

# A single base insertion in the putative transmembrane domain of the tyrosinase gene as a cause for tyrosinase-negative oculocutaneous albinism

(human albinism/skin pigmentation/insertional mutation/prenatal diagnosis/melanocyte culture)

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**ABSTRACT** We have determined a molecular defect to be the likely basis for inactivity of the tyrosinase (EC 1.14.18.1) from a patient with tyrosinase-negative oculocutaneous albinism. A single base (thymine) was inserted in exon 5 of the tyrosinase gene following codon 471 in the putative transmembrane coding region. This insertion caused a shift in the reading frame of 19 amino acids at the 3' end and introduced a premature termination signal that would be expected to truncate the protein by 21 amino acids at the carboxyl terminus. The albino tyrosinase was not recognized by antibodies directed to the carboxyl terminus of tyrosinase. Furthermore, as shown by gel electrophoresis of the immunoprecipitated protein, the tyrosinase was  $\approx 3$  kDa smaller than normal. Similar immunoprecipitation data were obtained when cloned normal and mutant tyrosinases were expressed in COS-1 cells.

Oculocutaneous albinism (OCA) is a syndrome that encompasses a group of individual inborn errors, each inherited as a Mendelian autosomal recessive trait and characterized by the absence or near absence of melanin pigmentation in the skin, hair, and eyes. To date, depending upon the classification method, there are 8 or 11 recognized forms of OCA (1, 2). No melanin is produced in the absence of tyrosinase, a copper-containing glycoprotein (3). Tyrosinase (monophenol, 3,4-dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) catalyzes three reactions in the melanin biosynthetic pathway: hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (dopa), oxidation of dopa to dopaquinone, and oxidation of 5,6-dihydroxyindole to indole-5,6-quinone (3-6). OCA is a serious disorder since the deficiency of melanin produces visual difficulties such as nystagmus, strabismus, photophobia, and astigmatism. The cutaneous photosensitivity results in a predisposition of the skin to cancer (1). The most severe form of OCA is tyrosinase-negative albinism, in which, by definition, tyrosinase activity is not detected in the hair bulbs (7). The incidence of this form of OCA in the United States is estimated at 1:39,000 in the Caucasian population and 1:28,000 in the Black population (1).

We have isolated a human tyrosinase cDNA (8) and used it to show that the tyrosinase gene is present as a single copy per haploid genome at the *TYR* locus on human chromosome 11 (9) and at the *c* locus on mouse chromosome 7 (8). The availability of tyrosinase cDNA enabled us and others to identify mutations in human and murine tyrosinase-negative albinos (10-14). In this report, we show a mutation in the tyrosinase gene of a tyrosinase-negative albino patient that

resulted in an inactive enzyme with an altered carboxyl terminus.

## MATERIALS AND METHODS

**Cell Cultures.** Tyrosinase-negative albino melanocytes from the proband and normal human control melanocytes were cultured from shaved skin biopsy samples as described (15). The melanocytes were grown in Ham's F-10 medium (American Biorganics, North Tonawanda, NY) supplemented with penicillin (200 units/ml), streptomycin (100  $\mu$ g/ml), L-glutamine (1 mM), newborn calf serum (2.5%), and calf serum (2.5%) (both from GIBCO), phorbol 12-myristate 13-acetate (85 nM, Chemsyn Science Laboratories, Lenexa, KS), 3-isobutyl-1-methylxanthine (0.1 mM, Sigma), insulin (5  $\mu$ g/ml), and bovine pituitary extract (40  $\mu$ g/ml). Jurkat and MOLT-3 are T-cell leukemia lines and were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10%) streptomycin (100  $\mu$ g/ml), and penicillin (100 units/ml). COS-1 cells were grown in Dulbecco's modified Eagle's medium containing fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100  $\mu$ g/ml).

**Southern Blot Hybridization.** High molecular weight human genomic DNA was prepared as described (8). DNA digested with *Taq* I restriction endonuclease was electrophoresed in 1% agarose gels at 4°C, transferred to GeneScreenPlus membrane (DuPont/NEN) as described by Southern (16), and hybridized to <sup>32</sup>P-labeled normal human tyrosinase cDNA Pmel34 (8) at 65°C. The blot was then washed twice in 2 $\times$  standard saline citrate (SSC) for 5 min at room temperature, twice at 65°C in 2 $\times$  SSC/1% SDS for 30 min, and twice with 0.1 $\times$  SSC for 30 min at room temperature. The blot was autoradiographed for 2 days at -80°C.

**RNA Blot Analysis.** Poly(A)<sup>+</sup> RNA samples from normal human melanocytes, the proband tyrosinase-negative albino melanocytes, human melanotic and amelanotic melanoma cells, and Jurkat and MOLT-3 were fractionated in formaldehyde/1.4% agarose denaturing gels (17) and transferred to GeneScreenPlus membrane. The blot was hybridized to nick-translated normal human tyrosinase cDNA Pmel34 in 50% (vol/vol) formamide/10% (wt/vol) dextran sulfate/1 M sodium chloride/1% (wt/vol) SDS containing 100  $\mu$ g of sheared salmon sperm DNA per milliliter.

**Construction and Screening of cDNA Library.** The normal tyrosinase cDNA library was constructed and screened as described (8). A cDNA library was also prepared from poly(A)<sup>+</sup> RNA derived from the proband's tyrosinase-negative albino melanocytes and cloned in a  $\lambda$ gt11 cloning

vector (18, 19). The latter library was screened with two different  $^{32}\text{P}$ -labeled oligomers, which represented the 5' and 3' ends of normal tyrosinase cDNA.

**DNA Sequencing.** Restriction fragments of cDNA from normal human melanocytes were subcloned into a M13 vector for sequence determination by the dideoxy chain-termination method (20). The albino tyrosinase cDNAs were sequenced by the double-stranded sequencing method (21) using various oligonucleotides corresponding to different regions of the tyrosinase cDNA. The 3' region of the *Bgl* II-*Eco*RI fragment of the albino tyrosinase cDNA was subcloned into the M13mp8 *Bam*HI/*Eco*RI site and sequenced by the dideoxy chain-termination method.

**Tyrosinase Assay.** Tyrosinase activity was determined in anagen hair bulbs as described by King and Witkop (22). Tyrosinase activity in melanocyte extracts was measured by the method of Pomerantz (23); 1 unit of enzyme is defined as the activity that catalyzes the oxidation of 1  $\mu\text{mol}$  of tyrosine per minute.

**Immunoprecipitation.** Melanocytes in culture were deprived of methionine and cysteine for 4 hr and pulsed for 15 min with  $\text{Trans}^{35}\text{S}$ -label (500  $\mu\text{Ci}/\text{ml}$ ; ICN). The cells were lysed in phosphate-buffered saline (PBS: pH 7.4)/1% (vol/vol) Nonidet P-40/0.1% (wt/vol) SDS/0.1 mM phenylmethylsulfonyl fluoride and subjected to immunoprecipitation with either polyclonal anti-tyrosinase antibodies raised in rabbits against hamster tyrosinase ( $\alpha$ -tyr) (15) or with antibodies raised against a synthetic peptide spanning the carboxyl-terminal 15 amino acids of mouse tyrosinase ( $\alpha$ -PEP7, a gift from V. J. Hearing, National Cancer Institute, Bethesda, MD; ref. 24). Twelve of these 15 amino acids are identical in murine and human tyrosinase. Immune complexes were resolved by PAGE and fluorographed at  $-75^\circ\text{C}$  for 1 or 2 days ( $\alpha$ -tyr,  $\alpha$ -PEP7, respectively).

**Transfection of COS-1 Cells with Normal and Albino Tyrosinase cDNA.** COS-1 cells were grown to 30–50% confluency in Dulbecco's modified Eagle's medium as described above and transfected by the DEAE-dextran method (25) with 4  $\mu\text{g}$  of plasmid DNA containing normal and albino tyrosinase cDNA inserts. Normal tyrosinase cDNA clone Pmel34 lacks nucleotide A of the first ATG codon (8). Therefore, we constructed a new clone, Pmel34A, which contained the initiation ATG codon, by replacing the 5'-end *Eco*RI-*Hgi*AI fragment of Pmel34 with the corresponding fragment containing the first ATG codon. This construct and the albino cDNA were ligated to expression vector pXM (26) and used in the transfection of COS-1 cells.

Forty-four hours after transfection, the cells were incubated for 4 hr in methionine/cysteine-free medium and pulsed with  $\text{Trans}^{35}\text{S}$ -label (500  $\mu\text{Ci}/\text{ml}$ ) for 15 min. The cultures were washed twice with PBS, harvested by scraping, and lysed in PBS/1.0% Nonidet P-40/0.1% SDS with protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; leupeptin, pepstatin, chymostatin, and bestatin, each 1  $\mu\text{g}/\text{ml}$ ) for 2 hr on ice. The lysates were centrifuged at  $10,000 \times g$  for 10 min. The supernatants were used for immunoprecipitation as described above.

## RESULTS

***Taq* I Restriction Fragment Length Polymorphism of the Proband and His Parents.** The proband is a 1.5-year-old Caucasian boy with tyrosinase-negative OCA. His parents, both Caucasians of Irish descent, are of normal phenotype but are heterozygotes with respect to tyrosinase activity as determined by the hair-bulb tyrosinase assay (22). Southern blot analysis of the genomic DNA of this family revealed *Taq* I polymorphic fragments of either 2.4 kb (father and child) or 2.8 kb (mother and child) and four nonpolymorphic fragments of 0.6, 0.9, 1.4, and 5.0 kb (all three) (Fig. 1). This polymor-

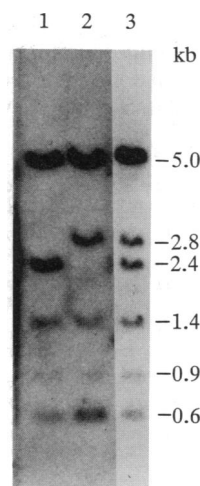


FIG. 1. Southern blot analysis of *Taq* I-digested genomic DNA of a tyrosinase-negative albino proband and his parents. High molecular weight genomic DNA was digested with *Taq* I, electrophoresed in a 1% agarose gel, transferred to GeneScreenPlus membrane, and hybridized to  $^{32}\text{P}$ -labeled normal human tyrosinase cDNA Pmel34 (8). Lane 1, father; lane 2, mother; and lane 3, proband. Marker sizes are shown in kilobases (kb).

phism does not correlate with OCA, since unrelated normal individuals have the same frequency of polymorphism at these two alleles (ref. 27 and unpublished data). However, this analysis indicates that the tyrosinase gene of the proband is not associated with deletions or rearrangements detectable at this level.

**RNA Blot Analysis.** To determine whether tyrosinase was transcribed normally, Northern blots of poly(A)<sup>+</sup> RNA from the proband melanocytes were compared with those from normal melanocytes (Fig. 2). There was no difference in size or abundance of the proband tyrosinase mRNA relative to the tyrosinase mRNA from normal melanocytes or melanoma cells, which suggests that at this level as well there were no major defects of transcription.

**Isolation and Sequencing of Albino Tyrosinase cDNA Clones.** The melanocytes of the proband were amelanotic but

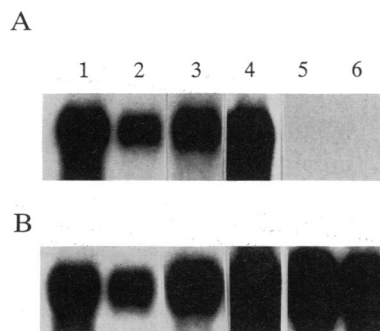


FIG. 2. Northern blot analysis of poly(A)<sup>+</sup> RNA derived from cultures of melanocytic and nonmelanocytic cell lines. (A) Poly(A)<sup>+</sup> RNA samples from normal human melanocytes (lane 1), the proband's tyrosinase-negative albino melanocytes (lane 2), human amelanotic melanoma cells (lane 3), human melanotic melanoma cells (lane 4), Jurkat T cells (lane 5), and MOLT-3 T cells (lane 6) were fractionated in a formaldehyde/1.4% agarose denaturing gel, blotted onto GeneScreenPlus membrane, and hybridized to  $^{32}\text{P}$ -labeled Pmel34. (B) The same blot was stripped and hybridized to nick-translated human  $\gamma$ -actin cDNA to show the amount of mRNA loaded in each lane. Tyrosinase mRNA was not detected in cells from nonmelanocytic origin (Jurkat and MOLT-3). The difference in the intensity of the tyrosinase bands in A is due to different amounts of poly(A)<sup>+</sup> RNA loaded onto the gel as evidenced by subsequent hybridization of the same blot to a  $\gamma$ -actin probe in B.

had residual tyrosinase activity, 5.7 microunits/mg of protein in comparison with 1673 microunits/mg in neonatal melanocytes pooled from normal donors, as measured *in vitro* by the method of Pomerantz (23). Since there was no decrease in the

amount or size of the tyrosinase mRNA or major deletion in the tyrosinase gene, we investigated the possibility of a pathogenic point mutation. We constructed a cDNA library from the poly(A)<sup>+</sup> RNA of the cultured albino melanocytes

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-18                               T CCTGCAGACC TTGTGAGGAC TAGAGGAAGA
1  ATG CTC CTG GCT GTT TTG TAC TGC CTG CTG TGG AGT TTC CAG ACC TCC GCT GGC CAT TTC
   Met Leu Leu Ala Val Leu Tyr Cys Leu Leu Trp Ser Phe Gln Thr Ser Ala Gly His Phe
   10
61  CCT AGA GCC TGT GTC TCC TCT AAG AAC CTG ATG GAG AAG GAA TGC TGT CCA CCG TGG AGC
   Pro Arg Ala Cys Val Ser Ser Lys Asn Leu Met Glu Lys Glu Cys Cys Pro Pro Trp Ser
   20
121 GGG GAC AGG AGT CCC TGT GGC CAG CTT TCA GGC AGA GGT TCC TGT CAG AAT ATC CTT CTG
   Gln Asp Arg Ser Pro Cys Gly Leu Ser Gly Arg Gly Ser Cys Gln Asn Ile Leu Leu
   30
181 TCC AAT GCA CCA CTT GGG CCT CAA TTT CCC TTC ACA GGG GTG GAT GAC CGG GAG TCG TGG
   Ser Asn Ala Pro Leu Gly Pro Gln Phe Pro Phe Thr Gly Val Asp Asp Arg Glu Ser Trp
   40
241 CCT TCC GTC TTT TAT AAT AGG ACC TGC CAG TGC TCT GGC AAC TTC ATG GGA TTC AAC TGT
   Pro Ser Val Phe Tyr Asn Arg Thr Cys Gln Cys Ser Gly Asn Phe Met Gly Phe Asn Cys
   50
301 GGA AAC TGC AAG TTT GGC TTT TGG GGA CCA AAC TGC ACA GAG AGA CGA CTC TTC CTG AGA
   Gly Asn Cys Lys Phe Gly Phe Trp Gly Pro Asn Cys Thr Glu Arg Arg Leu Leu Val Arg
   60
361 AGA AAC ATC TTC GAT TTG AGT GCC CCA GAG AAG GAC AAA TTT TTT GCC TAC CTC ACT TTA
   Arg Asn Ile Phe Asp Leu Ser Ala Pro Glu Lys Asp Lys Phe Phe Ala Tyr Leu Thr Leu
   70
421 GCA AAG CAT ACC ATC AGC TCA GAC TAT GTC ATC CCC ATA GGG ACC TAT GGC CAA ATG AAA
   Ala Lys His Thr Ile Ser Ser Asp Tyr Val Ile Pro Ile Gly Thr Tyr Gly Gln Met Lys
   80
481 AAT GGA TCA ACA CCC ATG TTT AAC GAC ATC AAT ATT TAT GAC CTC TTT GTC TGG ATG CAT ATA
   Asn Gly Ser Thr Pro Met Phe Asn Asp Ile Asn Ile Tyr Asp Leu Phe Val Trp Met His Ile
   90
541 TAT TAT GTG TCA ATG GAT GCA CTG CTT GGG GGA TAT GAA ATC TGG AGA GAC ATT GAT TTT TCT
   Tyr Tyr Val Ser Met Asp Ala Leu Leu Gly Gly Tyr Glu Ile Trp Arg Asp Ile Asp Ser
   100
601 GCC CAT GAA GCA CCA GCT TTT CTG CCT TGG CAT AGA CTC TTC TTG TTG CGG TGG GAA CAA
   Ala His Glu Ala Pro Ala Phe Leu Pro Trp His Arg Leu Phe Leu Leu Arg Trp Glu Gln
   110
661 GAA ATC CAG AAG CTG ACA GGA GAT GAA AAC TTC ACT ATT CCA TAT TGG GAC TGG CGG GAT
   Glu Ile Gln Lys Leu Thr Gly Asp Ala Leu Leu Asn Phe Thr Ile Ser Tyr Trp Asp Trp Arg Asp
   120
721 GCA GAA AAG TGT GAC ATT TGC ACA GAT GAG TAC ATG GGA GGT CAG CAC CCC ACA AAT CCT
   Ala Glu Lys Cys Asp Ile Cys Thr Asp Glu Tyr Met Gly Gly Gln His Pro Thr Asn Pro
   130
781 AAC TTA CTC AGC CCA GCA TCA TTC TTC TCC TCT TGG CAG ATT GTC TGT AGC CGA TTG GAG
   Asn Leu Leu Ser Pro Ala Ser Phe Phe Ser Ser Trp Gln Ile Val Cys Ser Arg Leu Glu
   140
841 GAG TAC AAC AGC CAT CAG TCT TTA TGC AAT GGA ACG CCC GAG GGA CCT TTA CGG CGT AAT
   Glu Tyr Asn Ser His Gln Ser Leu Cys Asn Gly Thr Pro Glu Gly Pro Leu Arg Ser Asn
   150
901 CCT GGA AAC CAT GAC AAA TCC ACA ACC CCA AGG CTC CCC TCT TCA GCT GAT GTA GAA TTT AGA
   Pro Gly Asn His Asp Lys Ser Thr Thr Pro Arg Leu Pro Ser Ser Ala Asp Val Glu Phe Arg
   160
961 TGC CTG AGT TTG ACC CAA TAT GAA TCT GGT TCC ATG GAT AAA GCT GCC AAT TTC AGC TTT
   Cys Leu Ser Leu Thr Gln Tyr Glu Ser Gly Ser Met Asp Lys Ala Ala Asn Phe Ser Phe
   170
1021 AGA AAT ACA CTG GAA GGA TTT GCT AGT CCA CTT ACT GGG ATA GCG GAT GCC TCT CAA AGC
   Arg Asn Thr Leu Glu Gly Phe Ala Ser Pro Leu Thr Gly Ile Ala Asp Ala Ser Gln Ser
   180
1081 AGC ATG CAC AAT GCC TTG CAC ATC TAT ATG AAT GGA ACA ATG TCC CAG GTA CAG GGA TCT
   Ser Met His Asn Ala Leu His Ile Tyr Met Asn Gly Thr Met Ser Gln Val Gln Gly Ser
   190
1141 GCC AAC GAT CCT ATC TTC CTT CTT CAC CAT GCA TTT GTT GAC AGT ATT TTT GAG CAG TGG
   Ala Asn Asp Pro Ile Phe Leu Leu His His Ala Phe Val Asp Ser Ile Phe Glu Gln Trp
   200
1201 CTC CAA AGG CAC CGT CCT CTT CAA GAA GTT TAT CCA GAA GCC AAT GCA CCC ATT GGA CAT CGA
   Leu Gln Arg His Arg Pro Leu Gln Glu Val Tyr Pro Glu Ala Asn Ala Pro Ile Gly His Arg
   210
1261 AAC CGG GAA TCC TAC ATG GTT CCT TTT ATA CCA CTG TAC AGA AAT GGT GAT TTC TTT ATT
   Asn Arg Glu Ser Tyr Met Val Pro Phe Ile Pro Leu Tyr Arg Asn Gly Asp Phe Ile
   220
1321 TCA TCC AAA GAT CTG GGC TAT GAC TAT AGC TAT CTA CAA GAT TCA GAC CCA GAC TCT TTT
   Ser Ser Lys Asp Leu Gly Tyr Asp Tyr Ser Tyr Leu Gln Asp Ser Asp Pro Asp Ser Phe
   230
1381 CAA GAC TAC ATT AAG TCC TAT TTG GAA CAA GCG AGT CGG ATC TGG TCA TGG CTC CTT GGG
   Gln Asp Tyr Ile Lys Ser Tyr Leu Glu Gln Ala Ser Arg Ile Trp Ser Trp Leu Leu Gly
   240
1441 GCG GCG ATG GTA GGG GCC GTC CTC ACT GCC CTG CTG GCA GGG CCT GTG AGC TTG CTG TGT CTT
   Ala Ala Met Val Gly Ala Val Leu Thr Ala Leu Leu Ala Gly Pro Val Ser Leu Leu Cys Leu
   250
   TGC CCT GCT GGC AGG GCC TGT GAG CTT GCT GTG
   Cys Pro Ala Gly Arg Ala Cys Glu Leu Ala Val
   260
1501 CGT CAC AAG AGA AAG CAG CTT CCT GAA GAA AAG CAG CCA CTC CTC ATG GAG AAA GAG GAT
   Arg His Lys Arg Lys Gln Leu Pro Glu Glu Lys Gln Pro Leu Leu Met Glu Lys Glu Asp
   270
1712 TCG TCA CAA GAG AAA GCA GCT TCC TGA
   Ser Ser Gln Glu Lys Ala Ala Ser ---
   280
1561 TAC CAC AGC TTG TAT CAG AGC CAT TTA TAA AAAGGCTTAGGCAATAGAGTAGGGCCAAAAAGCCTGACCT
   Tyr His Ser Leu Tyr Gln Ser His Leu ---
   290
1631 CACTCTAACTCAAAGTAATGTCCAGGTTCCAGAGAATATCTGCTGGTATTTCTGTAAAGACCATTTCGAAATTTGTAA
   1712 CCTAATACAAGTGTAGCCTTCTCCACTCAGGTAGAACACACCTGCTTTGCTGTCTTTTCACTCAGCCCTTTA
   1792 ACATTTCCCTAAGCCCATGTCTAAGGAAAGGATGCTATTGTAATGAGAACTGTTATTGTATGTGAATTAATA
   1873 GTGCTCTATTTTAAAAAA

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FIG. 3. Nucleotide sequence of normal human tyrosinase cDNA (Pme134A), the deduced amino acid sequence, and the changes predicted on the basis of the identified point mutation in the albino proband. Nucleotides are numbered from the first nucleotide of the ATG initiation codon. The deduced amino acids are shown below the nucleotide sequence and are numbered from the amino-terminal amino acid of mature tyrosinase. The amino acid residues of the putative signal peptide are indicated by negative numbers and are heavily underlined. The potential glycosylation sites are underlined in regular print. The putative transmembrane region is doubly underlined. The stop codons are indicated by three dashes. The nucleotide and deduced amino acid sequences downstream from the T insertional mutation (horizontal arrow) in the albino cDNA are shown in italics. The positions of five potentially harmless point mutations are shown by stars, and the nucleotide substitutions and consequent amino acid changes are indicated at right and are underlined.

in the  $\lambda$ gt11 cloning vector (18, 19). The cDNA library was screened with two oligonucleotide probes, representing the first and fourth exons of the tyrosinase gene to avoid isolating cDNAs of alternatively spliced RNA (28). From 300,000 plaques of recombinant phages screened, 8 independent clones hybridized to both probes. The cDNA inserts varied in size from 1.4 to 2 kb. Sequencing of five of these clones by a double-stranded-DNA sequencing method (21), using various oligonucleotide primers spanning different regions of the tyrosinase cDNA, showed that the clones were full-length. The albino nucleotide sequence was compared to that of Pmel34 (8) and to other cDNAs spanning the 5' and 3' portions of tyrosinase. Fig. 3 presents the sequence of Pmel34A, the corrected version of tyrosinase cDNA Pmel34 (8). The assignment of the signal sequence is based on tyrosinase protein sequence information (29). The mature tyrosinase is composed of 511 amino acids with seven potential glycosylation sites (Fig. 3). The albino tyrosinase sequence differs from Pmel34A by five nucleotide substitutions and one insertion. The substitutions were detected at nucleotide positions 498, 575, 923, 1205, and 1484, counted from the A of the initiation codon ATG. These substitutions would result in amino acid changes indicated at positions 148, 174, 290, 384, and 477. They can be regarded as polymorphism. However, an insertion occurred at the putative transmembrane region, where a T residue was found between nucleotides 1467 and 1468 after codon 471 (Fig. 4). This mutation alters the reading frame and introduces a premature termination signal, TGA (nucleotide positions 1528–1530) after amino acid 490 (Figs. 3 and 4). This mutation would be expected to result in tyrosinase that differs from the normal enzyme in the 19 amino acids at its carboxyl terminus and that lacks 21 carboxyl-terminal amino acids.

**Immunoprecipitation of Tyrosinase with Anti-Tyrosinase Antibodies.** The predicted structural alteration due to the T insertional mutation was corroborated by immunoprecipitation studies. The albino tyrosinase immunoprecipitated with anti-tyrosinase antibodies ( $\alpha$ -tyr) had faster electrophoretic mobility in polyacrylamide gels, with an estimated size 3 kDa smaller than the normal tyrosinase (Fig. 5 *Left*). Antibodies that recognize the carboxyl terminus of tyrosinase ( $\alpha$ -PEP7; ref. 24) did not immunoprecipitate the albino tyrosinase (Fig. 5 *Left*). These results indicate that the tyrosinase of our proband has a major defect at the carboxyl terminus. The same results were obtained with tyrosinase immunoprecipitated from COS-1 cells transiently expressing normal or albino tyrosinase cDNA. Anti-tyrosinase antibodies immunoprecipitated tyrosinase from both normal and albino cDNA-transfected cells, with the albino tyrosinase again displaying faster mobility, and  $\alpha$ -PEP7 failed to immunoprecipitate the albino tyrosinase (Fig. 5 *Right*). Untransfected and pXM (vector)-transfected COS-1 cells did not produce

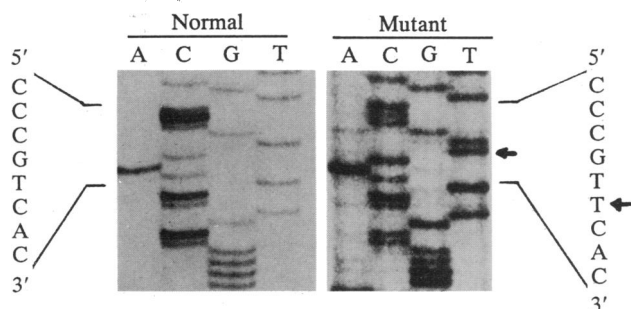


FIG. 4. Portion of sequencing gel containing the T insertional mutation. The normal sequence represents Pmel34A and the mutant sequence is that of the albino tyrosinase cDNA. The mutation site is indicated by arrows. The sequence is labeled 5' and 3' in reference to the orientation of the tyrosinase gene.

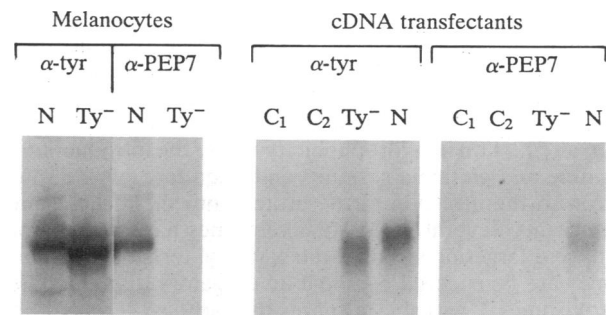


FIG. 5. PAGE analysis of pulse-labeled immunoprecipitated tyrosinase protein. (*Left*) Tyrosinase from normal (N) and albino ( $Ty^-$ ) melanocytes. (*Right*) Tyrosinase from normal (N) and albino ( $Ty^-$ ) cDNA-transfected COS-1 cells. Controls: C<sub>1</sub>, nontransfected; C<sub>2</sub>, pXM DNA-transfected.

immunoreactive tyrosinase (Fig. 5 *Right*, lanes C<sub>1</sub> and C<sub>2</sub>, respectively).

## DISCUSSION

Our observations support the conclusion that the single base insertion in the putative transmembrane region of this albino tyrosinase produces a protein that is altered in its carboxyl terminus and renders the enzyme inactive. The eight isolated tyrosinase cDNA clones have the same T insertion, and we could not detect  $\alpha$ -PEP7-immunoprecipitable tyrosinase even after long fluorographic exposure (5 days, data not shown). If the proband were heterozygous for the T insertion, one should detect  $\approx 50\%$  of the tyrosinase with the  $\alpha$ -PEP7 antibodies. This result may indicate that both alleles of the proband harbor the same mutation. The other alternative is that one allele produces no mRNA or an unstable mRNA, since the parents were unrelated. We could not find differences in the mRNA levels of the albino and normal controls. However, tyrosinase is an inducible enzyme (30), and the components of the medium, such as phorbol ester, alter the level of tyrosinase (15). Therefore, the level of expression of tyrosinase in the cultured melanocytes does not necessarily reflect the situation *in vivo*.

The mutation observed by us is different from the mutation reported by Tomita *et al.* (12), where a single base C insertion in exon 2 of an OCA individual caused a shift of the reading frame and introduced a premature termination signal after amino acid 298. In that case, it was not demonstrated that the albino melanocytes produced the predicted truncated tyrosinase. Another tyrosinase gene mutation was reported by Giebel *et al.* (13) in 6 out of 30 unrelated tyrosinase-negative albinos, where a change from C to T (CCT  $\rightarrow$  CTT) at codon 81 (codon 63 of the Pmel34A sequence, Fig. 3) would result in a substitution of leucine for proline. This Pro  $\rightarrow$  Leu mutation is not at any of the putative functional domains such as the transmembrane, copper binding, or glycosylation sequences. Nevertheless, the proline and the five amino acids preceding it are conserved in both tyrosinase and the melanocyte-specific *b*-locus protein catalase B (8, 31, 32), which indicates an important function for this domain. Spritz *et al.* (14) also reported two missense substitutions in an albino: one in one of the two putative copper binding sites and the other in a potential glycosylation site. Whether these mutations can be held responsible for the inactivity of the enzyme(s) remains to be validated at the protein level.

The nucleotide substitutions detected at positions 498, 575, 923, 1205, and 1484 in albino tyrosinase cDNA putatively alter the amino acids at positions 148, 174, 290, 384, and 477. Four of these substitutions are also seen in other normal tyrosinase sequences (refs. 14, 33, and 34; unpublished data).

The fifth, a Met → Ile change at position 148, was not reported before; but because of the conservative nature of the substitution, it may represent another site of polymorphism.

The mutation observed in our proband in the putative transmembrane domain would cause, in addition to truncation, a reduction in hydrophobicity due to the introduction of arginine and glutamic residues and, therefore, would interfere with the insertion of the protein into the melanosomal membrane. Several peroxisomal enzymes have been shown to have a targeting signal at the carboxyl terminus. Melanosomes are considered by some investigators to be modified peroxisomes.<sup>†</sup> Tyrosinase shares the carboxyl-terminal Ser-His-Leu peroxisomal targeting sequence with some peroxisomal enzymes (35). Therefore, if tyrosinase uses the carboxyl-terminal Ser-His-Leu as the melanosomal targeting signal, the truncation would abolish the signal.

Our results and those of others (12–14) have shown several kinds of mutations that can produce tyrosinase-negative OCA. The heterogeneity of mutations at the *TYR* locus make efficient prenatal diagnosis and carrier detection dependent on the availability of a panel of probes.

<sup>†</sup>Moellmann, G. & Halaban, R., Fourteenth International Pigment Cell Conference, Oct. 31–Nov. 4, 1990, Kobe, Japan, p. 90 (abstr.).

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