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Molecular Analysis of the DNA Segments Cross-Hybridizable to the Tyrosinase Gene in Patients Affected with Oculocutaneous Albinism

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TAKEDA, A., MATSUNAGA, J., TOMITA, Y., TAGAMI, H. and SHIBAHARA, S. *Molecular Analysis of the DNA Segments Cross-Hybridizable to the Tyrosinase Gene in Patients Affected with Oculocutaneous Albinism.* Tohoku J. Exp. Med., 1989, **159** (4), 333-340 —— The human tyrosinase gene is greater than 35 kb and is organized in four introns and five exons [Tomita et al. (1989) *Biochem. Biophys. Res. Commun.*, 164, 990-996]. Using the full-length cDNA encoding human tyrosinase and its exon-specific fragments as hybridization probes, we show that overall structural organization of the tyrosinase gene is unchanged in three patients affected with tyrosinase-negative oculocutaneous albinism (OCA). Moreover, we are able to show the presence of additional DNA segments cross-hybridizable to exon 4 or exon 5 of the tyrosinase gene in the genome of three OCA patients and a healthy individual. Namely, the exon 4-specific probe detected two bands in the DNA digested with *Eco*RI or *Hin*dIII and the exon 5-specific probe detected two bands in the *Bgl* II-digested DNA, in spite of the facts that no recognition sites for these enzymes are present in each exon. We have then isolated two phage clones harboring the distinct DNA segments, hybridizable to the exon 5 probe. Southern blotting analysis of each cloned DNA digested with *Bgl* II showed two different hybridization patterns as those detected in genomic DNA digested with *Bgl* II, confirming that there are at least two DNA segments hybridizing to the exon 5 sequence in human genome. ———— tyrosinase; melanin pigments; tyrosinase gene; inherited disease; oculocutaneous albinism

Albinism comprises a heterogenous group of heritable disorders of melanin formation. Oculocutaneous albinism (OCA), mostly inherited in an autosomal recessive fashion, is characterized by hypopigmentation in the eyes, skin and hairs, which leads to visual disturbances caused by optic neurologic defects and also predisposes the patients to skin cancer (for review, see Witkop et al. 1989).

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Abbreviations: OCA, oculocutaneous albinism; DOPA, dihydroxyphenylalanine.

OCA is classified into two maror types: tyrosinase-positive and tyrosinase-negative OCA, and the latter has been suggested to involve mutations in the tyrosinase gene (Witkop et al. 1989). Tyrosinase (EC 1.14.18.1), an essential enzyme in melanin biosynthesis, is a bifunctional copper-containing enzyme responsible for the conversion of tyrosine to dihydroxyphenylalanine (DOPA) and of DOPA to dopaquinone (Lerner et al. 1949; Shimao 1962; Pomerantz 1966). Recently, human tyrosinase cDNAs have been isolated (Kwon et al. 1987; Shibahara et al. 1988) and were shown to encode the protein possessing two catalytic activities of tyrosinase (Bouchard et al. 1989; Takeda et al. 1989). Subsequently, we have isolated and characterized the tyrosinase gene of one patient (S.S.) affected with tyrosinase-negative OCA, and identified a single base insertion in its exon 2, resulting in a nonsense mutation (Tomita et al. 1989). Here we demonstrate that the overall structural organization of the tyrosinase gene is unchanged in three patients affected with tyrosinase-negative OCA. Moreover, we provide evidence for the presence of additional DNA segments in human genome, hybridizing to either exon 4 or exon 5 sequences of the tyrosinase gene.

Experimental Procedures

Preparation of Genomic DNA

Peripheral lymphocytes of three patients, S.S., M.T. and F.S., affected with tyrosinase-negative OCA were transformed with Epstein-Barr virus and used as sources of genomic DNA as described previously (Tomita et al. 1989). Control DNA was prepared from the placenta of phenotypically normal individual.

Southern blotting analysis

Genomic DNA was digested with restriction enzymes and subjected to Southern blotting analysis as described (Southern 1975). The probe used was the *Sal*I(*Pst*I)/*Xba*I(*Nde*I) fragment (59/1892) containing a full-length human tyrosinase cDNA, derived from the expression plasmid pRHOHT2 (Takeda et al. 1989), and labeled with [α-^{32}p] dCTP by the random priming method (Feinberg and Vogelstein 1983). The numbers in parentheses, shown together with restriction enzymes, indicate the 5′-terminal nucleotide generated by cleavage. Both sites for *Pst*I and *Nde*I were eliminated during the construction of the expression plasmid pRHOHT2 and shown within parentheses (Takeda et al. 1989). Three exon-specific probes were prepared from the pRHOHT2 and used for hybridization (see Fig. 1A): the *Sal*I(*Pst*I)/*Taq*I fragment (59/454) as an exon 1 probe, the *Hinc*II/*Bgl*II fragment (1262/1412), containing 5 bp of the exon 3 and a 145 bp-segment of the exon 4, as an exon 4 probe, and the *Bgl*I/*Xba*I(*Nde*I) fragment (1557/1892) as an exon 5 probe. Two other hybridization probes specific to either exon 2 or exon 3 were prepared from the human tyrosinase cDNA, pHT γ 1 (Shibahara et al. 1988): the *Ava*I/*Hinf*I fragment (960/1065) as an exon 2 probe and the *Sph*I/*Hinc*II fragment (1169/1262) as an exon 3 probe.

Cloning of genomic DNA encoding human tyrosinase

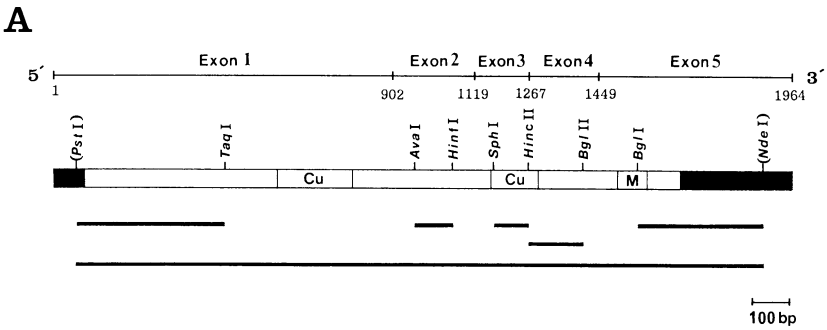
Genomic DNA library was constructed in EMBL3 phage using *Mbo*I-partial digests of DNA prepared from the patient, F.S. The library was screened for DNA segments encoding tyrosinase by *in situ* plaque hybridization (Benton and Davis 1977). The probe used was the *Sal*I(*Pst*I)/*Xba*I(*Nde*I) fragment (59/1892) containing a full-length human tyrosinase

cDNA. Several phage clones were thus isolated, and two of them, λ AFT9 and λ AFT12, carry the genomic DNA segments hybridizing to the exon 5 probe.

Results and Discussion

Recently, Barton et al. (1988) reported that the tyrosinase cDNA hybridized to two sites on the human chromosome 11, a major site of hybridization on the long arm and a minor site on the short arm, and that the sequence on the short arm may represent a truncated pseudogene or a related gene. They indicated that the 8.5-, 5.1- and 2.1-kb *Pst*I fragments are derived from the long arm (region 11q14 →q21, the tyrosinase locus), and the 7.5- and 4.4-kb *Pst*I fragments are located on the proximal short arm (region 11p11.2), although they were unable to exclude the possibility that the tyrosinase locus on 11q also contains the 7.5- and 4.4-kb *Pst*I fragments (Barton et al. 1988).

In order to analyze their observations more precisely, we also carried out Southern blotting analysis using the genomic DNA of normal individual and three patients affected with tyrosinase-negative OCA. Five restriction fragments of about 8.5, 7.5, 5.1, 4.4 and 2.1 kb were detected in *Pst*I-digested DNA using the full-length cDNA probe (Fig. 1), which is consistent with the report of Barton et al. (1988). Since the human tyrosinase gene is organized in five exons (Tomita et al. 1989) and no *Pst*I sites are present in the full-length cDNA probe used (Shibahara et al. 1988; Takeda et al. 1989), the presence of five *Pst*I fragments suggests that each signal may represent one of five exons of the tyrosinase gene. We therefore analyzed the genomic DNA digested with *Pst*I using five exon-specific probes (Fig. 1), revealing a 2.1 kb-band with the exon 1 probe, a 5.1 kb-band with exon 2 probe, a 8.5 kb-band with exon 3 probe, a 4.4 kb-band with the exon 4 probe (see Fig. 2), and a 7.5 kb-band with exon 5 probe (see Fig. 3A). Each probe specific to one of exons 1, 2 and 3 also hybridized to a single fragment in DNA digested with other enzymes, such as *Eco*RI, *Hin*dIII and *Bgl*II (data not shown), suggesting that the exons 1, 2 and 3 of the tyrosinase gene are present as a unique sequence in human genome. In contrast, the exon 4 probe hybridized to two fragments of about 5.4 kb and 4.5 kb in the *Eco*RI-digested DNA (indicated



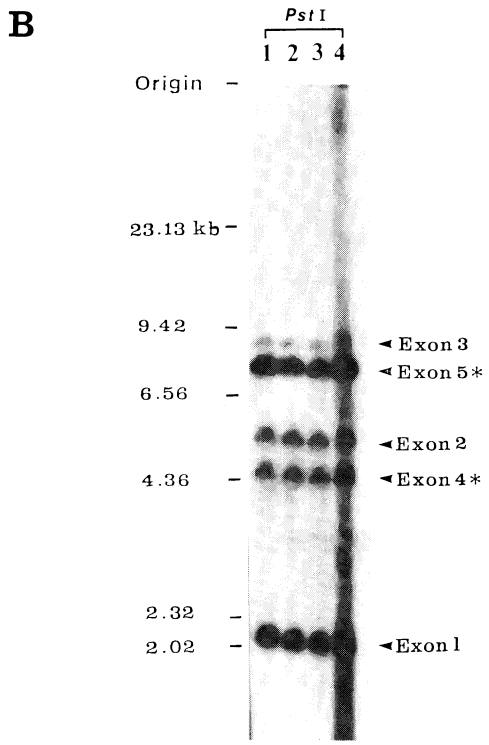


Fig. 1. Southern blotting analysis of the tyrosinase gene using full-length cDNA probe. A. Schematic representation of the human tyrosinase cDNA and strategy for preparation of exon-specific probes. The nucleotide residues are numbered from the transcription initiation site (Takeda et al. 1989). The numbers shown indidcated the first residue of each exon, except for the number at the 3′ end, representing the last residue of the cDNA (Shibahara et al. 1988). The 5′- and 3′-untranslated regions are indicated by closed boxes, and the protein-coding region is indicated by an open box. Two putative copper-binding sites (Cu) and a putative transmembrane region (M) are also indicated. Shown are the restriction enzymes used for preparation of the hybridization probes. Both *Pst*I and *Nde*I are shown within parentheses, since their recognition sites were converted to those of *Sal*I and *Xba*I, respectively in the expression plasmid, pRHOHT2 (Takeda et al. 1989), from which three probes were prepared: the full-length cDNA probe, exon 1-specific probe and exon 5-specific probe. Thick horizontal bars represent either exon-specific probes or the full-length cDNA probe. B. Five *Pst*I fragments hybridizable to the full-length cDNA probe. Each lane contained 10 μg of genomic DNA digested with *Pst*I. Sources of DNAs were transformed lymphocytes from patients with tyrosinase-negative OCA (lanes 1, F.S.; 2, M.T.; and 3, S.S.) and the placenta of phenotypically normal individual (lane 4). The hybridization probe used was the *Sal*I(*Pst*I)/*Xba*I(*Nde*I) fragment (59/1892), a full-length cDNA probe. The positive bands, representing each exon, were determined using five exon-specific probes (original data not shown). Asterisks indicate the bands, composed of at least two hybridizing fragments. Size markers are λ DNA digested with *Hin*dIII.

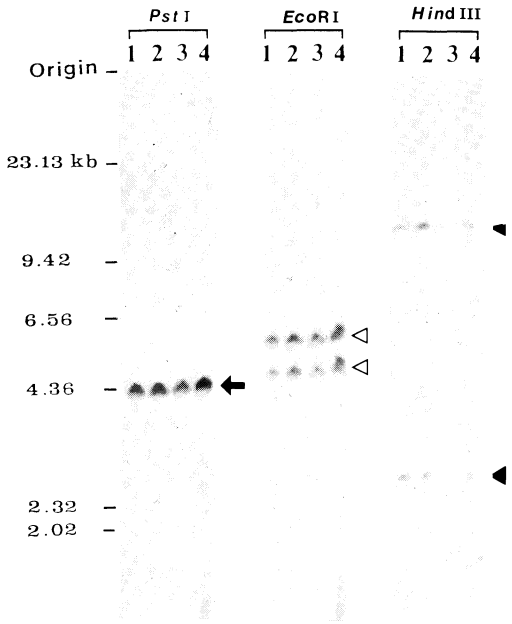


Fig. 2. Southern bloting analysis of the exon 4 and its cross-hybridizable segment. Each lane contained 10 μg of genomic DNA digested with the restriction enzymes indicated. Sources of DNAs and size markers are the same as those in Fig. 1. Lanes 1, F.S.; 2, M.T.; 3, S.S.; and 4, normal individual. The hybridization probe was the *Hin*cII/*Bgl*II fragment (1262/1412), an exon 4 probe. An arrow indicates a single *Pst*I-band of 4.4 kb, composed of two hybridizing fragments, while each triangle indicates a single hybridizing fragment.

by open triangles in Fig. 2) and to two fragments of about 13 kb and 2.7 kb in the *Hin*dIII-digested DNA (closed triangles in Fig. 2), while *Pst*I-digestion gave rise to a single band of 4.4 kb (an arrow in Fig. 2). Since no recognition sites for *EcoRI or Hin*dIII are present in the exon 4 sequence (Shibahara et al. 1988) and one 4.4-kb *Pst*I fragment was shown to be located on the region 11p11.2, different from the tyrosinase locus of 11q14→q21 (Barton et al. 1988), we propose that the *Pst*I band of 4.4 kb consists of two species of DNA segments containing either exon 4 of the tyrosinase gene or its cross-hybridizable sequence. Such an assumption is also supported by the fact that the intensity of the 4.4-kb *Pst*I band is stronger than that of other bands with *Eco*RI or *Hin*dIII. Moreover, using the exon 5 probe, we detected a single band of 7.5 kb in the *Pst*I-digested DNA and two bands of about 6.8 kb and 1.8 kb in the *Bgl*II-digested DNA (Fig. 3A). Again, one 7.5-kb *Pst*I fragment is located on the region 11p11.2 (Barton et al. 1988) and the signal intensity of the *Pst*I fragment was stronger than that of the *Bgl*II frag-

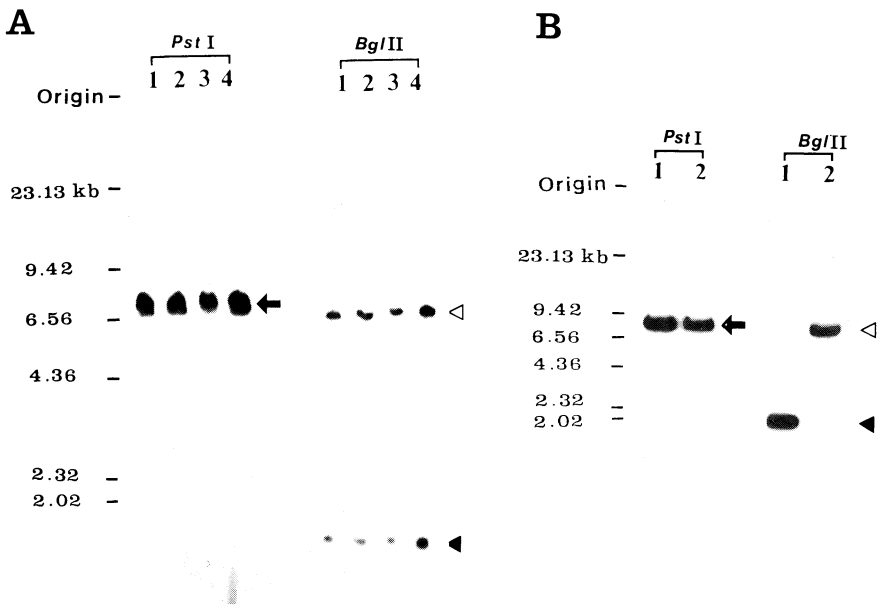


Fig. 3. Two distinct DNA segments hybridizing to the exon 5-specific probe. The *Bgl*I/*Xba*I(*Nde*I) fragment (1557/1892), an exon 5 probe, was hybridized to the genomic DNA (A) or cloned DNA (B) digested with *Pst*I or *Bgl*II. In B, each lane contained 0.5 μg of cloned genomic DNA, derived from the λ AFT12 (lane 1) or the λ AFT9 (lane 2). Other conditions are the same as described in Fig. 1. An arrow in A indicates a single *Pst*I-band of 7.5 kb, composed of two hybridizing fragments, while an arrow in B indicates a single *Pst*I-band of 7.5 kb, representing either exon 5 (lane 1) or its cross-hybridizable segment (lane 2). A closed triangle indicates a 1.8-kb *Bgl*II band, representing the DNA segment carrying the exon 5, and an open triangle indicates a 6.8-kb *Bgl*II band cross-hybridized (in A and B).

ments. In addition, the exon 5 sequence contains no recognition sites for *Bgl*II (Shibahara et al. 1988). These results also suggest that there are at least two DNA segments in human genome, hybridizing to the exon 5 probe. Subsequently, we were able to isolate two genomic clones, λ AFT9 and λ AFT12, which carry the distinct DNA segments of the patient F.S., hybridizable to the exon 5 probe. The exon 5 probe detected a 1.8-kb band in the *Bgl*II-digested DNA of λ AFT12 (Fig. 3B, lane 1) and a 6.8-kb band in the *Bgl*II-digested DNA of λ AFT9 (Fig. 3B, lane 2), while both two clones yielded a single 7.5-kb *Pst*I band (indicated by an arrow in Fig. 3B). These results are in good agreement with those of genomic DNA blotting analysis (Fig. 3A). Moreover, restriction mapping analysis of the phage clone, harboring the exon 5 of the tyrosinase gene of the other patient S.S (Tomita et al. 1989), reveals a 1.8 kb *Bgl*II fragment (data not shown),

indicating that the exon 5 is carried by λ AFT12. Considering all these observations, we conclude that there are at least two species of the DNA segments hybridizing to the exon 5 probe in human genome.

The data presented here indicated that the tyrosinase gene, on human chromosome 11q, consists of at least five *Pst*I-fragments of 8.5, 7.5, 5.1, 4.4 and 2.1 kb, each of which carries one of five exons, except for the 4.4- and 7.5-kb *Pst*I fragments, containing additional DNA segments cross-hybridizable to the exon 4 and exon 5 probe, respectively. These two *Pst*I-fragments of 4.4 kb and 7.5 kb, cross-hybridized to exon 4 probe or exon 5 probe are located on the proximal short arm of chromosome 11, which is different from the tyrosinase locus (Barton et al. 1988) and may represent parts of other functional genes or truncated pseudogenes. The biological significance of these related sequences remains to be elucidated.

Genomic DNA blotting analysis also reveals no apparent differences in sizes and strength of hybridization signals between three patients' DNA and control DNA (Figs. 1-2 and 3), indicating that the tyrosinase gene and its cross-hybridizable DNA segments are present in the genome of these three patients and suggesting that there are no gross rearrangement nor gross deletions and insertions of DNA segments in their tyrosinase gene. Therefore, the OCA-phenotype of these patients may be a consequence of the small change(s) in the tyrosinase gene or the defect in the regulatory factor required for the expression of the tyrosinase gene. Such an assumption was verified in the tyrosinase gene of the patients, S.S. and M.T., which carries a single base insertion causing a nonsense mutation (Tomita et al. 1989). We are currently attempting to isolate genomic clones for the entire tyrosinase of the other patient F.S. to examine whether the tyrosinase gene involves mutation(s).

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