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EXPRESSION OF TYROSINASE GENE IN AMELANOTIC MUTANT MICE

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In order to study the molecular aspects of albinism, we analyzed the expression of tyrosinase gene in some amelanotic mutant mice by Northern blotting using mouse tyrosinase cDNA as a probe. It hybridized with a species of 2.1 kilobase RNA prepared from the skin of wild-type mice and two albino strains in the same quantity. In black-eyed white mouse, however, no RNA transcript encoding tyrosinase was detected. Our results suggest that these albinism is due to a point mutation in the structural region of the tyrosinase gene, not to a deficiency of tyrosinase gene expression and that the black-eyed white mouse has a deficiency in the gene expression possibly related to melanocyte differentiation.

In mammals, the coloration of hair, skin, and eyes is mainly dependent on the presence of melanin, the specific product of melanocytes in the hair follicles, epidermis, and retinal pigmented epithelium. This pigment is synthesized by the bifunctional enzyme tyrosinase (EC.1.14.18.1), a copper-containing glycoprotein (1,2). Although there are a number of loci which influence melanogenesis in mice, the c-locus on chromosome 7 has been considered to be the most important in this metabolism because mutation at this locus results in albinism. Several allelic c-locus mutations characterized by reduced tyrosinase activity have been reported (3,4). Based on these genetical implications as well as biochemical findings, it has been hypothesized that this locus contains the structural gene for tyrosinase (5,6). However, some investigators have argued that the c-locus is a regulatory gene and that the structural gene can be found at other loci (7). Further understanding of the mechanisms of albinism seems to require an analysis of tyrosinase gene expression in albinos. If the c allele at the c-locus was the product of a point mutation in the structural region of the gene, quasinormal mRNA could be found in animals homozygous for c. In contrast, if the c-locus is a regulatory gene, no (or very little) mRNA could be found in c/c animals. In this sense, it is also interesting to compare the albino mutants at the c-locus and the white mutants at the mi-locus. In contrast to the albino in which amelanotic melanocytes are present, no melanocyte is found in black-eyed white mouse (mibw/mibw). Recently, we cloned

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To examine tyrosinase gene expression in melanocytes, we isolated mRNA from the skin of 3.5-day-old newborn mice since the number of the dopa-positive cells per area shows a peak at this time after birth (21). Northern blot analysis of wild-type skin showed that the cDNA probe hybridized with a single band corresponding to a mRNA species of the same size as that observed with TM10 cells. This result indicates that the tyrosinase gene is expressed as a 2.1 kb mRNA in melanocytes *in vivo* as well as in cultured melanocytes.

Our studies on *c*-locus mutants were carried out with two strains of albino mice which completely lack the ability to convert tyrosine to dopa, or dopa to melanin. Northern blot analysis showed that tyrosinase mRNA is expressed in both strains in the same quantity and length. This result seems to indicate that these albino alleles have no deficiency in tyrosinase gene expression at the transcriptional level and supports the hypothesis that the site of mutation is in the structural region of the tyrosinase gene. Unlike the case of albinos, no RNA transcript encoding tyrosinase was detected in the black-eyed-white skin (mi^{bw}/mi^{bw}). Lack of differentiation potency of melanocyte in the mutant has been demonstrated at the cellular level (22). Our results seem to confirm the genetic deficiency of the differentiation at molecular level. Precise genetic mechanism involved in melanocyte differentiation can be clarified by using cooperatively functioning marker genes during melanocyte differentiation.

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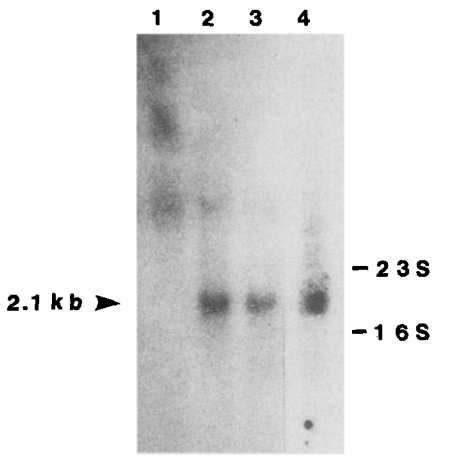

Fig.2 Analysis of tyrosinase gene expression in the dorsal skin of the wild-type and mutant mice (3.5-day-old) by Northern blot hybridization. Each mRNA (10ug) was fractionated on a 6% formaldehyde denaturing 1.3% agarose gel, transferred, hybridized, and washed as described in METHODS. Exposure time was 68 hr. 1, C57BL/6J-mi^{bw}; 2, BALB/c; 3, C57BL/6J; 4, ICR. The location of 23S and 16S ribosomal RNA are shown at right.

the bands in the wild type and albinos were indistinguishable. A minor band of higher molecular weight was detectable in all strains. This may have resulted from the non-specific hybridization of the probe with 28S ribosomal RNA.

DISCUSSION

Several isozymes (multiple forms) with different molecular weights have been reported for the mouse tyrosinase. Judging from the results of partial proteolysis and/or hydrolysis of sugar-chain of the enzyme, it can be assumed that the multiple forms are due to post-translational modifications of the enzyme protein(9,10,16). Although removal of carbohydrate residues from tyrosinase reduced its molecular weight, the treatment did not affect enzymatic activity (17,18). It has also been demonstrated that the different isozymes are immunologically similar and possess the same amino acid contents (16,19). In our study, the cDNA probe characterized the mouse tyrosinase mRNA as a species with a length of 2.1 kb. This result together with earlier findings suggest that tyrosinase is synthesized as a single polypeptide from 2.1 kb mRNA and then glycosylated to form several isozymes. It is known that hamster tyrosinase contains four moles of asparagine-linked sugar chain (20). In mouse tyrosinase cDNA, we have so far found six potential glycosylation signals. Multiple forms may result from glycosylation at different sites and/or different modes in nascent polypeptide.

and sequenced cDNA for mouse tyrosinase (8). Using this cDNA clone as a probe, we examined the tyrosinase gene expression at the messenger RNA level in wild-type mice, in two strains of mice with mutation at the c-locus and in black-eyed white mice by Northern blot analysis.

The tyrosinase of mice has been shown to occur in multiple forms separable by acrylamide-gel electrophoresis (9,10). Since it is possible that these multiple forms are produced by the processing of mRNA, it is necessary to determine whether the cDNA probe is capable of detecting the existence of multiple forms of mRNA by hybridizing with these possible multiple forms. For this purpose, we used mouse tyrosinase cDNA clone Tyrs-33 with a length of 1738bp (Fig.1-A). This clone was screened from lambda gt.11 cDNA library derived from the cultured melanocyte cell line TM10 (11) using anti-mouse tyrosinase rabbit antiserum as well as oligonucleotide probes deduced from the amino acid sequence of purified enzyme. In preparation of the antiserum and for determination of the amino acid sequence, we used purified T1-like tyrosinase protein which is considered to be a peptide which is common to all of the multiple forms (8,12,13). Therefore, the cDNA probe used in our study is assumed to be suitable for detecting the gene transcripts.

METHODS

Mouse strains. C57BL/6J (C/C, a/a) mice were raised in our laboratory. The original stocks were derived from the Jackson Laboratory, Bar Harbor, ME. C57BL/ 6J-mi^{bw} (C/C, mi^{bw}/mi^{bw}) mice were developed by introducing the gene mi^{bw} into the strain C57BL/6J genome by crossing and repeated backcrossing. BALB/c (c/c) and ICR (c/c) mice were raised in our laboratory which were originally purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan.

Radiolabeling of cDNA. Mouse tyrosinase cDNA Tyrs-33 obtained from lambda gt 11 cDNA library (8) was radiolabeled with [^{32}P] dCTP by the random hexamer method using a multiprime kit (Amersham Corp.,UK.) and [alpha-^{32}P] dCTP (Amersham Corp.,UK. ; 3,000 Ci/m mol) according to manufacturer's directions.

Isolation of RNA and Northern blot analysis. The materials used in this study were dorsal skin from 3.5-day-old mice and cell clots obtained by injecting cultured melanocyte cell line TM 10 (11) into C57BL/6J mice. Total RNA was isolated by the guanidine isothiocyanate-hot phenol method (14) and poly(A)RNA was purified from total RNA by using oligo(dT) cellulose (15). Five or ten microgram of poly(A) RNAs were electrophoresed in a 6% formaldehyde-1.3% agarose gel in TAE buffer (40mM Tris-acetate, 2mM EDTA, pH 7.5) after denaturation of the RNA at 65°C for 5 min in 50% formamide - 6% formaldehyde - TAE buffer. The RNA was electrophoretically transferred to a nyron membrane Hybond-N (Amersham Corp., UK.). The filters were hybridized at 42°C for 20 hr. in a solution containing 5 x SSPE (1 x SSPE; 180mM sodium chloride, 10mM sodium phosphate, pH7.7, and 1mM EDTA), 50% (V/V) formamide, 5x Denhardt's solution, 0.5% SDS and denatured salmon sperm DNA (100 microgram/ ml). Filters were washed twice in 2 x SSPE and 0.1% SDS at 42°C for 30 min., then in 1 x SSPE and 0.1% SDS at 42°C for 30 min. The final wash was carried out in 0.1 x SSPE at room temperature. Then it was exposed to Fuji New RX film at -80°C with double intensifying screen. The mRNA sizes were determined by comparison with E. coli rRNA (23 S, 16 S).

RESULTS

Characterization of the mRNA encoding mouse tyrosinase was made by Northern blot analysis of polyadenylated [poly(A)$^+$]RNA from TM10 cells (Fig.1-B). This cell line is derived from cutaneous melanocytes of black mouse C57BL/6J(genotype: a/a,C/C) and expresses complete multiple forms of tyrosinase (data not shown). Autoradiography revealed that tyrosinase mRNA was detected as a single RNA species with an approximate length of 2.1 kilobases. This result suggests that tyrosinase is synthesized as a unique polypeptide and that the multiple forms may result from post-translational modifications such as glycosylation, or differential conjugation with other proteins.

Expression of tyrosinase gene in dorsal skin of 3.5-day-old wild-type mice and mutant mice were examined (Fig.2). The C57BL/6J (C/C) strain is black and is wild type concerning the c-locus. BALB/c and ICR are albino strains with genotypes of c/c. Both strains possess no melanin in skin, hair, and eyes. The black-eyed white mutant C57BL/6J-mi^{bw} mice (genotype; a/a, mi^{bw}/mi^{bw}, C/C) were also examined. This mutation is characterized by the absence of melanocyte in the skin. Autoradiography showed the existence of a predominant single band corresponding to that of a mRNA species and equivalent to that observed in TM10 cells in wild-type mice as well as both albino mice, whereas no signal was detected in the black-eyed white mice. The intensity of

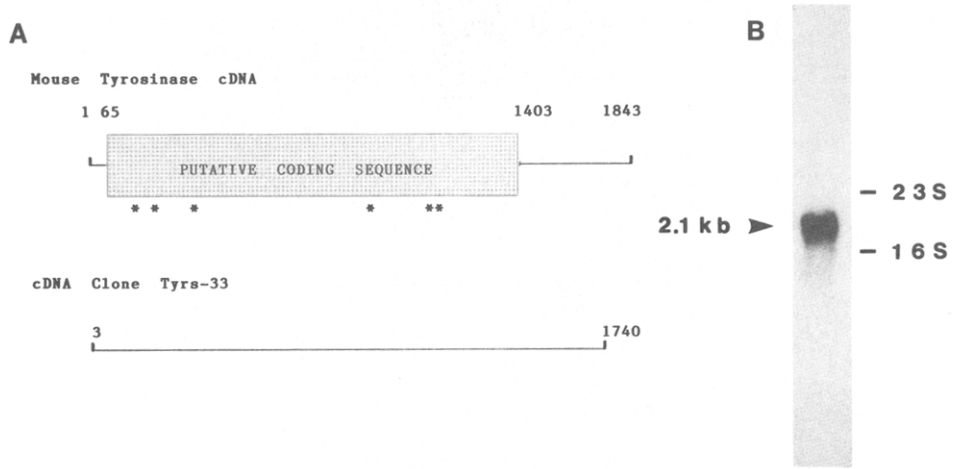

Fig.1 (A) Map of mouse tyrosinase cDNA clone Tyrs-33. The number at the termini indicate the position of them in total mouse tyrosinase cDNA. Asterisks show the position of the sequences that correspond to amino acid sequences obtained from purified T1-like tyrosinase (8). (B)Northern blot analysis of tyrosinase mRNAs in TM10 cells. Five microgram of poly(A)RNA was applied. The conditions of electrophoresis, hybridization and wash are described in METHODS. Exposure time was 13 hr. The mRNA sizes were determined by comparison with E. coli rRNA (23 S , 16 S). The location of ribosomal RNA are shown at right.

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