in the transfer of tyrosinase into the

melanosomes. Thus, tyrosinase is composed of 513 amino acids with a molecular weight of 57,872, which is in good agreement with the reported value of *de novo* form of mouse tyrosinase (33).

As tyrosinase is a glycoprotein (2,42-44) and hamster melanoma tyrosinase contains 4 moles of asparagine-linked sugar chains (44), we examined the possible glycosylation sites. There are seven potential glycosylation signals, Asn-X-^{Ser}Thr (45), where X is any amino acid except proline (46) (underlined in Fig. 4). Therefore, the possible glycosylation sites are the asparagine residues at position 72,80,157,280,326,361 and 509.

We found the similarity of deduced amino acid sequence of mouse tyrosinase (amino acid residues 77-470) to the entire amino acid sequence of Neurospora tyrosinase (47) (Fig. 5). The homology is about 26% aligned and is statistically significant (see Fig. 5 legend). For these calculations, gaps were counted as one substitution regardless of their length (31). If we take into account the changes among chemically similar amino acids, the homology is about 45%. This result suggests that both tyrosinases are evolutionally related, which is consistent with the fact that melanins are widespread pigments found in all living organisms. We tried to identify the copper-binding sites, as both tyrosinases contain two copper atoms per enzyme molecule (2,48). Lerch (47) suggested that the histidine residue at position 306 of Neurospora tyrosinase is one of copper ligands. He also pointed the presence of a common sequence, Leu-His-His, in Neurospora tyrosinase (amino acid residues 304-306) and in human ceruloplasmin (49) (amino acid residues 255-257), which is also a copper-containing protein. Interestingly, the histidine residue at position 380 of mouse tyrosinase is aligned to the histidine residue at position 306 of Neurospora tyrosinase, a possible copper ligand. Mouse tyrosinase contains the sequence of Leu-Leu-His (amino acid residues 378-380) instead of Leu-His-His and the preceding sequence (amino acid residues 363-377) of Leu-Leu-His shows the good similarity to the corresponding sequence of Neurospora tyrosinase. Particularly, the histidine residue at position 368 of mouse tyrosinase is aligned to the histidine residue at position 289 of Neurospora tyrosinase, which was also suggested as another copper ligand(50). Therefore, we tentatively

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77  CND*NFSGHNCGTCRPGWRGAACNQKILTVRRNLLDLSPEEKSHFVRALDMAKRTTHPQF
   :  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
1  STDIKFAITGVPT*TPSSNGA*****VPLRRELRLQQNYPEQFNLYL*LGLR****DF

136 VIATRRLEDILGPDGNTPQFENISVYNYFVWTHYYSVKTKFLGTGQESFG****DVDFSH
   :  :  +  :  :  :  :  :  :  :  :  +  :  +  +  :  :  +
49  QGLD***EAKLDS*****YYQVA*GIHGMPFKPWAGVPSDTDWSQ

192 EGPA*****FLTWHRYHLLQLERDMQEMLQEPSFSLPYWNFATGKNVC**DV
   :  +  :  :  :  :  :  :  :  +  +  +  :  :  +  :  :  +
85  PGSSGPGGYCTHSSILFITWHRPYLALYEQALYASVQAVAKQFPVEGGLRAKYVAAKDF

237 CTDDL*MGSRSNFDSLISPNVSFQWRVVCESLEEYDTL*GTLCNSTEGGPIRRNPA*G
   +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
145 RPYFDWASQPP*KGTLAPPELSSRTIQVVDVDGRTKSINNPLHRT*FHPV**NPSPG

294 NVGRPAVQRLPEPQDVTQCL**EVRVFDTPPFYSNSTDSFRNTVEGYSAPTGYDP***
   :  +  +  :  :  +  +  :  :  :  +  +  :  :  :  +  :  +  +  +
201 NFS*AAWSRYPSTVRYPNRLPGASRDERIAPILADELASLRNNVSLLLSYKDFDAFSYN

348 *****AVRSLHNLHLFLNGTGGQTHLS*****PNDPIFVLLHTFTDAVFDEW***
   :  :  +  +  :  :  +  +  :  :  +  +  :  :  +  +  :  :  +  +
260 RWDPNNTNPGDFGSLAVHNEIHDRTGGNGHMSSLEVSAPDPLFWLHHVNVDRLSWIQDL

391 *****LRRYNADISTFPLENAPIGHNRQYNMVPFWP*PVTN**TEMFVTAPDNLGYAY*E
   +  :  :  :  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
320 NPNSFMTPRPAPYSTFVAQEG*ESQSKSTPLEPFWDKSAANFWTSEQVKDSITFGYAYPE

442 VQ*WPGQEFT*VSEIITI**AVVAALLLVAAIF
   :  :  +  +  :  :  +  :  :  +  +  :  :  +  +
379 TQKW***KYSSVKYQAAIRKSVTALY*GSNVF

```

Fig. 5. Alignment of the deduced amino acid sequence of mouse tyrosinase with the amino acid sequence of *Neurospora* tyrosinase
The sequences are shown by the single letter code. The sequence at top line indicates the portion of mouse tyrosinase (amino acid residues 77-470), which is aligned with the entire sequence of tyrosinase from *Neurospora crassa* (47) (amino acid residue 1-407). We obtained the following values: $s = 6.40$; $\tau = 2.29$; $s.d. = 0.75$; and $A = 5.48$, which corresponds to a probability of $P = 2.1 \times 10^{-8}$ to find the actual alignment shown here. The symbol, :, indicates the identical amino acids found in both sequences and the symbol, +, indicates the chemically similar amino acids. The histidine residues at 368 and 380, the putative copper-binding ligands, are indicated by asterisks.

assign two histidine residues at position 368 and 380 as copper ligands in mouse tyrosinase (marked with asterisks in Fig. 5).

Using tyrosinase cDNA as a hybridization probe, we are

isolating the tyrosinase gene in order to study the regulation of its expression, which will be a unique system for the study of neuronal differentiation. Furthermore, we are analyzing the molecular basis of albinism.

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