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MOLECULAR BASIS OF MOUSE HIMALAYAN MUTATION

Byoung S. Kwon[1]*, Ruth Halaban[2] and Chaya Chintamaneni[1]

[1]Department of Microbiology & Immunology and The Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46223

[2]Department of Dermatology, Yale University Medical School, New Haven, Connecticut 06510

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Summary. Many different coat-colors result from the c-locus mutation in the mouse. One of these interesting mutants is a Himalayan, which produces temperature-sensitive tyrosinase, and the basis of this sensitivity remains unknown. We cultured Himalayan mouse melanocytes from the skin and constructed a cDNA library; then, we isolated the Himalayan tyrosinase cDNAs and determined the nucleotide sequence. The tyrosinase gene in the Himalayan mouse contains an A -> G change at nucleotide 1259 that alters a histidine residue to an arginine residue at amino acid 420. This histidine residue and the surrounding amino acids are conserved in their evolution from mouse to human. Interestingly, the residue with its surrounding eight amino acids are aligned between mouse b-protein and human tyrosinase. These results indicate the possibility that the altered residue at amino acid 420 of mouse tyrosinase may be important in stabilization of the tyrosinase molecule, or in interaction with other molecules, such as tyrosinase inhibitors.

Tyrosinase (monophenol, L-dopa, oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing enzyme which plays a key role in melanin biosynthesis (1). Tyrosinase catalyzes the oxidation of tyrosine to L-dopa (3.4-dihydroxyphenylalanine) and the dehydrogenation of L-dopa to dopaquinone, constituting the first two steps in the melanin biosynthetic pathway (2,3,4).

Cutaneous melanin is found in the skin, in the hair-follicles, and in the eyes. Melanin synthesis takes place in the intracellular organelle--melanosomes of melanocytes--and is transferred to surrounding keratinocytes through the dendritic process (5,6). Melanocytes in the skin and hair follicles are differentiated from neural crest, while those in the retina are from the optic cup (7).

Recently, several reports, including those from this laboratory, have described the isolation of mouse tyrosinase cDNA, and this progress has allowed us to study the molecular basis of human and mouse albinism. We (8,9) isolated a human tyrosinase cDNA and showed that the tyrosinase gene mapped to the mouse albino locus. Yamamoto

*To whom correspondence should be addressed.

Abbreviations: MSH, melanocyte-stimulating hormone; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

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et al. (10) reported the isolation of a mouse tyrosinase cDNA; however, when we (11) and Muller et al. (12) isolated a full-length mouse tyrosinase cDNA and Ruppert et al. (13) characterized the mouse tyrosinase gene, it was determined that Yamamoto's cDNA had resulted in the deletion of exon 3, and was, in fact, aberrant. A putative mouse tyrosinase cDNA isolated by Shibahara (14) has been shown to map to the mouse brown (b)-locus, and apparently encodes a melanocyte protein that is not tyrosinase (15). It is interesting to note that b- and tyrosinase proteins seem to be immunologically cross-reactive (15).

Functional analysis of the brown (b)- and c-locus cDNAs, performed after transfection of plasmid carried these clones into tyrosinase-negative cells, demonstrated that the c-locus, and not the b-locus, directed the synthesis of a protein with tyrosinase activity (12). Chromosomal mapping of mouse and human tyrosinase genes (9,16), the analyses by Muller et al. (12), and those demonstrating structural alterations in tyrosinase proteins in various c-locus mutants (17), show that the c-locus encodes the structural gene for tyrosinase, and that tyrosinase is present as a single-copy gene at mouse chromosome 7 and human chromosome 11.

Halaban et al. (17) have been successful in culturing mouse melanocytes from several mouse strains, including a Himalayan mouse mutant. These cultured melanocytes were used to study RNA-expression and immunoprecipitable tyrosinase.

In the mouse, the c-locus mutation results in many different coat-colors. One of these interesting mutants is a Himalayan (18), which produces temperature-sensitive tyrosinase (19,20); however, the basis for temperature-sensitivity in this enzyme has remained a puzzle.

This report will outline the molecular cloning of Himalayan tyrosinase cDNA, and localize the mutation-site of Himalayan tyrosinase by the comparison of nucleotide sequences between Himalayan and normal littermate tyrosinase-cDNAs. We hope, through this study, to provide insight into the nature of human tyrosinase gene mutation, as well as to suggest possible models for elucidating the underlying mechanisms involved in human albinism.

Materials and Methods

Mouse melanocyte cultures

Three littermates, C57BL/6J-C/C, C57BL/6J-C/c^h, and C57BL/6J-c^hc^h (Himalayan), were obtained from the Jackson Laboratory, Bar Harbor, Maine. Pure cultures of the melanocytes of Himalayan and C57BL/6J mouse-strains were established from the dermis of one-day-old mice by a method described elsewhere (21). Briefly, melanocytes were grown in Ham's F-10 medium containing 48 nM TPA (12-O-tetradecanoylphorbol acetate); 0.1 mM N^6-2'-dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP); 50 ug protein/ml human placental extract; 1 mM L-glutamine; 200 units/ml penicillin; 100 ug/ml streptomycin, and 10% newborn calf serum. Melanotropin (melanocyte-stimulating hormone) was also added at 0.2 uM. Melanotic Cloudman S-91, mouse melanoma cells and its amelanotic subline HPRT were grown in Ham's F-10 medium, as described (22).

Southern Blot Analysis

High molecular-weight DNA of mouse-spleens was prepared as previously described (23). Restriction endonuclease digests of DNA were electrophoresed in 0.8% agarose gel at 4°C. The gel was denatured with 0.5M NaOH, 1M-NaCl and neutralized with 1 M

tris. HCl (pH8.0), 1 M NaCl. The DNA in the gel was transferred to GeneScreenPlus as described by Southern (24), and hybridized to [^{32} P]-labeled mouse tyrosinase-cDNA. The filters were then washed 3 times at room temperature for 10 min. each in 2 X SSC and 0.1% SDS, and twice at 65°C for 45 min. each in 0.1 X SSC, 0.1% SDS.

RNA Blot Hybridization

Poly (A)$^+$ mRNA (2 ug) were fractionated on 1.2% agarose-formaldehyde gels, transferred to GeneScreenPlus (NEN, Boston, MA), and hybridized with [^{32}P]-labeled mouse tyrosinase cDNA (11). Filters were prehybridized at 42°C in 50% formamide, 5 X SSC (1 X SSC-150 mM NaCl, 15 mM sodium citrate pH 7.0), 0.1% SDS, 250 ug/ml of salmon sperm DNA and 10% dextran sulfate. Hybridization was carried out in the same buffer with nick-translated cDNA probes. Filters were washed at room temperature for 15 min in 2 X SSC, 0.1% SDS and at 42°C for 30 min in 0.1 X SSC, 0.1% SDS with several changes.

cDNA libraries and screening

Double-stranded cDNA was prepared from poly (A)$^+$ mRNA derived from Himalayan mouse melanocytes, as previously described (22). The cDNA was inserted into the unique EcoRI site of λgt11 cloning vector (25). The recombinant phage DNA was packaged in vitro as described by Grosvel et al. (26). The cDNA library was screened with [^{32}P]-labeled mouse tyrosinase-cDNA.

DNA sequencing

DNA restriction fragments, subcloned in M13 vectors (27), were sequenced by the dideoxy chain-termination technique (28).

Polymerase chain reaction and oligonucleotide hybridization

Genomic DNA of C57BL/6-C/C and Himalayan mouse was amplified by the PCR-technique (29), with Taq-DNA polymerase (Perkin-Elmer Cetus) and oligonucleotide primers, 5' GAACAATaGCTGCGAAGGCAC 3' and 5' TGACTCTTGGAGGTAGCTGTA 3', specific for mouse tyrosinase gene exon 4. A modification (lower-case letter) creates the Taq-I site. The amplified 173 base-pair segments were cleaved with Taq-I, ligated into ACC-I site M13mp18 vector, and were sequenced.

The amplified DNA segments were also run on 1.2% agarose gel, denatured, and transferred to GeneScreenPlus membrane, and were hybridized with [^{32}P]-labeled oligonucleotide probe specific to the Himalayan mouse-allele, 5' CTGTTACGGCCG 3' at 30°C in tetramethylammonium chloride solution (30).

Results

The Soutnern Blot method of analysis was applied to the tyrosinase gene restriction fragment length polymorphism linked to the Himalayan-mouse mutation. As shown in Fig. 1 (left panel) the 17 kb EcoRI-band is co-segregated with the Himalayan DNA. When the C57BL/6J-C/C littermate mouse DNA is digested with EcoRI, four bands (12.0 kb, 8.0 kb, 4.5 kb and 0.6 kb) are detected. The 12.0 kb band represents the second exon of the mouse gene; the 8.0 kb represents the third and fifth exons as a doublet; the 4.5 kb band, as the first exon and the 0.6 kb, as the fourth exon (13).

The 12.0 kb band resolves as the 17 kb band in the Himalayan homozygote (c^h/c^h), while the heterozygote C57BL/6J-C/c^h littermate shows both 12 kb and 17 kb bands. As shown in Fig. 1 (right panel) the Hind III enzyme does not detect polymorphisms in the three littermates. Enzymes Msp1 and PvuII did not produce polymorphisms (not shown). These results indicate that the three mice are littermates; however, the nature of the EcoRI polymorphism linked to the Himalayan mutation can not yet be defined. To investigate whether the 17kb band is typical for Himalayan mouse DNA, we analyzed EcoRI polymorphisms of several strains of mice. Mouse strains of Balb/c, C57BL/6, $B_{10}D_2$ and AKR/J origin generated 12kb bands, and mouse strains of CBA/J, C3A/J, and DBA/2J produced 17kb bands. The original Himalayan mutant was derived from a litter

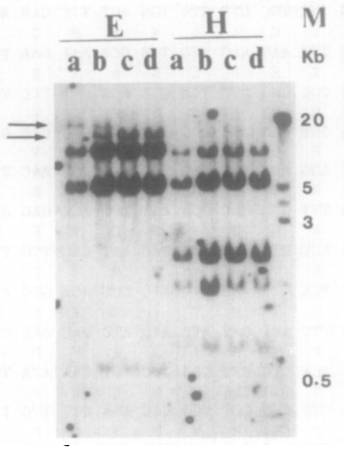

Figure 1. EcoRI restriction fragment length polymorphism of Himalayan mouse mutant.
Genomic DNA from Himalayan mouse (lane a), C57BL/6-C/c^h (lane b) and C57BL/6-C/C (lane c), and $B_{10}D_2$ (lane d) were digested with EcoRI (left panel) or Hind III (right panel). The DNAs were fractionated on a 0.8% agarose gel, transferred to GeneScreenPlus membrane, and hybridized to mouse tyrosinase cDNA, MTY811. Arrows indicate two polymorphic bands, 17kb and 12kb. "M" indicates the molecular size-markers.

of DBA/2J and AKR/J cross (18). Therefore, the 17kb band seen in the Himalayan mouse DNA may not be responsible for the Himalayan mouse phenotype; instead, it may be indicative of the origin of the mouse strain (DBA/2J).

Northern blot analysis (Fig. 2) shows the abundance and size of the Himalayan mouse tyrosinase RNA to be similar to those of the C57BL/6 mouse, indicating that the Himalayan mutation lies in the coding region of the tyrosinase gene. This prediction was further supported by a report by Halaban et al. (17), who showed that the Himalayan tyrosinase was similar in size to the de novo form of normal tyrosinase; however, only a fraction of the tyrosinase was fully processed in that study.

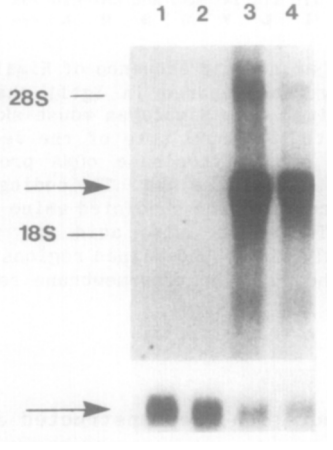

Figure 2. Northern blot analysis of Himalayan melanocyte RNA.
Five ug of poly(A)$^+$ RNA from melanotic Cloudman S-91 (lane 1), amelanotic subline of Cloudman S-91 (lane 2), Himalayan (lane 3), and C57BL/6 (lane 4) melanocytes was run on 1.2% denaturing agarose gel, blotted and hybridized to MTY811 mouse tyrosinase cDNA probe (upper arrow). The same filter hybridized to Pmel 14-2 as a control (21) (lower arrow). Positions of 28S and 18S mRNA markers are each indicated.

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   1 ATG TTC TTG GCT GTT TTG TAT TGC CTT CTG TGG AGT TTC CAG ATC TCT GAT GGC CAT TTT   60
   1  M  F  L  A  V  L  Y  C  L  L  W  S  F  Q  I  S  D  G  H  F                    20
  61 CCT CGA GCC TGT GCC TCC TCT AAG AAC TTG TTG GCA AAA GAA TGC TGC CCA CCA TGG ATC  120
  21  P  R  A  C  A  S  S  K  N  L  L  A  K  E  C  C  P  P  W  M                    40
 121 GGT GAT GGG AGT CCC TGC GGC CAG CTT TCA GGC AGA GGT TCC TGC CAG GAT ATC CTT CTG  180
  41  G  D  G  S  P  C  G  Q  L  S  G  R  G  S  C  Q  D  I  L  L                    60
 181 TCC AGT GCA CCA TCT GGA CCT CAG TTC CCC TTC AAA GGG GTG GAT GAC CGT GAG TCC TGG  240
  61  S  S  A  P  S  G  P  Q  F  P  F  K  G  V  D  D  R  E  S  W                    80
 241 CCC TCT GTG TTT TAT AAT AGG ACC TGC CAG TGC TCA GGC AAC TTC ATG GGT TTC AAC TGC  300
  81  P  S  V  F  Y  N  R  T  C  Q  C  S  G  N  F  M  G  F  N  C                   100
 301 GGA AAC TGT AAG TTT GGA TTT GGG GGC CCA AAT TGT ACA GAG AAG CGA GTC TTG ATT AGA  360
 101  G  N  C  K  F  G  F  G  G  P  N  C  T  E  K  R  V  L  I  R                   120
 361 AGA AAC ATT TTT GAT TTG AGT GTC TCC GAA AAG AAT AAG TTC TTT TCT TAC CTC ACT TTA  420
 121  R  N  I  F  D  L  S  V  S  E  K  N  K  F  F  S  Y  L  T  L                   140
 421 GCA AAA CAT ACT ATC AGC TCA GTC TAT GTC ATC CCC ACA GGC ACC TAT GGC CAA ATG AAC  480
 141  A  K  H  T  I  S  S  V  Y  V  I  P  T  G  T  Y  G  Q  M  N                   160
 481 AAT GGG TCA ACA CCC ATG TTT AAT GAT ATC AAC ATC TAC GAC CTC TTT GTA TGG ATG CAT  540
 161  N  G  S  T  P  M  F  N  D  I  N  I  Y  D  L  F  V  W  M  H                   180
 541 TAC TAT GTG TCA AGG GAC ACA CTG CTT GGG GGC TCT GAA ATA TGG AGG CAG ATT GAT TTT  600
 181  Y  Y  V  S  R  D  T  L  L  G  G  S  E  I  W  R  D  I  D  F                   200
 601 GCC CAT GAA GCA CCA GGG TTT CTG CCT TGG CAC AGA CTT TTC TTG TTA TTG TGG GAA CAA  660
 201  A  H  E  A  P  G  F  L  P  W  H  R  L  F  L  L  L  W  E  Q                   220
 661 GAA ATT CGA GAA CTA ACT GGG GAT GAG AAC TTC ACT GTT CCA TAC TGG GAT TGG AGA GAT  720
 221  E  I  R  E  L  T  G  D  E  N  F  T  V  P  Y  W  D  W  R  D                   240
 721 GCA GAA AAC TGT GAC ATT TGC ACA GAT GAG TAC TTG GGA GGT CGT CAC CCT GAA AAT CCT  780
 241  A  E  N  C  D  I  C  T  D  E  Y  L  G  G  R  H  P  E  N  P                   260
 781 AAC TTA CTC AGC CCA GCA TCC TTC TTC TCC TCC TGG CAG ATC ATT TGT AGC AGA TCA GAA  840
 261  N  L  L  S  P  A  S  F  F  S  S  W  Q  I  I  C  S  R  S  E                   280
 841 GAG TAT AAT AGC CAT CAG GTT TTA TGC GAT GGA ACA CCT GAG GGA CCA CTA TTA CGT AAT  900
 281  E  Y  N  S  H  Q  V  L  C  D  G  T  P  E  G  P  L  L  R  N                   300
 901 CCT GGA AAC CAT GAC AAA GCC AAA ACC CCC AGG CTC CCA TCT TCA GCA GAT GTG GAA TTT  960
 301  P  G  N  H  D  K  A  K  T  P  R  L  P  S  S  A  D  V  E  F                   320
 961 TGT CTG AGT TTG ACC CAG TAT GAA TCT GGA TCA ATG GAT AGA ACT GCC AAT TTC AGC TTT 1020
 321  C  L  S  L  T  Q  Y  E  S  G  S  M  D  R  T  A  N  F  S  F                   340
1021 AGA AAC ACA CTG GAA GGA TTT GCC AGT CCA GTC ACA GGG ATA GCA GAT CCT TCT CAA AGT 1080
 341  R  N  T  L  L  G  F  A  S  P  L  T  G  I  A  D  P  S  Q  S                   360
1081 AGC ATG CAC AAT GCC TTA CAT ATC TTT ATG AAT GGA ACA ATG TCC CAA GTA CAG GGA TCG 1140
 361  S  M  H  N  A  L  H  I  F  M  N  G  T  M  S  Q  V  Q  G  S                   380
1141 GCC AAC GAT CCC ATT TTT CTT CTT CAC CAT GCT TTT GTG GAC AGT ATT TTT GAA CAA TGG 1200
 381  A  N  D  P  I  F  L  L  H  H  A  F  V  D  S  I  F  E  Q  W                   400
1201 CTG CGA AGG CAC CGC CCT CTT TTG GAA GTT TAC CCA GAA GCC AAT GCA CCT ATC GGC CGT 1260
 401  L  R  R  H  R  P  L  L  E  V  Y  P  E  A  N  A  P  I  G  R                   420
1261 AAC AGA GAC TCT TAC ATG GTT CCT TTC ATA CCG CTC TAT AGA AAT GGT GAT TTC TTC ATA 1320
 421  N  R  D  S  Y  M  V  P  F  I  P  L  Y  R  N  G  D  F  F  I                   440
1321 ACA TCC AAG GAT CTG GGA TAT GAC TAC AGC TAC CTC CAA GAG TCA GAT CCA GGC TTT TAC 1380
 441  T  S  K  D  L  G  Y  D  Y  S  Y  L  Q  E  S  D  P  G  F  Y                   460
1381 AGA AAT TAT ATT GAG CCT TAC TTG GAA CAA GCC AGT CGT ATC TGG CCA TGG CTT CTT GGG 1440
 461  R  N  Y  I  E  P  Y  L  E  Q  A  S  R  I  W  P  W  L  L  G                   480
1441 GCA GCA CTG GTG GGA GCT GTT ATT GCT GCA GCT CTC TCT GGG CTT AGC AGT AGG CTA TGC 1500
 481  A  A  L  V  G  A  V  I  A  A  A  L  S  G  L  S  S  R  L  C                   500
1501 CTT CAG AAG AAG AAG AAG AAG AAG CAA CCC CAG GAG GAA AGG CAG CCA CTC CTC ATG GAC 1560
 501  L  Q  K  K  K  K  K  K  Q  P  Q  E  E  R  Q  P  L  L  M  D                   520
1561 AAA GAC GAC TAC CAC AGC TTG CTG TAT CAG AGC CAT CTG TGA
 521  K  D  D  Y  H  S  L  L  Y  Q  S  H  L ---                                    533
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Figure 3. Nucleotide and amino acid sequence of Himalayan mouse tyrosinase cDNA. A complementary cDNA library was prepared in λgt11 phage, with oligo d(T)-primed double-stranded cDNA synthesized from Himalayan mouse-skin melanocyte poly(A)$^+$ RNA, then ligated with linkers into the EcoRI site of the vector. The cDNA library was screened with [^{32}P]-labeled mouse tyrosinase cDNA probe, MTY811. The complete sequences of two cDNA inserts were determined. The coding region of the cDNA sequence is shown. The single-letter codes of the predicted amino acid sequence is shown below the nucleotide sequence. The boxed amino acid and triplet code indicates the Himalayan mutation (CAT -> CGT) site. Underlined regions show the oligomer sequences used in the PCR reaction. The putative transmembrane region was indicated by thick underline.

To localize the Himalayan mutation, we constructed a cDNA library from Himalayan melanocyte RNA; then, we isolated the Himalayan tyrosinase cDNA. Two cDNAs were fully sequenced and compared with published C57BL/6 mouse tyrosinase cDNA.

As shown in Fig. 3, there is a single nucleotide change A -> G at nucleotide 1259, which resulted in an amino-acid change, His to Arg, at amino-acid residue 420. The

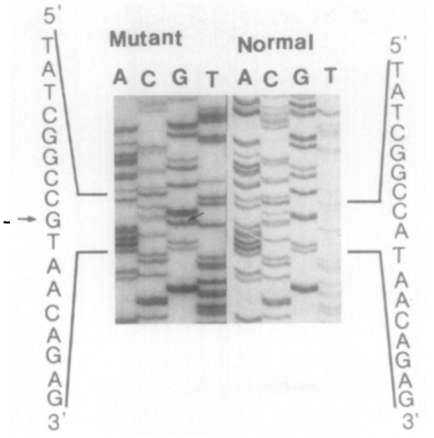

Figure 4. Nucleotide sequence of mutation site of Himalayan mouse tyrosinase gene.
Genomic DNA from C57BL/6-C/C and Himalayan mouse was amplified by the PCR technique with Taq DNA polymerase and oligonucleotide primers specific for exon 4 of the mouse tyrosinase gene. The amplified 173-base-pair segment was cleaved with Taq-I, ligated into the ACC-I site of M13mp18 vector and sequenced. "Normal" indicates C57BL/6-C/C sequence, and "mutant" indicates Himalayan mouse sequence with A -> G change (arrows). The sequence is labeled 5' and 3' in reference to the orientation of the tyrosinase gene.

rest of the sequences were exactly the same as the mouse tyrosinase cDNA sequence from B16 melanoma, published by Muller et al. (12). This histidine residue and surrounding amino acids bear witness to the evolutionary conservation from mouse to human tyrosinase. Interestingly, the histidine residue and surrounding eight amino acids are aligned between mouse b-protein and human tyrosinase (9). These results may indicate that the histidine residue at amino acid 420 of mouse tyrosinase plays an important role in stabilizing tyrosinase molecule, or in interacting with other molecules such as tyrosinase inhibitor (20) for enzyme activity.

To ensure that the mutation which we had identified was the bona fide Himalayan mutation, and not an artifact from cDNA cloning, we amplified, by the polymerase chain-reaction method, the 4th exon sequence of mouse tyrosinase, which contained the mutation site. Two synthetic 21-base oligomernucleotides were then used as specific primers to amplify a 173-bp segment of the 4th exon of the gene. Both C57BL/6J-C/C and C57BL/6J-$c^h c^h$ were sequenced. As shown in Fig. 4, the nucleotide change A -> G in Himalayan genomic DNA was confirmed in this experiment. The PCR-amplified segments of C57BL/6-C/C, C57BL/6C/c^h and Himalayan DNA were blotted onto Genescreen Plus membrane and hybridized with the Himalayan allele-specific probe. In Fig. 5, DNA of only Himalayan and heterozygote littermates was hybridized with the probe. This result validates our finding of an A -> G mutation in the Himalayan mouse tyrosinase gene.

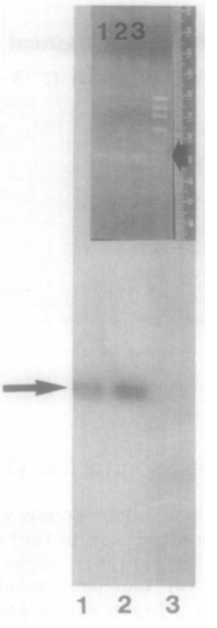

Figure 5. Genotype analysis of Himalayan mouse mutant.
Genomic DNA from Himalayan mouse (lane 1), C57BL/6-C/c^h (lane 2) and C57BL/6-C/C (lane 3) was amplified by using the PCR technique and oligonucleotide primers specific for exon 4 of mouse tyrosinase gene. The amplified 173-base-pair segment was run on 1.2% agarose gel, transferred to GeneScreenPlus membrane, and hybridized to the Himalayan mutation-specific probe. The inset shows the three amplified 173-base-pair fragment in agarose gel (thick arrow). Bottom arrow indicates the specific hybridization to Himalayan mouse mutation (A → G).

Discussion

Ever since the first report of Himalayan mouse mutants which were mapped to the c-locus, the basis for temperature-sensitivity in Himalayan tyrosinase has been an intriguing question. Coleman (19) suggested that the Himalayan mouse tyrosinase is heat-labile at normal body temperature as a consequence of conformational changes in the enzyme protein. Kidson and Fabian (20) found a low molecular-weight tyrosinase-inhibitor from Himalayan mouse-skin homogenates, and proposed that temperature controls the affinity of tyrosinase for its inhibitor, causing Himalayan tyrosinase to bind more strongly to the inhibitor at normal body temperatures than at lower body temperatures. This change in affinity of the enzyme for the inhibitor is modulated by temperature-induced conformational changes of either the enzyme or the inhibitor, or both. This hypothesis is reasonable, since there have been reports on the existence of tyrosinase inhibitors in mouse melanomas (31,32,33) and albino mice (34). Coleman and Kidson and Fabian all have suggested that the basis for temperature-sensitivity in Himalayan tyrosinase is conformational change in the enzyme induced by normal body temperature.

The finding in this laboratory of a single amino-acid change in the highly-conserved region between tyrosinase and the b-protein may suggest that the mutated

region is important for the stabilization of enzyme structure, and that body temperature may change the conformation of the Himalayan enzyme. This postulation is further supported by the following observations: 1) the histidine residue at 420 does not appear to be involved as a critical site for enzyme function, such as the copper-binding sites (35); 2) The histidine residue may not be in the catalytic site of the enzyme, because the residue is located near the transmembrane region.

Halaban et al. (17) recently observed that the majority of the immunoprecipitable Himalayan tyrosinase is not N-glycosylated, and this observation suggests the possibility that temperature-sensitivity may result from the N-glycosylation deficiency. We have localized the mutation site of Himalayan tyrosinase. Now, we are in a position to test, by mutagenesis and in vitro expression, whether the temperature-induced low enzyme activity is due not only to conformational change, but also to N-glycosylation deficiency.

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