

Identification of the albino mutation of mouse tyrosinase by analysis of an *in vitro* revertant

(polymerase chain reaction/coat color/inbred strains/melanocytes)

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ABSTRACT From within an albino melanocyte line grown *in vitro* we identified and cloned cells that apparently had reverted to wild type. We sequenced a part of the tyrosinase gene, encompassing a candidate mutation, from wild-type, albino, and revertant cell DNAs. The revertant cells contain, on one chromosome, a perfect base reversion to the wild-type sequence of this candidate mutation, proving that this is the sole defect in the tyrosinase gene of albino mutant mice. The revertant cells readily regain the albino phenotype after freezing and thawing. Taking advantage of a *Dde* I restriction site created by the albino mutation, we demonstrated that the regained phenotype is due to allele loss involving the wild-type chromosome. The *Dde* I site also allowed us to show that all inbred albino mice carry the same mutation and so must be derived from the same progenitor.

The albino (*c*) mutation of mice is one of the oldest and most widespread known. Anecdotal reports claim that the mutation was known in ancient Greece and China (1). Mutations in many other species have also produced pink-eyed white animals and are believed to be equivalent (2), while various alleles causing partial reductions in pigment have been described in mice (3).

Albino mice have a normal number of melanocytes in the skin and eyes (3). These cells produce premelanosomes, the organelles in which melanin pigment is normally deposited to produce melanosomes, but albino premelanosomes fail to become melanized (4, 5). The activity of tyrosinase (monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1), the essential and only well-characterized enzyme of melanin synthesis, is reduced or abolished by mutations at the *c* locus on chromosome 7 (3), where it is encoded (6, 7). A number of different mouse tyrosinase cDNA sequences have been characterized, including several splicing variants (8–12), but only one of these has been shown to confer functional tyrosinase activity on cultured cells (13). In one sequence variant, cysteine-85 is replaced by serine (10). Recently, the origin of this variant has been identified as the BALB/*c* strain, which carries the albino tyrosinase gene, and it is thus a candidate for the albino mutation (7). However, as only a small part of this BALB/*c* gene has been sequenced, it is possible that this variant is only a neutral substitution in the protein and the mutation may be elsewhere in the gene. We have identified and made use of an *in vitro* revertant of albino to define the mutation precisely.

Reversion at this locus is very rare. Attempts have been made to assess the frequency of spontaneous or radiation-induced reversion (14–16), but no case was observed in these studies involving over 3 million mice. One phenotypically revertant mouse was mentioned, but that mouse was outside

the study (15) and was not used in breeding studies to distinguish true reversion from suppression.

Cultured lines of albino mouse melanocytes have been established by three separate groups (17–19). The melan-*c* line (19) was derived from the LAC-MF1 mouse breed, which is partially outbred from various Swiss stocks but is homozygous for albino (*c*) and black (*B*). We have identified and subcloned an apparent revertant to wild type at the *c* locus in this line and have used the polymerase chain reaction (PCR) to amplify and sequence from wild-type, melan-*c*, and revertant DNA the part of the tyrosinase gene containing the candidate Cys → Ser mutation.

MATERIALS AND METHODS

Cell Culture. Melanocytes were grown as described previously in a supplemented Eagle's minimal essential medium at pH 6.9 (19, 20) containing 5% (vol/vol) fetal bovine serum and 0.1 mM 2-mercaptoethanol. B16C3 melanoma cells (21) and XB2 keratinocytes (22) were obtained from John Kreider and Jim Rheinwald, respectively, and grown as described (20, 21, 23). Frozen cell stocks in liquid nitrogen were prepared as described (19). Recovery of melan-*c*R cells was low, 1–10%.

Enrichment and Cloning of Pigmented Cells. Groups of pigmented melanocytes were trypsinized separately, each with about 1000 surrounding albino cells, by using plastic cloning rings, and each cell suspension was transferred to a 16-mm culture well and made up to 0.5 ml in growth medium with soybean trypsin inhibitor (30 μg/ml). The resulting cultures, containing about 1–2% pigmented cells, were subcultured, expanded, and resuspended for cloning. XB2 keratinocytes were previously growth-inactivated with mitomycin C for use as feeder cells and were plated in 6-mm wells. Single pigmented cells were transferred each to a 6-mm well containing feeder cells, by use of a drawn-out Pasteur pipette and mouth tube. The first subculture after cloning was onto XB2 feeder cells in 16-mm wells.

DNA Methods. All mouse strain DNAs were purchased from The Jackson Laboratory, except for MF1 DNA, a gift from Robin Lovell-Badge (National Institute for Medical Research, London), and DDK DNA, which was prepared at the Medical Research Council Mammalian Development Unit (London). DNA was prepared by suspending cells in 100 mM NaCl/50 mM Tris-HCl, pH 8/10 mM EDTA, followed by treatment with proteinase K (100 μg/ml) in 0.5% SDS. After phenol extraction and chloroform extraction, the DNA was precipitated with ethanol and dissolved in 10 mM Tris, pH 7.5/1 mM EDTA.

Restriction endonuclease digestions were performed according to manufacturers' instructions. Electrophoresis was in 4% NuSieve GTG agarose (FMC) gels in 40 mM Tris, pH 7.5/1 mM EDTA containing ethidium bromide (0.5 μg/ml).

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Abbreviations: PCR, polymerase chain reaction; TRP-1, tyrosinase-related protein 1.

PCR. Oligonucleotide primers were synthesized (Applied Biosystems model 381A): TCCGAATTCAAAGGGGTG-GATGACCG (bases 293–312 plus a terminal *EcoRI* site) and GACACATAGTAATGCATCC (bases 633–615; ref. 13). PCR was carried out essentially as described (24). Each reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.01% Nonidet P-40, primers at 3 μg/ml, all four dNTPs at 0.1 mM and 2.5 units of *Thermus aquaticus* DNA polymerase in 50 μl, with 200 ng of genomic DNA. The reaction was cycled 30 times through 90 sec at 92°C, 90 sec at 53°C, and 120 sec at 72°C.

Direct Sequencing. Amplified DNA for sequencing was purified by electrophoresis in 1% low-gelling-temperature agarose gels. The ethidium bromide-stained bands were excised and melted. A fraction was subjected to further PCR amplification for 30 cycles using one primer at 3 μg/ml and the other at 30 ng/ml to generate single-stranded molecules. These were desalted by passage through Sephadex G-50 columns, concentrated by precipitation, and used as substrate for sequencing with the limiting primer and Sequenase (United States Biochemical), used according to the manufacturer's instructions. Analysis was in 6% polyacrylamide sequencing gels.

RESULTS

***In Vitro* Reversion of the Albino Cell Line.** The establishment of the melan-c albino melanocyte line has been described (19). Groups of black cells were first observed in this line, at low frequency, at the 16th passage after cloning of the line. Such a group is illustrated in Fig. 1 *a* and *b*. The frequency of the pigmented cells then was estimated at 2–3 per 10⁵ (1 in 3–5 × 10⁴) from the number of groups detected and the plating density.

The pigmented cells were enriched and cloned. From 93 pigmented cells plated, 75 progressively growing clones were

obtained. Three of these clones, one from each of the original marked colonies, were subcultured and expanded separately for preparation of frozen stocks. One of these sublines was selected for characterization and is designated melan-cR (R for dominant phenotypic reversion). Each subline consisted entirely of pigmented cells, but otherwise the cell morphology and growth were similar to those of melan-c cells (19). Given the rarity of reversion of the mutation *in vivo*, we suspect, but are unable to prove, that all the clones derive from a single revertant clone that was unnoticed and passaged prior to passage 16.

The revertant cell line readily regains the albino phenotype. After the cloned melan-cR stock had been frozen for storage and thawed, twice in succession, unpigmented cells were found in it at a substantial frequency (over 20%; Fig. 1 *c* and *d*). The survival of pigmented cells is poor after frozen storage. Recovery of melan-cR cells has been particularly low (≤10%; see *Materials and Methods*), whereas that of unpigmented cells is usually well over 90%. Freezing would thus considerably enrich any unpigmented cells present.

This reemergence of phenotypically albino cells represents a further genetic alteration, since albino cells were absent from the original clonal colony of melan-cR cells, when they could readily have been detected. We isolated and cloned a number of these unpigmented cells, which we designate melan-cR-W1, -W2, etc. (W for white clones) and abbreviate here to W1, W2, etc. In addition, cells were noticed that exhibited much less pigment than the melan-cR line. Several lines of these "grey" cells were cloned and designated melan-cR-G1, -G2, etc. (shortened to G1, G2).

PCR Amplification and Sequencing of a Candidate Mutant Region of the Tyrosinase Gene. The tyrosinase cDNA sequence published by Kwon *et al.* (10) contained one difference in encoded protein when compared to other murine tyrosinase sequences. The G³⁹⁰ → C base substitution (num-

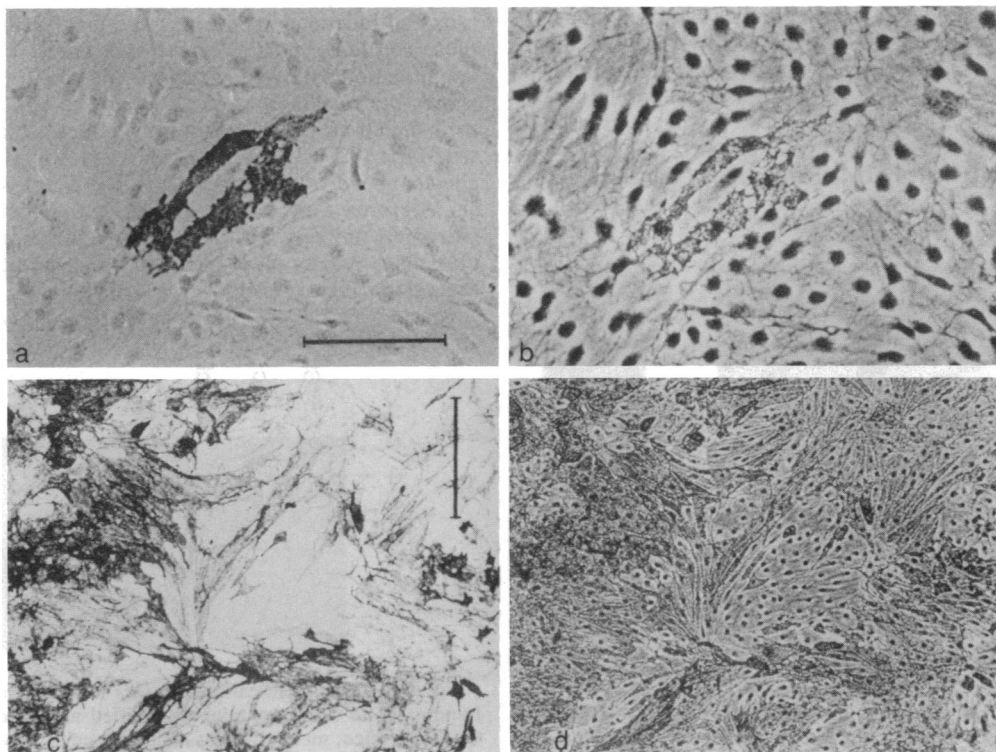


FIG. 1. melan-c and melan-cR cells. (*a*) Group of pigmented cells as first observed in the melan-c line, viewed with partial bright-field optics to show both pigmented and unpigmented cells. This group of four cells shows that even a single pigmented cell is prominently visible. (Bar = 100 μm.) (*b*) Same field as *a*, viewed with phase-contrast optics to show that the pigmented and unpigmented cells have similar morphology. (*c*) Melan-cR cells after two rounds of frozen storage and thawing, with bright field. Substantial numbers of unpigmented cells have appeared, seen as white patches. (Bar = 300 μm.) (*d*) Same field as *c*, with phase contrast, showing that the white patches contain unpigmented cells.

being as in ref. 13) results in substitution of serine for Cys⁸⁵ in the encoded protein. Those authors later reported (7) that this portion of the tyrosinase sequence was obtained from a genomic DNA clone derived from BALB/c (albino) mice. The Cys⁸⁵ → Ser mutation is therefore a candidate for the defect of tyrosinase in albino mice. Cysteine is found in the same position in human tyrosinase and in the murine tyrosinase-related protein 1 (TRP-1), suggesting that it is an important residue. Further, another conserved cysteine, located only 3 residues away in TRP-1, is mutated in the coat-color variant brown (25). This is strong circumstantial evidence to suggest that Cys⁸⁵ → Ser may cause the albino mutation, but functional or genetic proof has so far been lacking.

We used the PCR to amplify a region of the tyrosinase gene (bases 232–633) from wild-type (C57BL/6), melan-c, and melan-cR DNA. Following a second, linear amplification to generate single-stranded molecules, the primers were used to sequence the DNAs in each direction. Fig. 2 shows regions of the sequencing autoradiographs. The C57BL/6 sequence contains G at base 390, while melan-c contains C, confirming the previously reported difference between wild-type and albino sequences. DNA from melan-cR contains two sequences, with both G and C at position 390. Sequencing the other strand of melan-cR shows the same two bases at 390. The melan-cR cells are therefore heterozygous, +/c, for the albino mutation, and are phenotypically wild-type.

This demonstrates that the genetic event that has resulted in pigmentation of the melan-cR cells is a perfect base reversion to wild type on one chromosome 7. This is proof that the variant sequence is the sole cause of the mutation, and any other differences that may be found within the amino acid sequence are neutral.

All Inbred Albino Mice Have the Same Base Change. The albino mutation introduces a *Dde* I restriction enzyme site (CTNAG) into the tyrosinase gene. We used this site to analyze PCR-amplified DNA from a number of inbred mouse strains. The region we amplified from wild-type DNA contains two *Dde* I sites, resulting, after digestion, in fragments of 165, 113, and 63 base pairs. DNA from the albino mutant tyrosinase gene has three *Dde* I sites, giving restriction fragments of 130, 113, 63, and 35 base pairs.

Fig. 3 shows the results of *Dde* I digestion of the amplified

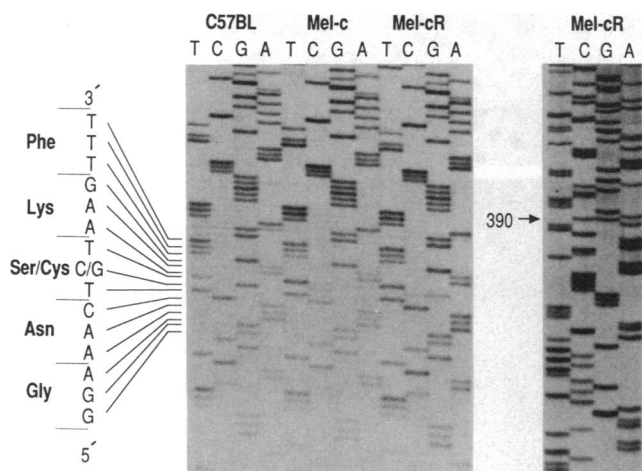


FIG. 2. Direct PCR sequence analysis of the tyrosinase gene. (Left) Autoradiograph reading the sense sequence from C57BL/6, melan-c, and melan-cR amplified DNA. Sequence lanes are labeled. The 15 bases around and including the variant base are shown to the left, together with the encoded amino acid sequence. C57BL/6 DNA has a G at base 390, melan-c has C, and melan-cR has both. (Right) Antisense sequence of melan-cR, showing two bases at position 390 (arrow).

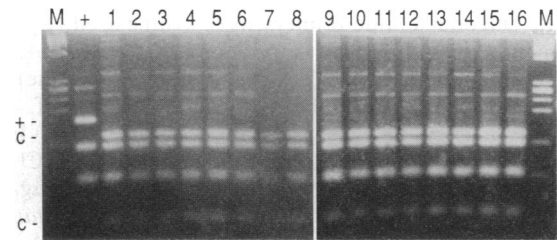


FIG. 3. PCR amplification and *Dde* I analysis of several mouse strain DNAs, showing all albino mutations are the same. Lane M, ϕ X174 DNA cut with *Hae* III; lane +, DNA from C57BL/6; numbered lanes are *Dde* I-digested DNA samples from the albino strains as follows: 1, A/J; 2, AKR/J; 3, BALB/cJ; 4, BUB/BnJ; 5, DA/HuSn; 6, DDK; 7, HRS/J; 8, LG/J; 9, MA/MyJ; 10, NZW/LacJ; 11, PL/J; 12, RF/J; 13, RIIS/J; 14, SJL/J; 15, SWR/J; 16, MF1. Fragment labeled + is 165 base pairs and diagnostic for the wild-type allele, the fragments labeled c are 130 and 35 base pairs and are diagnostic for the albino allele of tyrosinase.

DNA from C57BL/6, which is wild type (lane 1), and from 15 other inbred mouse strains, plus the MF1 outbred stock, all of which carry the albino mutation. All the albino mice (listed in the legend to Fig. 3) have the same *Dde* I restriction pattern and therefore all have the same G³⁹⁰ → C mutation. As it is highly unlikely that the same mutation has arisen twice among laboratory mice, we can conclude that all of these inbred strains (representing most branches of the inbred-strain family tree) carry a mutant tyrosinase gene derived from the same progenitor.

Reacquisition of the Albino Phenotype by melan-cR Is Due to Allele Loss at the Tyrosinase Locus. The reappearance of unpigmented cells in the melan-cR population seems to be a frequent occurrence, although the selective pressure imposed by freezing and thawing the cells makes determination of the actual frequency impossible. It is therefore likely that the three unpigmented clones we isolated, W1, W2, and W3, arose as independent events. We analyzed the DNA from these cell lines, plus a "grey" cell line, G1, by using PCR amplification followed by *Dde* I digestion as above. Fig. 4 shows the results of such an analysis. The amplified DNA from melan-cR cells contain the fragments diagnostic both of wild-type (C57BL/6 on this gel) and albino (melan-c here) alleles, as we would expect from the direct sequence analysis. All three white clones have only the albino pattern. It is extremely unlikely that the wild-type chromosome of melan-cR cells would acquire a second point mutation identical to that on the albino chromosome, which would be necessary to generate a *Dde* I site. We suggest that the wild-type tyrosi-

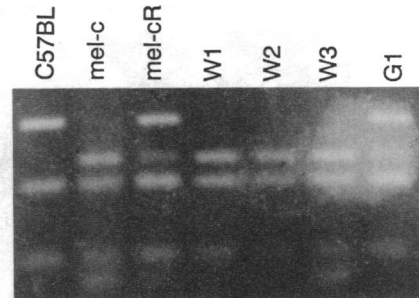


FIG. 4. PCR amplification and *Dde* I analysis of cell-line DNA. Lanes (left to right) contained amplified and digested DNA from C57BL/6 mice; melan-c cells; melan-cR cells; three cell lines, W1, W2, and W3, that have regained an albino phenotype, and one cell line, G1, that has acquired a "grey" phenotype. The 165-base-pair diagnostic wild-type fragment is labeled +, and the 130-base-pair albino fragment is c. The additional, lower molecular weight band in melan-c, melan-cR, and W3 is an inconsistently seen product of unknown origin.

nase gene has not been mutated but has undergone allele loss, which we discuss below.

The grey cell line G1 contains both alleles at the tyrosinase locus. The modification of the pigmentation phenotype may well involve the tyrosinase locus, possibly through a less severe genetic change, or more probably through an epigenetic change such as methylation.

DISCUSSION

Distinguishing between neutral polymorphic variation at a locus and a change causing a mutation can be problematic. This paper and an earlier work (25) show the power of a genetic approach. Tyrosinase and TRP-1 have overall about 40% amino acid identity, including 15 conserved cysteines. The previous study (25) used a revertant at the brown locus to determine that only one of two amino acid differences from wild type in the brown mutant TRP-1 was the cause of the brown phenotype. This mutation, Cys⁸⁶ → Tyr, alters one of the conserved cysteine residues. In this paper we have shown that the albino mutation of tyrosinase is due to a mutation at residue 85, which is also a cysteine. So, the classic mutation in each of these two genes (which both result in the null phenotype) involves a conserved cysteine, in both cases located at the end of the first of two cysteine-rich regions found in tyrosinase and related proteins (see ref. 26).

These cysteine residues must be important for tyrosinase and TRP-1 function. The active site of tyrosinase, however, is thought to reside at two histidine-rich, copper-binding domains (13). Tyrosinases of the prokaryote *Streptomyces* and the lower eukaryote *Neurospora* also contain these copper-binding sites but do not contain cysteine-rich regions (27, 28). The acquisition of the cysteine domains is a more recent evolutionary event whose function is not known.

All albino inbred strains examined carry the same tyrosinase mutation. Previous work (25, 29) showed that all brown inbred strains have the same mutation in TRP-1. Thus two of the widespread coat-color mutations are each derived from single progenitors. These observations are a reflection of the limited gene pool from which laboratory mice have been drawn, which can be traced back to animals acquired by a small group of workers early this century (30). The pigmentation variants among these animals existed prior to the use of mice for scientific purposes, amongst the mouse fanciers, who bred mice for their aesthetic value.

To our knowledge, the reversion described here is the only extant example of reversion at the *c* locus. The only previous reported example was a pigmented mouse that was not test-bred and may have been due to suppression rather than reversion. The pigmented cells observed here initially were found at 1 in 3–5 × 10⁴, probably reflecting a single event when the population was about this size (at passage 2). This occurrence is at least 2 orders of magnitude higher than the observed frequency in mice, but in the absence of further revertants we cannot with certainty ascribe this to luck or to a higher mutability of cultured cells than of mouse germ cells.

The reacquisition of the albino phenotype at high frequency reflects two factors: (i) the heavy selection pressure against pigmented cells imposed by freezing and thawing, which may be due to leakage of toxic melanin precursors from melanosomes, and (ii) the heterozygous nature of the dominant revertant, which allows the albino mutation to be expressed following loss of the wild-type allele. This loss might take the form of a deletion of part of the wild-type chromosome 7 but more likely is a loss of the whole chromosome through nondisjunction, leading to a monosomic cell line, or is the result of mitotic recombination between homologous chromatids such that a daughter cell becomes homozygous for the albino mutation. The selection for loss of pigmentation might also explain the incidence of "grey" cells

in the culture, which retain their wild-type tyrosinase allele. In these cases the selection may act on cells that have undergone some heritable epigenetic change, such as methylation or chromatin conformation, either at the wild-type tyrosinase gene or at some other locus that affects cell pigmentation.

In human cells heterozygous for a mutation at the thymidine kinase locus, when selection for loss of thymidine kinase activity is imposed, the wild-type allele is lost much more frequently than it is mutated (31). This allele loss by chromosome loss, loss and reduplication, or local homozygosity by mitotic recombination or gene conversion is very significant in tumorigenesis (32). Proof that the melan-cR-W cells have undergone allele loss by one of these methods will require analysis of other polymorphic loci along the length of chromosome 7, to identify the region that has become homozygous. Although outbred, the MF1 strain from which the cells originated is homozygous at most loci. Further study of the allele-loss phenomenon would be better carried out with cells with more heterozygosity, which might be useful model systems.

Coat-color genetics has long been a valuable system for the study of germ-line mutagenesis *in vivo*. Mutagenesis *in vitro* has utilized selectable enzyme or cell-surface markers, and there are few loci at which mutagenesis has been studied both *in vivo* and *in vitro*. Cultured melanocytes may provide a means of screening large numbers of cells for novel somatic mutations in genes for which germ-line mutagenesis data are available.

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