Conserved cysteine to serine mutation in tyrosinase is responsible for the classical albino mutation in laboratory mice

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ABSTRACT

Albinism, due to a lack of melanin pigment, is one of the oldest known mutations in mice. Tyrosinase (monophenol oxygenase, EC 1.14.18.1) is the first enzyme in the pathway for melanin synthesis, and the gene encoding this enzyme has been mapped to the mouse albino (*c*) locus. We have used mouse tyrosinase cDNA clones and genomic sequencing to study the albino mutation in laboratory mice. Within the tyrosinase gene coding sequences, a G to C transversion at nucleotide 308, causing a cysteine to serine mutation at amino acid 103, is sufficient to abrogate pigment production in transgenic mice. This same base pair change is fully conserved in classical albino strains of laboratory mice. These results indicate that a conserved mutation in the tyrosinase coding sequences is responsible for the classical albino mutation in laboratory mice, and also that most albino laboratory mouse strains have been derived from a common ancestor.

INTRODUCTION

Tyrosinase (monophenol oxygenase, EC 1.14.18.1) is a copper containing glycoprotein that catalyzes the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (L-dopa), and the dehydrogenation of L-dopa to dopaquinone (1). Dopaquinone is a precursor in the synthesis of black and yellow melanin (eumelanin and pheomelanin, respectively) (2,3,4). The absence of melanin results in oculocutaneous albinism (5,6). In mice, mutations at the *c* (albino) locus on chromosome 7 have been found to be associated with diminished tyrosinase activity (7) and recently Kwon et al. (8) showed that the mouse *c*-locus encodes tyrosinase.

Mouse tyrosinase cDNA clones have recently been isolated by three different laboratories (9,10,11). Two of these clones,

Tyrs-J (9) and pmcTyr-1 (11), were isolated from cDNA libraries derived from pigmented cells of the C57BL/6 mouse strain. These cDNAs were shown to be functional both *in vitro* (11,12) and *in vivo* (13,14). In contrast, the coding region of MTY811c (10) was created by ligation of promoter/exon 1 sequences from an albino (BALB/c) mouse genome to 3' cDNA sequences isolated from Cloudman S-91 melanoma cells (DBA/2 origin). The coding sequences of Tyrs-J and MTY811c were found to differ at only two sites (compare TyBS and Ty811B in Fig. 1) (12). At nucleotide 308, Tyrs-J encodes a G while MTY811c shows a C resulting in a cysteine to serine change at amino acid 103. This region of MTY811c was isolated from the BALB/c genome. The other nucleotide difference is located at nucleotide 956, which is a C in Tyrs-J and a T in MTY811c. This substitution predicts a glycine to valine amino acid change. Because the region of MTY811c that encodes amino acid 103 was isolated from an albino mouse genome, because this cysteine is a conserved amino acid of mammalian tyrosinase genes (11), and because this amino acid substitution has previously been proposed as a possible candidate for the albino mutation (15, 16), we tested whether a cysteine to serine conversion at amino acid 103 was sufficient to inactivate tyrosinase minigenes in transgenic mice. We also sequenced the corresponding region of the genome for other albino strains of laboratory mice to determine if the G to C transversion at nucleotide 308 was conserved.

METHODS

Tyrosinase minigene constructions

MTY811H (B. S. Kwon, unpublished) contains the MTY811c tyrosinase cDNA (10) plus BALB/c genomic sequences located upstream from the mouse tyrosinase gene. A 4.1 kb fragment of MTY811H was isolated after complete digestion with *EcoRI* and partial *HindIII* digestion. This fragment was subcloned into

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a *HindIII/EcoRI* digest of pBluescript KS(–) (Stratagene) to give pTy811B (Fig. 1). In order to use the cDNA part of Tyrs-J, a 1.85 kb *XhoI/EcoRI* cDNA fragment from Tyrs-J (12) was used to replace the analogous cDNA region of MTY811H. Subsequently, the homologous 4.1 kb *HindIII-EcoRI* fragment was subcloned into pBluescript KS(–) to give pTyBS (Fig. 1). To generate chimeric constructs, pTyBS and pTy811B were both digested with *ScaI*. Both plasmids contain one *ScaI* site in the tyrosinase sequences and one in Bluescript. The 5' tyrosinase sequences of pTy811B were ligated to the 3' sequences from pTyBS to give pTy811D, and *vice versa* for pTy811C. The *ScaI* site in Bluescript is located within the *amp^r* gene, allowing the desired orientation of ligation to be selected by culture on ampicillin plates. pTy811C has the Tyrs-J coding region at amino acid 103, while pTy811D has the MTY811c sequences (see Fig. 1). These coding regions were confirmed by double-stranded dideoxy sequencing. The *ScaI* ligation sites were also sequenced and shown not to contain any insertions, deletions, or base pair changes. Note that pTy811C is not the same as MTY811c.

Generation of transgenic mice

Transgenic mice were generated by the standard technique of microinjection into single cell mouse embryos (17). FVB/N female mice mated to FVB/N males were used as embryo donors. ICR females bred to vasectomized BDF1 males were used as embryo recipients and surrogate mothers.

Southern hybridizations

Tail DNAs from potential transgenic mice were isolated as described previously (17). DNAs of common laboratory mouse strains were purchased from Jackson Laboratory (Table 2, all strains except FVB/N and ICR/Hsd). Aliquots (10 μ g) of genomic DNA were digested with *PstI* or *EcoRI*, then electrophoresed through a 0.8% agarose gel and transferred to a nylon membrane (Zeta-probe, Bio-Rad). A 1.9 kb of *BglII-EcoRI* fragment of the mouse tyrosinase cDNA clone MTY811c (10) was labelled with ³²P-dCTP by the random primer method (18) and used as a hybridization probe. Hybridizations were done in 5 $\times$ SSC, 5 $\times$ Denhardt's and 45% formamide at 42°C overnight (1 $\times$ SSC is 150 mM NaCl, 15 mM Na citrate). Hybridization membranes were washed to a final stringency of 0.1 $\times$ SSC, 0.1% sodium dodecyl sulfate at 65°C.

Polymerase chain reaction and DNA sequencing

The first exon of the tyrosinase gene was amplified directly from genomic DNA by use of the polymerase chain reaction (PCR) (19) with oligonucleotide primers, 5'-GGGGTTGCTGGAAGAA-GAAGTCTGTG-3' (nucleotides –73 to –49 in tyrosinase exon 1) and 5'-AACTCTCTATATAGTGCATCTT-3' (antisense to sequences of tyrosinase intron 1). Amplification conditions were: denaturation for 30 sec at 92°C, 1 min annealing 62°C and 2 min polymerization at 72°C for 30 cycles. The reaction products were diluted with an equal volume of H₂O and an equal volume of 7.5M ammonium acetate, followed by precipitation with 2.5 volumes of ethanol. After centrifugation, precipitates were washed in 75% ethanol, dried, and resuspended in 8 μ l of H₂O. The sequencing primer 5'-TGCTAAAGTG-AGGTAAGAAAAGAAC-3' (nucleotides 423 to 399 in tyrosinase exon 1) was end-labelled with γ -³²P-ATP using polynucleotide kinase, then purified using a NENSORB™20 column as described by the manufacturer (New England Nuclear). PCR-amplified template DNA was alkali denatured, annealed

with the end-labelled primer, and subjected to dideoxy sequencing (Pharmacia ³²P sequencing kit) using the manufacturer's protocol.

RESULTS

A cysteine to serine substitution at amino acid 103 inactivates tyrosinase minigenes

To investigate the importance of the amino acid differences between Tyrs-J and MTY811c, four different tyrosinase minigenes were made (TyBS, Ty811B, Ty811C and Ty811D) (see Fig. 1). All four constructs have the same 2.25 kb of upstream regulatory sequences including the first 65 base pairs of exon 1. Attached to this region, the four constructs have coding sequences derived from either MTY811c or Tyrs-J. TyBS contains an *XhoI-EcoRI* fragment from clone Tyrs-J; it encodes cysteine at amino acid 103 and glycine at amino acid 346. Ty811B contains the corresponding *XhoI-EcoRI* fragment from MTY811c encoding serine at amino acid 103 and valine at amino acid 346. Ty811C and Ty811D are chimeric constructs, each containing one end from Tyrs-J and the other end from MTY811c, ligated at a central *ScaI* site (see Fig. 1). Ty811C codes for a cysteine at amino acid 103, while Ty811D codes for a serine.

Each construct was isolated as a *KpnI-EcoRI* fragment and microinjected into one-cell stage fertilized FVB/N (albino) embryos. Embryos that survived injection were implanted into pseudopregnant ICR (albino) females and the offspring were screened for integration of the tyrosinase minigenes by Southern hybridizations to tail DNAs (Fig. 2, summary in Table 1). Head-to-tail integration of the microinjected DNA yields a diagnostic hybridization band at 4.1 kb after digestion with *PstI* (Fig. 2).

All nine mice that were transgenic for Ty811B or Ty811D were albino (Table 1). By contrast, the TyBS and Ty811C constructs each produced pigmented transgenic mice (Table 1). The only difference between TyBS and Ty811D, and similarly between Ty811C and Ty811B is a cysteine to serine change at amino acid 103.

Fig. 1. Tyrosinase minigene constructs (TyBS, Ty811B, Ty811C and Ty811D). All four constructs have an identical 2.25 kb tyrosinase promoter (single line) plus 65 bp of tyrosinase exon 1 (to the *XhoI* site). The cDNA regions are derived from MTY811c (open box) or from Tyrs-J (shaded).

Pigmented transgenic mice

FVB/N mice are an inbred strain of mice that are homozygous mutants at the albino locus, but are wild-type at the agouti, brown and dilute loci (P. A. Overbeek, unpublished). Full expression of a functional tyrosinase minigene would be expected to convert the albino phenotype to an agouti pigmentation. However, the transgenic mice obtained with both the TyBS and Ty811C constructs showed considerable variation in their pigmentation. The coat colors were found to range from grayish (similar to the pigmentation of chinchilla (c^{ch}) or dilute (d) mutants) to brownish (see Fig. 3). None of the mice were black. Similarly, the intensity of the pigmentation varied from barely visible to near normal (Fig. 3A and 3C). One TyBS mouse and four Ty811C mice were mottled (Fig. 3A and 3C). All four TyBS transgenic mice and eight out of the 14 Ty811C transgenic mice had dark eyes at birth and were immediately identifiable as transgenic mice. Three other Ty811C mice showed light pigmentation in both fur and eyes by 14 days of age (Fig. 3C, mice 4, 5, & 6). None of the initial TyBS or Ty811C mice showed normal agouti pigmentation. However, pigmented male and female offspring of the mottled TyBS female show normal agouti pigmentation (Fig. 3B), implying that the founder mouse is mosaic and also that the TyBS minigene is sufficient to produce normal agouti pigmentation.

A conserved mutation in albino mouse strains

Our tyrosinase minigene study suggests that a G to C mutation at nucleotide 308 is sufficient to inactivate the tyrosinase gene. To determine whether a similar mutation is present in other albino mouse strains, the corresponding region of the genome was sequenced for a collection of pigmented and albino mouse strains. The albino strains were selected to include inbred and outbred mice of various origins. The first exon of the tyrosinase gene was amplified using the polymerase chain reaction (PCR) (19). The region surrounding nucleotide 308 was then sequenced directly by use of a ^{32}P end-labelled internal primer (Fig. 4A). All of the classical albino strains examined (see Table 2) were found to have a C rather than a G at nucleotide 308 of the coding strand of tyrosinase (for example, see Fig. 4B). By comparison, all pigmented mouse strains examined (Table 2) have a G at this position. Both the c/c and c/c^{ch} variants of strain 129/J were also sequenced (Fig. 4C). Heterozygous c/c^{ch} mice show both C and G nucleotides at position 308, while homozygous c/c mice encode only serine. The G to C transversion exhibits 100% concordance with the classical albino phenotype.

Mouse strain CE/J, which has the c^e (extreme dilute) mutation at the albino locus, encodes a cysteine at amino acid 103 (Fig. 4C), indicating that the c^e mutation lies elsewhere in the tyrosinase gene. We also sequenced the more recently established

Fig. 2. Southern hybridization analysis of tail DNAs from TyBS and Ty811D founder mice. Tail DNAs were digested with *Pst*I, fractionated by agarose gel electrophoresis, transferred to a Zeta-probe membrane and hybridized with a tyrosinase cDNA probe. The tyrosinase minigenes have a single *Pst*I restriction site. DNAs from the first six newborns injected with TyBS are shown in the left panel; DNAs from the eight newborns injected with Ty811D are shown on the right. FVB and C3H: albino and pigmented non-transgenic control mice. TyBS mice: lanes 1 and 2, DNAs from albino mice; lanes 3–6, DNAs from pigmented mice. All of the Ty811D mice were albino. Exposure time for TyBS lanes 4 to 6 was 3 hours. All other lanes were exposed for 18 hours.

Table 1.

Tyrosinase minigene construct	No. of newborn mice	No. of transgenic mice	No. of pigmented mice
TyBS	6	4	4
Ty811B	23	3	0
Ty811C	39	14	11
Ty811D	8	6	0

Pigmentation in transgenic mice generated with the different tyrosinase minigenes. Injections of Ty811B and Ty811D generated a total of 9 transgenic founder animals, none of which were pigmented. Injections of TyBS and Ty811C yielded 18 transgenic mice, 15 of which (83%) were pigmented.

Fig. 3. Photographs of pigmented TyBS and Ty811C mice. A. TyBS transgenic mice. Mice 1–5 correspond to Fig. 2, TyBS lanes 1–5. Two mice (4 and 5) show a grayish coat color. One mouse (#3) is mottled. B. The mottled TyBS founder mouse (panel A, mouse #3) and one of its offspring. C. Ty811C pigmented founder mice. One mouse (#2) shows a grayish coat color. Three mice (1, 7, and 9) have brownish fur. Three mice (4, 5, and 6) show light pigmentation. One of these mice (#4) has darkly pigmented ears and tail similar to himalayan (c^h) mutants. Four mice (3, 8, 10 and 11) are mottled.

albino mouse strains C3H/HeJ- c^{9J} , CBA/N- c^{10J} , and C57BL/10SuJ- c^{11J} . These strains all have a G at nucleotide 308 (Table 2) indicating that their mutations lie elsewhere in the

tyrosinase gene. The c^{11J} strain was found to have an A rather than a G at nucleotide 230, resulting in an Arg to Leu change at amino acid 77.

Fig. 4. Sequencing of the classical albino mutation. Schematic of PCR amplification and sequencing. Two oligonucleotide primers were designed to amplify the whole coding region of tyrosinase exon 1 by PCR. Sequencing was performed using an internal primer with an antisense orientation. B. Nucleotide sequences of albino (SJL/J, SWR/J and AKR/J) and pigmented (C3H/HeJ and DBA/2J) mouse strains. The antisense sequences for the nucleotides that encode amino acids 102–104 are shown. The corresponding coding sequences are AA-CTGTAAG (asn cys lys) for the pigmented strains and AACTCTAAG (asn ser lys) for the albino strains. The coding sequence G to C transversion is located at nucleotide 308. The arrow indicates the site of the conserved mutation. C. Nucleotide sequences of other *c*-locus mutants. 129/J *c/c* is homozygous for the classical albino mutation. 129/J *c/c^{ch}* is heterozygous for the albino and chinchilla mutations and shows both C and G bands at nucleotide 308. The *c^e* (extreme dilution) mutation in strain CE/J does not alter the cysteine at amino acid 103.

DISCUSSION

Classical albino mutation

The ability of tyrosinase minigenes to induce pigmentation in transgenic mice has been reported previously (13,14). The previous tyrosinase constructs encoded a cysteine at amino acid 103 and were linked to regulatory sequences from the genomes of pigmented mouse strains (C57BL/6 and 129/J respectively). Our tyrosinase minigenes were linked to 5' upstream regulatory sequences from the genome of an albino (BALB/c) strain of mice. Both the TyBS and Ty811C constructs produced pigmented transgenic mice, implying that the BALB/c promoter region is functional and that albinism in BALB/c mice is not due to a defective promoter. The minigenes TyBS, Ty811B, Ty811C and Ty811D were constructed to test whether specific point mutations within the coding sequences of tyrosinase could inactivate the gene. The absence of pigmentation in mice transgenic for Ty811B

Table 2. Coat color and nucleotide 308 comparison in pigmented and albino mouse strains.

Mouse strain	Coat color	Nucleotide 308 (sense strand)
Pigmented strains		
AU/SsJ	Black	G
C3H/HeJ	Agouti	G
C57BL/6J	Black	G
C57BR/cdJ	Brown	G
C57L/J	Leaden	G
C58/J	Black	G
CBA/J	Agouti	G
DBA/2J	Dilute brown	G
NZB/B1NJ	Black	G
P/J	Pink-eyed fawn	G
SM/J	Light-bellied	G
WB/ReJ-W	Agouti	G
	Black	G
Classical albino strains		
A/HeJ	Albino	C
AKR/J	Albino	C
BALB/cByJ	Albino	C
FVB/N	Albino	C
NZW/LacJ	Albino	C
SJL/J	Albino	C
SWR/J	Albino	C
129/J	Albino (<i>c/c</i>)	C
	Chinchilla (<i>c/c^{ch}</i>)	C and G
ICR/Hsd*	Albino	C
Other strains		
C3H/HeJ- <i>c^{9j}</i>	Albino ⁺	G
CBA/N- <i>c^{10j}</i>	Albino ⁺	G
C57BL/10SuJ- <i>c^{11j}</i>	Albino ⁺	G
CE/J (<i>c^R</i>)	Extreme dilute	G

* Outbred strain

⁺ Albino mutations identified in pigmented strains of mice

and Ty811D might be due to: 1) lack of expression of the transgenes, 2) artifacts introduced during the cloning procedure, or 3) inactivation of the tyrosinase genes by the cysteine to serine mutations. Our tyrosinase minigenes were constructed to give transcripts that were identical to the endogenous transcripts in order to eliminate any artifacts due to inappropriate sequences. We have not tried to document directly Ty811B or Ty811D expression. However, since the two constructs differ by only single nucleotides from TyBS and Ty811C, the frequencies of expression are expected to be equivalent. Over 80% of the founder mice that carry TyBS or Ty811C show expression and pigmentation. Thus, we feel that the lack of pigmentation in all nine Ty811B and Ty811D families is not likely to be due to lack of expression in every family. In order to check for cloning artifacts, important regions of Ty811B and Ty811D were sequenced. The only base pair changes detected were the G to C mutations at nucleotide 308. Thus, the most likely explanation for the absence of pigmentation in the mice transgenic for Ty811B and Ty811D is that the cysteine to serine changes at amino acid 103 are sufficient to inactivate the tyrosinase minigenes.

The region of the genome that encodes cysteine 103 was sequenced in a variety of pigmented and albino strains of mice (Table 2). All of the classical albino strains showed an identical G to C transversion at nucleotide 308, resulting in 100% concordance between classical albinism and the presence of a serine at amino acid 103. Our experimental results are fully consistent with the proposal that a missense mutation at amino

acid 103 is the cause of the classical albino mutation in laboratory mice.

Tyrosinase promoter

Our tyrosinase minigenes contain 2.25 kb of 5' upstream regulatory sequences. This is a shorter regulatory region than that used by either Tanaka et al. (13) or Beerman et al. (14). The shorter promoter can function at most sites of integration in the genome (83% pigmentation in the TyBS and Ty811C founder mice) and can be sufficiently active to yield full agouti pigmentation (Fig. 3B). Integration of a high number of copies of the tyrosinase minigene is not required to give agouti pigmentation. The founder mouse for the fully pigmented family had the lowest copy number of the original set of TyBS transgenic mice (Fig. 2), and the fully pigmented offspring (Fig. 3B) carry only 2–4 copies of TyBS per genome (data not shown). The tyrosinase promoter can function in both melanocytes (neural crest origin) and pigment epithelial cells of the retina (neuroectodermal origin). In addition, the promoter appears sufficient to provide appropriate developmental regulation of gene expression, including induction of pigmentation in the retinal pigment epithelial cells by day 13 of embryonic development (data not shown).

Coat color

Mice that are transgenic for the tyrosinase minigenes TyBS and Ty811C show considerable variation in their pigmentation. We have generated over 50 additional pigmented transgenic families with the TyBS construct, and the additional families show the same variability of pigmentation (data not shown). Some of the families show full agouti pigmentation (e.g. Fig. 3B), implying that the tyrosinase minigene is sufficient to produce normal levels of both eumelanin and pheomelanin. Further mating studies have revealed that the tyrosinase minigene can give normal black or brown pigmentation on the appropriate non-agouti genetic backgrounds (P. A. Overbeek, personal observation). Less intense pigmentation is seen in most families. Of particular interest is the fact that some families show pigmentation similar to chinchilla (c^{ch}) mutants, while other families have dark ears and light bodies, a phenotype also produced by the himalayan (c^h) mutation. The chinchilla mutation lies in the tyrosinase coding sequences at amino acid 464 (14). Himalayan mutants have a temperature sensitive tyrosinase and have been reported previously to have a His to Arg amino acid change at position 420 (20). For the transgenic mice, the variable patterns of pigmentation are likely to reflect different levels of transgene expression from different sites of integration in the genome. Considered together, these observations suggest that nearly identical mutant pigmentation patterns can occur as a result of alterations in either the coding sequences, or the transcriptional regulation, of the tyrosinase gene. About 15% of the families transgenic for TyBS or Ty811C do not show pigmentation, perhaps due to rearrangements of the transgenic DNA during integration or integration into inactive regions of the genome.

Common ancestor

The classical albino strains of mice that we examined included both inbred and outbred strains. All had an identical G to C transversion at nucleotide 308. In contrast, the more recently identified albino mutations c^{9J} , c^{10J} , and c^{11J} did not show the same mutation. The c^{11J} albinism may be due to an Arg to Leu change at amino acid 77. These sequencing results imply that mutations other than the G to C transversion at nucleotide 308

can cause albinism. Since the classical albino strains of mice all exhibit the same G to C transversion (Table 2) and the same *EcoRI* haplotype (data not shown), the data imply that these mice are all derived from a common ancestor. Although classical albino strains of laboratory mice were derived by a variety of investigators at different times and different locations (17), they all appear to be offspring of a single albino ancestor.

Jackson and Bennett (21) have reached similar conclusions based on their studies of pigmented revertants of a cultured line of albino melanocytes and studies of a *DdeI* polymorphism at nucleotide 308.

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