The Molecular Basis of *brown*, an Old Mouse Mutation, and of an Induced Revertant to Wild Type

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ABSTRACT

The murine *b* locus encodes the tyrosinase related protein, TRP-1, a putative membrane-bound, copper-containing enzyme having about 40% amino acid identity with tyrosinase. The protein is essential for production of black rather than brown hair pigment. We show that skin of mutant *brown* mice contains the same amount of TRP-1 mRNA as wild type. On sequencing the coding region of the mutant mRNA we find four nucleotide differences from the wild-type (*Black*) sequence. Two of these differences result in different amino acid residues encoded by the *brown* allele. By sequencing the TRP-1 gene from a mouse in which a reversion from *brown* to *Black* has been induced by ethylnitrosourea we are able to show that only one of these amino acid changes, which substitutes a tyrosine for a conserved cysteine, is the cause of the brown phenotype. This mutation is adjacent to another cysteine at which, in the analogous position in tyrosinase a mutation results in the albino phenotype. The sequence of the revertant is the first report of DNA sequence of an ethylnitrosourea-induced genetic change in mouse.

THE origins of a few mouse mutations go back to the mouse fancy, when mice were kept solely for aesthetic rather than scientific purposes. These old mutations are mostly, unsurprisingly, pigmentation or coat-color variants, which may have been isolated from wild populations. When genetic studies were initiated on the mouse, it was to these mutations and their wild-type alleles to which attention was first drawn. As a result of studies on these mutations, and on the many more isolated in this century, the genetics of pigmentation is the best understood complex developmental system in the mouse (SILVERS 1979; JACKSON 1985). In addition, six of these old mutations are used in the specific locus mutation tests (RUSSELL 1951; SEARLE 1974). In these experiments new alleles of *non-agouti, brown, albino, dilute, pink-eyed dilution* and *piebald* (plus the morphological mutation *short-ear*) are isolated from the offspring of mutagenized or control wild-type animals crossed with tester stocks homozygous for all seven mutations. Consequently, there exists a large body of knowledge as to the mutation rates of the different loci, under different treatments, as well as a large number of new mutant alleles at all the loci.

There has also been work to examine reversion rates of 4 of these old mutations. Two mutations, *non-agouti* and *dilute*, spontaneously revert or further mutate at a relatively high frequency (4.2×10^{-6} and 3.9×10^{-6} respectively), although only *dilute* reverts to true phenotypic wild type (SCHLAGER and DICKIE 1967, 1971). Two others, *albino* and *brown*, revert very rarely; both have spontaneous reversion rates of less than 1 in 10^6. Only one apparent albino revertant mouse (discussed in SCHLAGER and DICKIE 1971) and an apparent albino revertant cell line (I. J. JACKSON and D. C. BENNETT, in press) have been noted. Spontaneous reversion of the *brown* mutation has never been documented, despite analysis of several million animals. Nor have revertants of *albino* or *brown* been found following radiation mutagenesis, although *non-agouti* and *dilute* revert at higher frequency following irradiation (FAVOR, NEUHAUSER-KLAUS and EHLING 1987a). Only a single *brown* revertant mouse stock has ever been recovered, which was isolated following chemical mutagenesis with ethylnitrosourea (ENU) [FAVOR, NEUHAUSER-KLAUS and EHLING (1987b) and our unpublished results].

This accumulated knowledge and the resource of mutations makes the development of pigmentation an attractive candidiate for the application of molecular genetics. DNA closely linked to *non-agouti* (SIRACUSA *et al.* 1987; LOVETT *et al.* 1987), and from within dilute (JENKINS *et al.* 1981; COPELAND, HUTCHISON and JENKINS 1983), has been cloned. The cDNA product of the albino locus, which encodes the enzyme tyrosinase, was also recently cloned (KWON *et al.* 1987, 1989; YAMAMOTO *et al.* 1987, 1989; RUPPERT *et al.* 1988; MULLER *et al.* 1988). We have shown that another cDNA, pMT4 (SHIBAHARA *et al.* 1986) encodes a protein evolutionarily related to tyrosinase,

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tyrosinase-related protein (TRP-1) which maps at or close to the *brown* locus (JACKSON 1988). The restriction fragment length variant used to localize the TRP-1 gene is associated with the mutation carried in all common *brown* laboratory inbred strains, demonstrating a common origin for this mutation in the mouse fancy.

The amino acid similarities between human and mouse tyrosinases and the mouse TRP-1 sequence define a number of regions likely to be important for function of the two proteins. All have putative signal sequences at their N termini and hydrophobic transmembrane domains near their C termini. They all contain two regions which show extensive similarity to the copper binding sites of Neurospora and Streptomyces tyrosinases, and one of these sites is similar to one of the copper binding sites of arthropod hemocyanins [reviewed in MULLER *et al.* (1988) and HEARING and JIMENEZ (1989)]. Mammalian tyrosinases, like the lower eukaryotic and bacterial enzymes, contain two copper atoms. In addition they are localized on the inner face of the melanosome, the intracellular site of melanin synthesis. It is highly likely that TRP-1 is also a membrane-bound copper-containing enzyme. The mammalian proteins also have a similar number of apparently conserved cysteine residues. Both mouse and human tyrosinase have 17 cysteines in identical positions (human tyrosinase has one additional cysteine residue in the cytoplasmic domain). Fifteen of these are also found in equivalent positions in TRP-1; the two additional residues in tyrosinase being one in the signal sequence, and one at the C-terminal end of the transmembrane domain. Within the central portion of the molecules most of the amino acid similarity between TRP-1 and tyrosinase is located around the putative copper-binding domains and in two domains defined by the cysteine residues (see DISCUSSION and Figure 4).

We have determined the nucleotide sequence of the coding region of the TRP-1 gene from mutant *brown* mice, and find a number of differences from the wild-type (C57BL/6) sequence. Two of the nucleotide differences change the coding potential of the gene; both change amino acids which are also found in tyrosinase, one changes an arginine to histidine, the other changes one of the conserved cysteines to tyrosine. As a point mutagen (ENU) was able to induce a reversion of *brown*, we suspected that only one of these changes was the cause of the mutation. We sequenced the variant regions in the revertant TRP-1 gene, and find that only the cysteine is restored.

The importance of this region of the molecule to the protein's function is further underlined by the change in the albino mutation being localized also to a conserved cysteine residue of tyrosinase, only 3 residues away from a position equivalent to the *brown* mutation of TRP-1.

MATERIALS AND METHODS

RNA preparation: RNA was prepared from fresh or frozen neonatal mouse dorsal skin by homogenization in 3 M LiCl, 6 M urea (LOVELL-BADGE 1987). The RNA was precipitated overnight at 4°, centrifuged, washed in LiCl/urea and resuspended in 10 mM Tris (pH 7.5) and 0.1% sodium dodecyl sulfate (SDS). The RNA was deproteinized by digestion with 100 μg/ml proteinase K and extraction with phenol and chloroform before ethanol precipitation. The RNA pellet was subsequently resuspended in RNAase-free water.

Northern blots: RNA was electrophoresed through 1% agarose gels containing 2.2 M formaldehyde in 40 mM MOPS (pH 7), 5 mM sodium acetate, 1 mM EDTA (LEHRACH *et al.* 1977) before blotting in 10 × SSC to nitrocellulose (Amersham Hybond-C) [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7)].

Following transfer of RNA, the filters were baked at 80°, and prehybridized briefly in 50% formamide, 4 × SSC, 10 × Denhardt's solution, 0.1% sodium pyrophosphate, 0.1% SDS and 100 μg/ml denatured salmon-sperm DNA, and hybridized at 47° for at least 16 hr in the same solution (1 × Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinyl pyrrolidine).

Hybridization probes were made by labeling the 1.6-kb *Hin*dIII fragment of pMT4 (SHIBAHARA *et al.* 1986) with ^{32}P by the method of FEINBERG and VOGELSTEIN (1983).

Oligonucleotides: Oligonucleotides were synthesized on an Applied Biosystems Inc. model 381A DNA synthesizer, deprotected by overnight incubation at 65° in 30% ammonium hydroxide before precipitation from 0.3 M sodium acetate with 70% ethanol.

Reverse transcription and polymerase chain reaction (PCR): One microgram of skin total RNA was used to make double-stranded cDNA using a kit manufactured by Boehringer Mannheim, according to their instructions. Portions of the double-stranded cDNA were amplified essentially as in SAIKI *et al.* (1988) in 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.01% NP-40, four deoxynucleotides at 50 μM, a pair of primers at 3 μg/ml and 2.5 units Taq polymerase in a volume of 50 μl, overlaid with paraffin oil.

The standard amplification cycle was; 90 sec at 92°, 90 sec at 53° and 120 sec at 72°, repeated 30 times.

Direct sequencing of PCR product: After preciptation of the PCR products they were electrophoresed through low-gelling temperature agarose gels, the ethidium bromide stained band cut out and equilibrated in PCR buffer before melting and a fraction subjected to further PCR amplification with either the original primer pair, or new primers, one at 3 μg/ml, the other at 30 ng/ml for a further 30 rounds to generate single-stranded molecules, which were desalted by passing through G50 Sephadex (Pharmacia). In some cases the products of the first amplification were digested with restriction enzyme (*Xba*I or *Pvu*II), overhanging ends filled in, and double-stranded non-phosphorylated linkers added. The result of this is to add a new primer at the restriction site, which is then used in the asymmetrical amplification.

Single-stranded DNA was sequenced, using either the limiting primer from the asymmetric amplification, or internal primers, and Sequenase kits (USB) with ^{35}S-dATP according to manufacturer's instructions, and analyzed on 6% acrylamide sequencing gels.

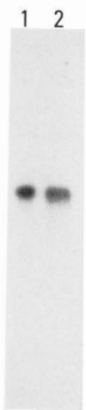

FIGURE 1.—Northern blot analysis of dorsal skin RNA from 3-day-old mice of wild-type and *brown* mutant strains, probed with TRP-1 cDNA. An aliquot of 10 μg total RNA was loaded in each lane. Lane 1, C57BL/6 mouse, wild type at the *b* locus. Lane 2, BALB/c mouse, *brown* mutant. In each case the hybridization reveals the 2500 nucleotide TRP-1 mRNA.

RESULTS

Mutant *brown* melanocytes produce normal levels of TRP-1 mRNA: We first considered the possibility that the *brown* mutation might be a defect in transcription or processing of the TRP-1 gene. We therefore compared the level of TRP-1 mRNA in total RNA extracted from the dorsal skin of 3-day-old mouse pups of BALB/c and of C57BL/6 strains. C57BL/6 is wild-type at the *b* locus, and BALB/c carries the *brown* mutation. Figure 1 shows a Northern blot of these RNAs probed with TRP-1 cDNA. The *brown* mutant mouse skin contains the same amount of TRP-1 mRNA as wild-type skin. The defect must be a small change or a point mutation in the TRP-1 mRNA sequence.

All common laboratory inbred strains which carry the *brown* mutation have the same variant TRP-1 restriction fragment, implying a common origin from a single progenitor *brown* mouse (JACKSON 1988). We therefore sequenced the BALB/c TRP-1 mRNA, in the knowledge that most laboratory *brown* mice will have the same sequence.

Nucleotide sequence of *brown* mutant TRP-1 mRNA: The PCR (SAIKI *et al.* 1988) was used to amplify fragments of double-stranded cDNA synthesized by oligo-dT priming of reverse transcriptase on total RNA from 3-day-old BALB/c mouse skin, followed by RNAase H treatment and second-strand synthesis with DNA polymerase I. It was assumed that the defect in the *brown* TRP-1 gene must lie in the protein it encodes, and we therefore concentrated only on the open reading frame (ORF) of the cDNA. We amplified both the whole ORF, using flanking primers, and smaller fragments within the ORF, and gel purified the amplified DNA. Primers within the ORF were then used to amplify single stranded DNA, by limiting the concentration of one of the priming pair, and the single stranded molecule was sequenced using the limiting primer to prime the dideoxynucleotide sequencing reaction. Some regions of the gene were amplified into single-stranded DNA using a novel procedure, in which a new priming site was ligated on to a restriction fragment terminus within

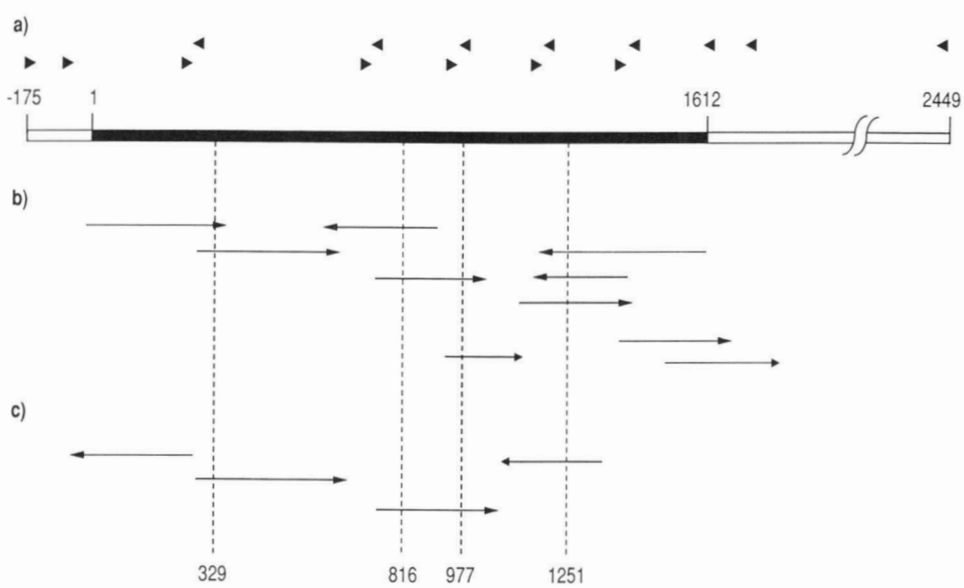

FIGURE 2.—Oligonucleotide primers and sequencing strategy for *brown* and *brown*-revertant TRP-1 cDNAs. a) Positions of oligonucleotides used for both PCR amplification of cDNA and for sequencing. The arrowheads represent the oligonucleotides, and point in a 5′ to 3′ direction. The TRP-1 mRNA is represented below, to scale, and numbered according to SHIBAHARA *et al.* (1986). The ORF is shaded dark, while the untranslated regions are open bars. b) Sequence analyses used to determine the sequence of *brown* TRP-1 cDNA, arrows pointing in the 5′ to 3′ direction. The four base differences are represented by vertical dashed lines, numbered with their position in the sequence. c) Sequence analyses of the *brown* revertant cDNA, showing the sequence reactions spanning each of the four base variants. Note that base 329 has reverted to wild type while the other three remain as in *brown*. Directions of arrows are as in b.

the ORF and this site was used to amplify single stranded cDNA. The single stranded DNA thus generated was directly sequenced using the limiting PCR primer, or internal primers. Figure 2 shows the primers used for the amplifications, and the regions sequenced of both mutant and revertant cDNA.

The sequence of the TRP-1 coding region of BALB/c mice has four differences from the published (C57BL/6-derived) sequence, at positions 329, 816, 977 and 1251) (Figure 3). We have confirmed that these are all genuine differences from wild type by resequencing the variant portions of the original pMT4 plasmid, and by sequencing genomic DNA clones of another wild-type mouse strain, 129 (I. J. JACKSON and D. CHAMBERS, unpublished results). All four differences are GC to AT pairs. Two (bases 816 and 977) could have arisen from changes of CpG dinucleotides to TpG or CpA. (The CG in each case is a contiguous dinucleotide in single exon in the genome.) The C on each strand of a CG dinucleotide is a substrate for DNA methylase, which gives rise to methylcytosine. Deamination of methylcytosine produces a thymidine/guanosine pair which, if replicated before repair, gives rise to an A being substituted for the G on one daughter strand, and so T for the methyl-C at the next replication (COULONDRE *et al.* 1978). This is a likely mechanism for the changes seen at bases 816 and 977, and implies that these CpG dinucleotides are methylated in germ-line DNA. Methylation of cytosine residues is not seen in the other dinucleotides and the other two GC to AT differences are unlikely to have occurred by this mechanism.

Two changes, at nucleotides 816 and 1251 (numbering as in SHIBAHARA *et al.* 1986) are in the third positions of codons, and do not change the encoded protein. The C to T change at base 816 eliminates a *Taq*I restriction site. This is the source of the restriction fragment length variant which was used to map the TRP-1 gene to the *brown* locus in recombinant inbred strains (JACKSON 1988). The other two changes alter the coding potential of the TRP-1 gene. The G to A transition at base 329 changes cysteine residue 86 to tyrosine, and the equivalent nucleotide change at 977 changes histidine-282 to arginine. The 4 nucleotide differences and the encoded amino acids are summarized in Figure 3.

The two amino acids changed between wild type and *brown* are conserved between wild-type TRP-1 and tyrosinases of mouse and human. In the absence of further information such a comparison would suggest that both might be important for TRP-1 function.

Nucleotide sequence of a *brown* revertant: The induction of a revertant to wild type by chemical mutagenesis of a *brown* strain indicated to us that only one nucleotide change caused the mutant phenotype.

FIGURE 3.—Sequence differences between wild-type (C57BL/6) and mutant *brown* (BALB/c) TRP-1 sequences. Portions of the C57BL/6 DNA sequence are shown, in lower case, with the variant bases in upper case, and the BALB/c variant below. The positions of the variant bases are indicated. The wild-type amino-acid sequence is shown above the DNA sequence, using single-letter code; except where the base variants alter the coding potential, in which case the three-letter code is used; the BALB/c amino acid being above the C57BL/6. The *Taq*I restriction site variant is indicated.

To confirm this, and to identify the crucial mutagenic change, we sequenced the revertant gene. ENU treatment (160 mg/kg) of 14,342 mice of the DBA/2 strain resulted in one revertant of the *brown* mutation to wild type. This is the only *brown* revertant ever identified.

Using the same PCR strategies, ds cDNA made from dorsal skin of 3-day-old homozygous *brown* revertant (b^+/b^+) was sequenced through the regions of all four *brown* base changes (Figure 2c). Three of the four variants remained the same. However, the variant A at base 329 has reverted to the original wild-type G, thus changing the codon from TAT to TGT, and restoring cysteine to the protein sequence. This A to G change is the only base substitution in the codon which will restore cysteine.

DISCUSSION

The work described here is definitive proof that TRP-1 is encoded by the *brown* locus. Furthermore,

the availability of the revertant mouse has enabled us to unequivocally show that the cysteine to tyrosine substitution seen in the *brown* TRP-1 sequence is the crucial mutagenic change and that the arginine to histidine change is probably neutral.

The sequence differences between *Black* and *brown* TRP-1: The origins of the other three nucleotide differences are unknown. We might speculate that the *brown* mutation arose in wild mice which were subsequently brought into captivity. They may be individual-specific differences, specific to the progenitor animal in which the *brown* mutation occurred, and fixed in the *brown* strains by selection of the coat-color phenotype. Alternatively they may be population-wide polymorphisms. The *Taq*I (base 816) variant is not seen in any laboratory mice, except *brown* strains, but may be found in certain wild populations. It might be possible to locate the source of the wild mouse in which the *brown* mutation originated by examining wild populations from diverse areas for this *Taq*I variant.

The arginine to histidine amino acid change is probably not common in non-*brown* mice; given the conservation of the arginine in tyrosinases. The function of TRP-1 in the revertant mice might even be subtly impaired, though the change could be chemically or functionally neutral. Further study is needed to compare the revertant pigmentation phenotype to true wild type. The presence of a second (albeit less severe) change in the mutant TRP-1 gene might result from a lack of selection pressure in the wild once the primary pathological change (to *brown*) has occurred.

It is also possible that the (unknown) mutagenic event which resulted in the *brown* mutation simultaneously caused a number of other base changes. PASTNIK *et al.* (1989), for example, noted that of 25 ENU-induced mutations in the Drosophila *vermillion* gene, three involved double base changes.

ENU mutagenesis: ENU is one of the most important experimental mutagens, and its mutagenicity has been well characterized in many systems, from bacterial to mammalian. Although the molecular nature of the ENU-induced changes have been characterized in bacteria and Drosophila, less is known about the nature of ENU-induced base changes in mammals.

ENU is mutagenic by its ethylation activity, usually of the nucleotide bases, but also of the phosphodiester backbone. The ethylated bases are misrepaired or misreplicated. In bacteria the changes are mostly (70%) GC to AT transitions, probably by unrepaired O^6-ethylguanosine mispairing with thymidine at replication (RICHARDSON *et al.* 1987), with almost all the rest being AT to GC transitions (probably due to error-prone repair of O^4-thymidine) (ZIELENSKA, BERANEK and GUTTENPLAN 1988) although transversions are also occasionally seen. In an extensive study of ENU-induced germ-line mutations at the *vermillion* locus of Drosophila, PASTNIK *et al.* (1989) showed that 61% of point mutations were of the form GC to AT, and 18% were AT to GC. The remaining 20% were transversions; AT to TA or CG and GC to CG or TA.

Almost all that is known about the molecular changes induced by ENU in mammalian systems comes from cultured cells or somatic tissues. Using an HSV-tk containing shuttle vector ECKHARDT *et al.* (1988) were able to isolate and sequence 46 ENU-induced mutations from a human lymphoblastoid cell line. Fifty percent of these were GC to AT changes, and 17% were AT to GC. Transversions made up the remaining 33%, which were largely AT to TA. Two ENU-induced mutants of the *Hprt* gene in mouse lymphoma cells in culture have been sequenced (VRIELING, SIMON and VAN ZEELAND 1988), and both affect AT pairs; one is a transition to GC, the other a transversion to TA. A recent report examined the effect of ENU mutagenesis on a *lacZ* transgene in mouse brain (GOSSEN *et al.* 1989) and found 4 of 4 changes were GC to AT transitions. In this case the cells mutated were not dividing, and the mutations cannot be due to misreplication, but rather to errors of repair.

Overall the spectrum of ENU mutagenesis in mammalian cells does not seem to be significantly different from *Escherichia coli* or the Drosophila germ line; eukaryotes may have a higher rate of transversions, but in all systems transitions predominate, and of these G to A is the major change. O^6-Ethyl-guanosine is the major premutagenic adduct. By contrast, no GC to AT changes have been found induced by ENU in the mouse germ line. The only ENU-induced germ-line mutations characterized to date have been in the globin genes, and their nature has been deduced from changes in the protein sequence (POPP *et al.* 1983; LEWIS *et al.* 1985; PETERS *et al.* 1985; PETERS 1986). All are AT changes; two transversions to TA and two transitions to GC.

The revertant sequence described here is the first report of DNA sequence analysis of an ENU-induced mutation in the mammalian germ line. It too is an AT to GC transition. We cannot draw any general conclusions from the study as the change for which we selected (the reversion of the *brown* phenotype) can only occur by this transition. Nevertheless this is direct evidence that these changes do occur, and at a frequency within our experimental range. It is possible that the mouse germ line has developed a better mechanism for repairing O^6-ethyl guanosine adducts, or that replication-linked mutagenic events predominate in the germ line.

The *albino* mutation of tyrosinase: Several different groups have reported cloning and sequencing of mouse tyrosinase cDNAs (YAMAMOTO *et al.* 1987,

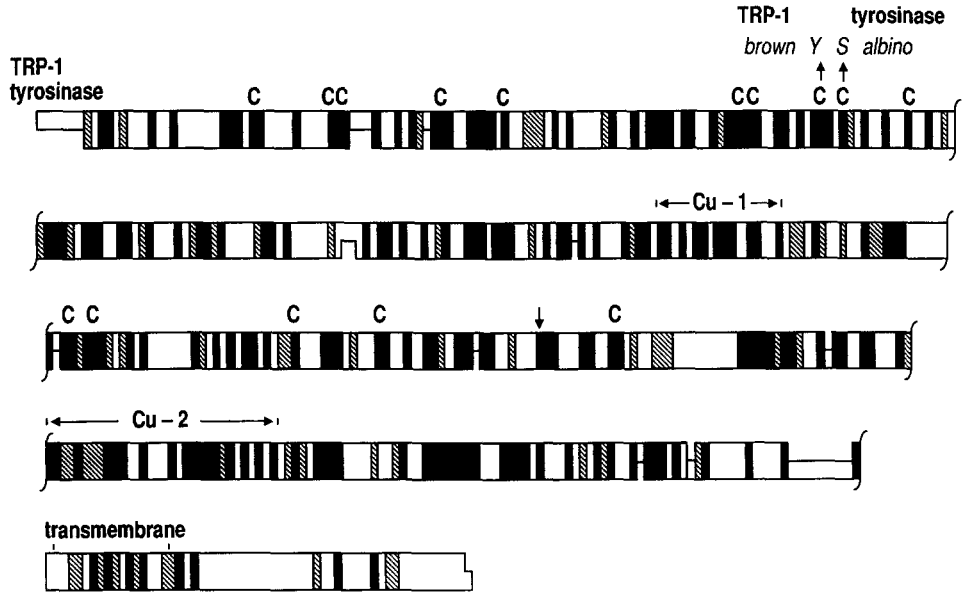

FIGURE 4.—Schematic comparison of the mouse TRP-1 and tyrosinase amino-acid sequences. The upper half of the bars represents TRP-1, the lower, tyrosinase. Where amino acids are identical the bar is shaded black, where they are functionally related the bar is hatched. Unrelated amino acids are indicated by an open bar, and the bar reduces to half width where gaps have been introduced to maximize similarity. The 15 cysteines in conserved positions are marked by a C above the bar. The sequence has been divided into five sections, to indicate five putative functional regions; two cysteine-rich regions, two copper binding domains, and the transmembrane region, with the cytoplasmic domain. The cysteine to tyrosine mutation causing the *brown* mutation of TRP-1, and the cysteine to serine mutation, the cause of the *albino* mutation of tyrosinase, are indicated using the single letter code.

1989; KWON *et al.* 1988; 1989; MULLER *et al.* 1988). All are essentially the same, except that of KWON *et al.*, in which Cys-103 is replaced by serine. This region of their sequence was obtained from BALB/c DNA, which carries the *albino* mutation in tyrosinase (KWON *et al.* 1989). Recently we have sequenced the tyrosinase gene from a melanocyte line which has reverted from *albino* to wild type, and shown that indeed the candidate mutation at residue 103 is the cause of the albino phenotype (I. J. JACKSON and D. C. Bennett, in press).

These data underline the importance of the cysteine residues in maintaining tyrosinase, and tyrosinase-related, protein function, previously inferred from their conservation. It is interesting that by contrast, the tyrosinase of Neurospora has only a single cysteine, and Streptomyces' tyrosinases have none. The more recently evolved cysteine domains in the mammalian proteins may serve a function not seen in the lower eukaryote and bacterial enzymes; they are not at the active center, defined by the copper-binding sites, but yet must have a function vital to the activity or possibly stability of the proteins, possibly within the context of the melanosome.

Figure 4 schematically illustrates the primary structure of mouse tyrosinase and TRP-1, with the pathological cysteine to tyrosine change of the *brown* mutation of TRP-1 and the pathological cysteine to serine change of the albino mutation of tyrosinase highlighted. The arrow indicates the position of the histidine-282 to arginine variant of TRP-1. The characterization of more mutations at both loci will play an important role in determining important domains and residues in this family of proteins, in particular for TRP-1, for which phenotype is the only assay.

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LITERATURE CITED

COPELAND, N. G., K. W. HUTCHISON, and N. A. JENKINS, 1983 Excision of the DBA ecotropic provirus in dilute coat-color revertants of mice occurs by homologous recombination involving the viral LTRs. Cell **33:** 379–387.

COULONDRE, C., J. H. MILLER, P. J. FARABAUGH and W. GILBERT, 1978 Molecular basis of base substitutions in *Escherichia coli*. Nature **274:** 775–780.

ECKHARDT, K. A., C. A. INGLE, D. K. KLINEDINST and N. R. DRINKWATER, 1988 Molecular analysis of mutations induced in human cells by *N*-ethyl-*N*-nitrosourea. Molecular Carcinogenesis 1:50–56.

FAVOR, J., A. NEUHAUSER-KLAUS, and U. H. EHLING, 1987a ENU and radiation-induced reverse mutations recovered in mice. Mutat. Res. **182:** 278.

FAVOR, J., A. NEUHAUSER-KLAUS and U. H. EHLING, 1987b Radiation-induced forward and reverse specific locus mutations and dominant cataract mutations in treated strain BALB/c and DBA/2 mice. Mutat. Res. **177:** 161–169.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

GOSSEN, J. A., W. J. F. DE LEEUW, C. H. T. TAN, E. C. ZWARTHOFF, F. BERENDS, P. H. M. LOHMAN, D. L. KNOOK and J. VIJG, 1989 Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutagenesis *in vivo*. Proc. Natl. Acad. Sci. USA **86:** 7971–7975.

HEARING, V. J., and M. JIMENEZ, 1989 Analysis of Mammalian Pigmentation at the molecular level. Pigment Cell Res. **2:** 75–85.

JACKSON, I. J., 1985 The genetics and biology of mouse melanocytes; mutation, migration and interaction. Trends Genet. **1:** 321–326.

JACKSON, I. J., 1988 A cDNA encoding tyrosinase-related protein maps to the mouse brown locus. Proc. Natl. Acad. Sci. USA **85:** 4392–4396.

JENKINS, N. A., N. G. COPELAND, B. A. TAYLOR and B. K. LEE, 1981 Dilute (*d*) coat colour mutation of DBA/2J mice is associated with the site of integration of an ecotropic MuLV genome. Nature **293:** 370–374.

KWON, B. S., A. K. HAQ, S. H. POMERANTZ and R. HALABAN, 1987 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.

KWON, B. S., A. K. HAQ, M. WAKULCHIK, D. KESTLER, D. E. BARTON, U. FRANKE, M. L. LAMOREUX, J. B. WHITNEY and R. HALABAN, 1989 Isolation, chromosomal mapping, and expression of the mouse tyrosinase gene. J. Invest. Dermatol. **93:** 589–594.

LEHRACH, H., D. DIAMOND, J. M. WOZNEY and H. BOEDTKER, 1977 RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:** 4743–4751.

LEWIS, S. E., F. M. JOHNSON, L. C. SKOW, D. POPP, L. B. BARNETT and R. A. POPP, 1985 A mutation in the mouse beta-globin gene detected in the progeny of a female mouse treated with ethylnitrosourea. Proc. Natl. Acad. Sci. USA **82:** 5829–5831.

LOVELL-BADGE, R. H., 1987 Introduction of DNA into embryonic stem cells, pp. 153–182 in *Teratocarcinomas and Embryonic Stem Cells; A Practical Approach*, edited by E. J. Robertson. IRL Press, Oxford.

LOVETT, M., Z.-Y. CHENG, E. M. LAMELA, T. YOKOI and C. J. EPSTEIN, 1987 Molecular markers for the agouti coat color locus of the mouse. Genetics **115:** 747–754.

MULLER, G., S. RUPPERT, E. SCHMID and G. SCHUTZ, 1988 Functional analysis of alternatively spliced tyrosinase gene transcripts. EMBO J. **7:** 2723–2730.

PASTNIK, A., C. VREEKEN, M. J. M. NIVARD, L. L. SEARLES and E. W. VOGEL,1989 Sequence analysis of *N*-ethyl-*N*-nitrosourea-induced vermilion mutations in *Drosophila melanogaster*. Genetics **123:** 123–129.

PETERS, J., 1986 Analysis of globin gene mutations induced by male mice by ethylnitrosourea. Mouse News Let. **76:** 74.

PETERS, J., S. J. ANDREWS, J. F. LOUTIT and J. B. CLEGG, 1985 A mouse beta-globin mutant that is an exact model of hemoglobin Ranier in man. Genetics **110:** 709–721.

POPP, R. A., E. G. BAILIFF, L. C. SKOW, F. H. JOHNSON and S. E. LEWIS, 1983 Analysis of a mouse alpha-globin gene mutation induced by ethylnitrosourea. Genetics **105:** 157–167.

RICHARDSON, K. K., F. C. RICHARDSON, R. M. CROSBY, J. A. SWENBERG and T. R. SKOPEK, 1987 DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*- nitrosourea. Proc. Natl. Acad. Sci. USA **84:** 344–348.

RUPPERT, S., G. MULLER, B. KWON and G. SCHUTZ, 1988 Multiple transcripts of the mouse tyrosinase gene are generated by alternative splicing. EMBO J. **7:** 2715–2722.

RUSSELL, W. L., 1951 X-ray induced mutation in mice. Cold Spring Harbor Symp. **16:** 327–336.

SAIKI, R. K., D. H. GELFAND, S. STOFFEL, S. J. SCHARF, R. HIGUCHI, G. T. HORN, K. B. MULLIS and H. A. ERLICH, 1988 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487–491.

SCHLAGER, G., and M. M. DICKIE, 1967 Spontaneous mutations and mutation rates in the house mouse. Genetics **57:** 319–330.

SCHLAGER, G., and M. M. DICKIE, 1971 Natural mutation rates in the house mouse; estimates for five specific loci and dominant mutations. Mutat. Res. **11:** 89–96.

SEARLE, A. G., 1974 Mutation induction in mice. Adv. Radiat. Biol. **4:** 131–207.

SHIBAHARA, S., Y. TOMITA, T. SAKAKURA, C. NAGER, B. CHANDHURI and R. MULLER, 1986 Cloning and expression of cDNA encoding tyrosinase. Nucleic Acids Res. **14:** 2413–2427.

SILVERS, W. K., 1979 *The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction*. Springer, New York.

SIRACUSA, L. D., L. B. RUSSELL, N. A. JENKINS and N. G. COPELAND, 1987 Allelic variation within the *Emv-15* locus defines genomic sequences closely linked to the *agouti* locus on mouse chromosome 2. Genetics **117:** 85–92.

VRIELING, H., J. W. I. M. SIMON and A. A. VAN ZEELAND, 1988 Nucleotide sequence determination of point mutations at the mouse HPRT locus using *in vitro* amplification of HPRT mRNA sequences. Mutat. Res. **198:** 107–113.

YAMAMOTO, H., S. TAKEUCHI, T. KUDO, K. MAKINO, A. NAKATA, T. SHINODA and T. TAKEUCHI, 1987 Cloning and sequencing of mouse tyrosinase cDNA. Jpn. J. Genet. **62:** 271–274.

YAMAMOTO, H., S. TAKEUCHI, T. KUDO, C. SATO and T. TAKEUCHI, 1989 Melanin production in cultured albino melanocytes transfected with mouse tyrosinase cDNA. Jpn. J. Genet. **64:** 121–135.

ZIELENSKA, M., D. BERANEK and J. B. GUTTENPLAN, 1988 Different mutational profiles induced by *N*-nitroso-*N*-ethyl urea: effects of dose and error-prone DNA repair and correlations with DNA adducts. Environ. Mol. Mutagen. **11:** 473–485.

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