Molecular Basis of the extreme dilution mottled Mouse Mutation
A COMBINATION OF CODING AND NONCODING GENOMIC ALTERATIONS

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Tyrosinase is the rate-limiting enzyme in melanin biosynthesis. It is an N-glycosylated, copper-containing transmembrane protein, whose post-translational processing involves intracytoplasmic movement from the endoplasmic reticulum to the Golgi and, eventually, to the melanosome. The expression of the tyrosinase (Tyr) gene is controlled by several regulatory regions including a locus control region (LCR) located 15 kb upstream from the promoter region. The extreme dilution mottled (Tyrc-em) mouse mutant (Tyrc-em) stock, whose molecular basis corresponds to a rearrangement of 5′-upstream regulatory sequences including the LCR of the Tyr gene. Tyrc-em mice display a variegated pigment pattern in coat and eyes, in agreement with the LCR translocation, but also show a generalized hypopigmented phenotype, not seen in Tyrc-m mice. Genomic analyses of Tyrc-em mice showed a C1220T nucleotide substitution within the Tyr encoding region, resulting in a T373I amino acid change, which abolishes an N-glycosylation sequon located in the second metal ion binding site of the enzyme. Tyrosinase from Tyrc-em displayed a reduced enzymatic activity in vivo and in vitro, compared with wild-type enzyme. Deglycosylation studies showed that the mutant protein has an abnormal glycosylation pattern and is partially retained in the endoplasmic reticulum. We conclude that the phenotype of the extreme dilution mottled mouse mutant is caused by a combination of coding and noncoding genomic alterations resulting in several abnormalities that include suboptimal gene expression, abnormal protein processing, and reduced enzymatic activity.

Tyrosinase (Tyr1 monophenol dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) (1, 2) is the rate-limiting enzyme in melanogenesis and catalyzes the conversion of L-tyrosine into L-dopaquinone in the presence of L-3,4-dihydroxyphenylalanine (3). Tyr is a transmembrane glycoprotein that undergoes a complex post-translational processing before reaching the melanosomes. In mice, Tyr processing includes N-glycosylation in at least four of six available glycosylation sites (4); translocation from the endoplasmic reticulum (ER) to the Golgi apparatus; binding of the copper cofactor to the two sites known as CuA and CuB; and finally, sorting to the melanosomes in its final catalytically active conformational state (5, 6). The relevance of Tyr post-translational processing is enhanced by the recent consideration of some types of ocularcutaneous albinism (OCA) as ER retention diseases. Oculocutaneous albinism type 1 (OCA1) is an autosomal recessive disease resulting from mutations in the human tyrosinase (Tyr) gene (7, 8), usually associated with abnormal processing of the enzyme (9–12). Related pigmentary disorders associated with mutations in other genes, such as OCA2 (13, 14), OCA3 (12), OCA4 (15), and more complex diseases as Hermansky-Pudlak and Chediak-Higashi syndromes (16–18) can also be accounted for by abnormalities in processing or intracellular trafficking of the corresponding proteins.

Mouse models for nearly all human pigmentary disorders have been reported (for review, see Ref. 19; listed in Refs. 20 and 21). Mutations in the mouse tyrosinase (Tyr) gene are mainly associated with alterations in the coding sequence, as in the albino (Tyr) (22), himalayan (Tyr-em) (23), dark eyed (Tyr-em 4wt) (24), and platinum (Tyr-p) (25) alleles. Some of these mutations in the Tyr gene are associated not only with low enzymatic activities but also with processing or trafficking defects (6, 11, 12, 25).

The Tyr gene is expressed exclusively in two cell types: melanocytes, derived from migrating neural crest cells, which eventually colonize iris, choroid, skin, and cochlea; and retinal pigmented epithelium (RPE) cells, derived from the optic cup (26, 27). In mice, the expression of Tyr is tightly regulated by a combination of proximal promoter elements (28–31) and distal elements, most remarkably a locus control region (LCR), located 15 kb upstream from the transcription start site (32–36). The presence of a LCR was inferred from the analyses of artificial chromosome-type transgenes in mice harboring the entire Tyr expression domain (37, 38) and from previous studies on the molecular basis of the chinchilla mottled mutant allele of the Tyr gene (Tyr-em) (39–41). Tyr-em mice displayed a variegated pigmentation in the coat, RPE cells, and choroid (39, 40). This phenotype was correlated with a genomic rearrangement.

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ment of ~30 kb 5' upstream Tyr regulatory sequences, which included the LCR (41). Interestingly, the subsequent analysis of transgenic mice carrying large Tyr genomic constructs, in which the LCR has been experimentally deleted, showed most similar variegated phenotypes (42, 43).

The extreme dilution mottled (Tyr<sup>-em</sup>) mutation arose spontaneously at the MRC Mammalian Genetics Unit in Harwell (UK) from a chinchilla mottled mouse stock. 2 Tyr<sup>-em</sup> mice display a variegated pattern of pigmentation in coat, but also an overall hypopigmented phenotype, compared with the presence of additional mutations in the Tyr gene. The molecular basis of the extreme dilution mottled mutation was not known. We decided to investigate this Tyr allele because we believed that it could contribute to the understanding of the Tyr gene regulation, including LCR function. We have rescued Tyr<sup>-em</sup> mice from frozen embryos and performed a series of phenotypic, genomic, and biochemical analyses that have resulted in the description of the molecular and functional basis of the extreme dilution mottled mutation.

**EXPERIMENTAL PROCEDURES**

**Animals**—The following types of mouse were used in this study: heterozygous wild-type pigment Tyr<sup>R2</sup>-<sup>YAC-tirosinase</sup> transgenic mice (Tyr<sup>R2-<sup>a</sup>; Tyr<sup>a</sup>/Tyr<sup>b</sup>) (line 1999; 37), heterozygous YRT4 -<sup>YAC-tirosinase</sup> transgenic mice (YRT4<sup>-<sup>a</sup>; Tyr<sup>a</sup>/Tyr<sup>b</sup>) (line 2138; 34, 42), homozygous or heterozygous extreme dilution mottled (Tyr<sup>-em</sup>) and chinchilla mottled mutant mice (Tyr<sup>-<sup>a</sup>; Tyr<sup>a</sup>/Tyr<sup>b</sup>) (39–41), and albino outbred NMRI (Harlan Interfauna Iberica, S. L., Barcelona, Spain) mice (Tyr<sup>-<sup>a</sup>; Tyr<sup>a</sup>/Tyr<sup>b</sup>). Tyr<sup>-em</sup> and Tyr<sup>-e<sup>+</sup></sup> mice were rescued from frozen embryos (embryos kept frozen since 1984 at the Frozen Embryo and Sperm Archive (FESA) of the MRC Mammalian Genetics Unit, in Harwell (UK) and sent to the Centro Nacional de Biotecnología (CNB; Madrid) with the following initial genotypes: Tyr<sup>-<sup>e<sup>+</sup></sup>+</sup>/Tyr<sup>-<sup>e<sup>+</sup></sup></sup>; Tyr<sup>-<sup>a</sup>+</sup>/Tyr<sup>-<sup>a</sup></sup>; a/a; Ednr<sup>b</sup>/Ednr<sup>b</sup>, respectively. Thereafter, extreme dilution mottled and chinchilla mottled mutant mice as well as YRT2 and YRT4 transgenic mice were bred and have been maintained in an albino outbred NMRI genetic background. Histological analysis of adult mouse retinae was performed as reported (42). All experiments complied with local and European legislation concerning vivisection and the experimentation and use of animals for research purposes.

**Quantification of Melanin and Tyrosinase Enzymatic Activity Assays**—Melanin contents were measured spectrophotometrically as described previously (44). Two types of tyrosinase assay were performed in whole eye extracts following described procedures (45, 46). The first one consisted of a tyrosine hydroxylase activity determination by a radiometric method based on the release of tritiated water from labeled [3<sup>H</sup>]tyrosine. The second assay measured melanin formation from L-[<sup>14</sup>C]tyrosine, and therefore provides an estimate of the rate of the complete melanogenic pathway. The tyrosine hydroxylase activity of Tyr<sup>-</sup> in whole cell extracts was determined using the same radiometric method described above, except that shorter incubation times (1 h instead of 14–16 h) and smaller volumes of extract (usually 5 μl of the cell lysates instead of 25 μl, used for whole eye extracts) were employed. One unit was defined as the amount of enzyme catalyzing the hydroxylation of 1 μmol of L-tyrosine/min, in the presence of a 50 μM concentration of the substrate in the presence of 4-dihydroxyphenylalanine as cofactor (47). For statistical analyses, where indicated, paired Student’s t tests were applied and statistical significance indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001 (StatView, SAS Institute, Inc., Cary, NC).

**Southern Blot Analyses**—For high resolution Southern blot analysis, megabase-size genomic DNA from mouse cells was prepared from splenocytes following described procedures (48). EcoRI and ApaI digests (0.5 units/μl) were run at 37 °C for 12–14 h on agarose plugs (Seaplaque LMP, Cambrex Bio Science Rockland, Inc., Rockland, ME) containing mouse genomic DNA. Plugs were resolved by horizontal gel electrophoresis in a 20 × 25-cm gel of 0.5% (w/v) Seakem Gold-agarose (Cambrex Bio Science Rockland, Inc.), 1× TAE buffer (40 μM Tris acetate, 2 mM EDTA), at 3 V/cm, 4 °C for 16 h. The gel was transferred onto a Hybond-N nylon membrane (Amersham Biosciences) by capillary blotting and hybridized with a randomly primed [α-<sup>32</sup>P]dCTP-labeled D probe (904 bp LCR internal probe, nucleotide positions 8574 to 9478, GenBank AF364302; 35) in hybridization buffer (0.25 M Na<sub>H</sub>PO<sub>4</sub>, pH 7.2, 7% (w/v) SDS, 1% (w/v) bovine serum albumin) for 16 h at 65 °C, washed at 65 °C in 20 mM Na<sub>H</sub>PO<sub>4</sub>, pH 7.2, 1% (w/v) SDS, 1 mM EDTA, pH 8.0, and finally autoradiographed and/or analyzed with a Phosphorimager (Molecular Dynamics).

Standard Southern blot analyses were carried out using previously described procedures (34). In brief, 15–20 μg of genomic DNA from mouse tail biopsies was digested with EcoRI or HindIII (Roche Applied Science), fractionated by horizontal electrophoresis in a 1% (w/v) agarose gel (Invitrogen), transferred by capillary blotting onto Hybond-N nylon membranes (Amersham Biosciences), and subsequently hybridized with a full-length mouse Tyr cDNA probe (pmcYr1; 28).

**Rescue of the Tyr<sup>-em</sup> cDNA and Preparation of a Tyr<sup>-em</sup> Expression Construct**—Several overlapping fragments comprising the entire Tyr<sup>-em</sup> coding region were obtained by PCR amplification of genomic DNA with Vent DNA polymerase (New England Biolabs, Beverly, MA) or by reverse transcription-PCR, from RNA extracted from eyes (RNeasy Mini Kit, Qiagen), using specific oligonucleotides designed according to reported mouse Tyr cDNA sequences (GenBank X12782 and M24560). PCR products were cloned into pmCR1 T/A vector (Invitrogen), sequenced, and analyzed (alignments) using MacVector (Accelyrs, Inc., San Diego). A detailed description of oligonucleotides and plasmids used is available upon request. A full-length Tyr<sup>-em</sup> cDNA was reconstituted, sequenced entirely (GenBank AJ262894), and cloned into a pcDNA3 expression vector (Invitrogen). DNA was obtained and confirmed from three independent Tyr<sup>-em</sup> homozygous mutant mice. The Tyr<sup>-</sup> expression construct, as well as those encoding the Tyr<sup>-</sup> and Tyr<sup>-M<sub>dl</sub></sup> mutant variants, all generated in pcDNA3, have been described previously (47).

**Culture and Transient Transfection of Mammalian Cells**—Human embryonic kidney (HEK 293T cells (American Type Culture Collection) were cultured at 37 °C and 5% CO<sub>2</sub> in 6-well plates (Falcon, BD Biosciences) with RPMI 1640 medium (Invitrogen), 10% (v/v) fetal calf serum (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were transiently transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s recommendations, and harvested 24 h after transfection. Whole cell crude extracts were prepared in 100 μl of 10 mM sodium phosphate buffer, pH 6.8, 0.1 mM EDTA, pH 8, 1% (v/v) Igepal CA-630, 0.1 mM phenylmethylsulfonyl fluoride (Roche Applied Science) as reported previously (47). The protein content of the extracts was determined by the bicinchoninic acid method, using bovine serum albumin as standard.

**Deglycosylation Analysis**—Endo H or Endo F digestions were made as described previously (47). In brief, 8 μg of total protein was incubated at 37 °C for 1 h in 4% (w/v) para-formaldehyde for 15 min at 25 °C and then permeabilized with methanol for 20 min at 4 °C. After blocking with 10% (v/v) fetal calf serum in PBS for 1 h at 25 °C, cells were incubated with 1:500 mouse monoclonal antibody against KDEL (Stressgen Biotechnologies, Inc., San Diego) (a generous gift from Dr. V. H. Vehar, National Institutes of Health) (50) following previously published procedures (51). Staining and detection was done with the ECL Plus chemiluminescent substrate (Amersham Biosciences).

**Immunocytochemistry**—HEK293T cells were transiently transfected with the Tyr<sup>-</sup>, Tyr<sup>-M<sub>dl</sub></sup>, Tyr<sup>-M<sub>sd</sub></sup> (47), and Tyr<sup>-em</sup> cDNA constructs, cloned in pcDNA3 vector, as described above. Immunocytochemistry was performed as described previously (12). Briefly, 24 h post-transfection, cells were fixed in 4% (w/v) para-formaldehyde for 15 min at 25 °C and then permeabilized with methanol for 20 min at 4 °C. After blocking with 10% (v/v) fetal calf serum in PBS for 1 h at 25 °C, cells were incubated with 1:500 mouse monoclonal antibody against KDEL (Stressgen Biotechnologies, Inc., San Diego) (a generous gift from Dr. V. H. Vehar, National Institutes of Health) in 2% (v/v) fetal calf serum in PBS overnight at 4 °C. Cells were washed three times in PBS and then incubated with 1:500 Alexa 488-labeled goat anti-rabbit (dilution, 1:500) and Alexa 594-labeled goat anti-mouse (dilution, 1:500; Molecular Probes, Invitrogen), for 1 h at 25 °C. Nuclei were counterstained with 1:200 TO-PRO3 in PBS (Molecular Probes). All preparations were examined in an Axiovert 200 confocal microscope (Carl Zeiss AG, Oberkochen, Germany), and images were processed with Laser Sharp 2000 program (BioRad).

**RESULTS**

**Phenotypic Analysis of extreme dilution mottled Mutant Mice**—The phenotype of adult heterozygous extreme dilution

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In the eye, both heterozygous and homozygous extreme dilution mottled mice exhibited a variegated pigmentation pattern at the RPE cells and irises (Fig. 1, B–G), similar but much lighter than that described previously in adult chinchilla mottled (Tyrc-em) mice (41) (Fig. 1, H–J). In contrast to chinchilla mottled mice, the choroid of extreme dilution mottled mice was weakly pigmented (Fig. 1, K–M).

Tyrosinase Activity and Melanin Content in the Eyes of extreme dilution mottled Mutant Mice—We measured the Tyr enzymatic activity of heterozygous extreme dilution mottled (Tyrc-em/Tyr^r) mutant mice in crude protein eye extracts and compared it with the corresponding enzymatic activities of albino mice (Tyr^r/Tyr^r, NMRI), heterozygous chinchilla mottled (Tyrc-em/Tyr^r) mice (39–41), YRT4 and YRT2 transgenic mice (34, 37). YRT2 animals are indistinguishable from wild-type pigmented mice (52, 53), whereas YRT4 mice display a variegated pigmentation phenotype in the eye and coat, associated with the experimental deletion of the locus control region (LCR) in the YAC-tyrosinase transgene (42).

The tyrosine hydroxylase and melanogenic activities of Tyr in extreme dilution mottled mice were intermediate between those of control albino NMRI and wild-type pigmented YRT2 transgenic mice but indistinguishable from those observed in chinchilla mottled or YRT4 transgenic mice (Fig. 2, A and B). Whole eye melanin contents in extreme dilution mottled mice were lower than in any other pigmented mouse analyzed and similar to albino NMRI mice (Fig. 2C).

Genomic Analysis of the Tyrc-em Locus—To gain insight into the potential alterations affecting the Tyrc-em locus we performed a series of genomic analyses (Fig. 3). First, we investigated using Southern blot analysis the presence and endogenous location of the LCR element in the Tyrc-em mutant allele on homozygous extreme dilution mottled genomic DNA samples digested with restriction enzymes EcoRI and ApaI, using an internal LCR-specific probe (probe D, Fig. 3A). The expected 3.7-kb EcoRI DNA fragment containing the LCR element (32) was found in all mice analyzed (Fig. 3B). Further, we observed that the Tyrc-em LCR was located approximately at −40 kb from the transcription start site, a distance similar to that observed previously in chinchilla mottled mice, further upstream from its endogenous normal position in wild-type mice, found approximately at −15 kb (35, 40) (Fig. 3A).

Second, we studied the entire genomic structure and integrity of the Tyr locus in the Tyrc-em mutant allele by Southern blot analysis, using a full-length Tyr cDNA probe (pmcTyr1; 28) and two analytical restriction enzymes: EcoRI and HindIII (28, 34). No obvious differences were observed in the DNA banding pattern of the Tyr gene between the mutant Tyrc-em and Tyrc-em alleles compared with the expected pattern of wild-type Tyr^+ locus (Fig. 3, A and C).

Third, we obtained the entire coding sequence of the Tyr gene in the extreme dilution mottled mutant mice by PCR and reverse transcription-PCR, using a number of specific oligonucleotides. Several overlapping DNA fragments comprising the whole Tyrc-em cDNA were obtained, aligned, and a full-length cDNA sequence was eventually assembled (GenBank AY526904). The analysis of the Tyrc-em cDNA sequence showed a single nucleotide mutation, cytidine (C) to thymidine (T), at position 1220, according to wild-type Tyr^+ cDNA (GenBank X12782). This point mutation would result in an amino acid substitution, threonine (T) to isoleucine (I), at position 373 in the predicted protein sequence compared with wild-type tyr protein (SwissProt P11344).

Processing and Deglycosylation Analysis of Tyr from the extreme dilution mottled Mouse Mutant—Tyr amino acid position 373 is located within the sixth potential N-glycosylation sequon of the...
enzyme, within the CuB binding site (47). This glycosylation sequon is conserved in mammalian tyrosinases (6), is glycosylated in Tyr (4, 47), and its occupancy has been reported to correlate with cofactor binding and acquisition of full enzymatic activity (4, 47), as well as proper processing. To investigate whether the T373I mutation impairs the processing and glycosylation of Tyr in extreme dilution mottled mutant mice (Tyr-em), we transiently transfected HEK293T cells with wild-type or Tyr-em expression constructs followed by Western blot analysis of detergent-solubilized extracts with a Tyr-specific antibody (αPEP7; 50, 51). HEK293T cells (54) have been shown to reproduce the normal Tyr post-translational processing observed in B16 mouse melanoma cells (47). For comparison, we also analyzed two tyrosinase mutants, Tyr36 and Tyr-MeB. Tyr36 bears a N371Q conservative substitution predicted to abolish the same 371NGT373 glycosylation sequon as the extreme dilution mottled mutation. Tyr-MeB is a chimeric construct consisting of the complete Tyr sequence except for the replacement of the entire CuB site (from residue His363 to residue His390) by the homologous fragment of the tyrosinase-related protein 1 (Trp1). The preparation and characterization of these mutants have been described (47). Tyr36 is partially active and is not glycosylated in one glycosylation sequon, whereas Tyr-MeB is totally inactive, underglycosylated in more than one site, and retained in the ER as judged by its endoglycosidase digestion pattern (47). Western blot analysis of extracts from HEK293T cells transfected with the wild-type Tyr expression construct showed a major broad band (apparent molecular mass ~78 kDa) and faster migrating bands (~69 kDa and lower), corresponding to complete and partially glycosylated forms, respectively (Fig. 4A). As expected, the electrophoretic mobility of the Tyr36 and Tyr-em mutants was very similar and higher than for the corresponding forms of wild-type Tyr. The mobility of the Tyr-MeB construct was even higher. This pattern is fully consistent with underglycosylation in one site for the Tyr36 and Tyr-em mutants. Interestingly, the intensity of the signal in Tyr-em samples was consistently lower than for the wild-type enzyme for comparable protein loads (37 ± 6% of controls, n = 3), indicative of lower levels of the mutant enzyme (Fig. 4B).

The processing of Tyr-em was assessed further in vitro by means of glycosidase digestion with Endo H and Endo F. Endo H removes N-linked high mannose-type carbohydrates characteristic of ER-resident, incompletely processed forms of N-linked glycoproteins. Upon further processing at the medial Golgi these proteins become resistant to Endo H. Hence, Endo H provides a way to distinguish immature forms of Tyr from the mature enzyme. In turn, Endo F digests all forms of N-glycans, independently of their degree of processing, thus allowing for the estimation of the size of the protein backbone (9, 11, 12, 47).

The wild-type Tyr+ protein was largely resistant to Endo H digestion, thus suggesting a mature, post-ER conformation (Fig. 4C). In contrast, the sensitivity to Endo H of Tyr-em was higher, consistent with incomplete or abnormal processing and significant retention in the ER. The electrophoretic mobility of the single band present after Endo F digestion, corresponding to the deglycosylated protein backbone with an apparent molecular mass of 55 kDa, was identical to wild-type. Taken together, these data suggested that Tyr-em is not properly glycosylated and therefore is likely retained, at least partially, in the ER.

The potential retention of Tyr-em in the ER was investigated further by confocal microscopy (Fig. 4D). HEK293T cells were transiently transfected with wild-type Tyr, Tyr-em, Tyr6 or Tyr-MeB and stained with the tyrosinase-specific αPEP7 antibody (12, 50) and with the ER-specific KDEL antibody (12). In cells expressing the wild-type form, no significant colocalization of tyrosinase and ER-specific signals was detected, as reported for melanocytes. Conversely, extensive colocalization of Tyr-MeB and the ER-specific marker was detected, consistent with previously reported biochemical analysis (47). Finally, a limited colocalization of the Tyr-em and Tyr36 mutants and the ER marker was seen.
FIG. 3. Genomic analysis of Tyr<sup>c-em</sup> locus. A, schematic representation of Tyr<sup>+</sup>, Tyr<sup>c-em</sup>, and Tyr<sup>c-m</sup> genomic loci. Restriction sites for Apal (A), EcoRI (E), and Hind III (H) are indicated. White boxes correspond to DNA probes, black boxes to the LCR element; exons and reconstituted cDNA of the Tyr gene are depicted as gray boxes. A bent arrow indicates the transcription start site. Dashed lines show putative ends for the genomic translocation described in Tyr<sup>c-em</sup> mutant mice (40). B, high resolution Southern blot from large size genomic DNA from wild-type (Tyr<sup>+</sup>), homozygous mutant extreme dilution mottled (Tyr<sup>c-em</sup>), and homozygous mutant chinchilla mottled (Tyr<sup>c-m</sup>) mice, digested with Apal and EcoRI restriction enzymes and hybridized with a LCR-specific probe (probe D; 35). C, Southern blot of mouse genomic DNA from the same three genotypes indicated in B digested with HindIII and EcoRI restriction enzymes and hybridized with a full-length Tyr cDNA probe (pmcTyr1; 28).

Residual Enzymatic Activity of Tyr<sup>c-em</sup>—The occupancy of the sixth potential N-glycosylation sequon has been correlated with full Tyr enzymatic activity (4, 47), but the residual activity in different mutants where this site is negated may be dependent on the particular amino acid substitution considered (47). To determine the residual activity of Tyr<sup>c-em</sup>, we measured in vitro the Tyr enzymatic activity and kinetic constants of the mutant protein and compared them with those of wild-type Tyr<sup>+</sup>, using whole cell extracts prepared from HEK293T-transfected cells. The Tyr<sup>c-em</sup> mutant protein displayed higher <i>K<sub>m</sub></i> and lower Tyr activity (−13% of wild-type) and <i>V<sub>max</sub></i> resulting in a catalytic efficiency (<i>V<sub>max</sub>/K<sub>m</sub></i>) reduced almost 3-fold compared with wild-type Tyr<sup>+</sup> (Table I).

DISCUSSION

The LCR plays a fundamental role in regulating the expression of the Tyr gene in melanocytes and RPE cells (34, 42). The analysis of mice carrying a series of Tyr mutant alleles or plasmid/YAC-Tyr transgenes has allowed the characterization of this regulatory element (32–35, 40, 42, 55; for review, see Ref. 43). In chinchilla mottled mice, a likely translocation of 5′ upstream regulatory sequences, including the LCR, from −15 kb to approximately −40 kb from the Tyr transcription start site, results in a variegated pigmented phenotype in the coat and RPE cells (40, 41). YRT4 transgenic mice, carrying a modified YAC-Tyr transgene devoid of the LCR, displayed a similar variegated distribution of melanin in the coat and eye, although with a lower pigmentation level, compared with chinchilla mottled mice (34, 42). The extreme dilution mottled mouse mutation originated from a chinchilla mottled mouse stock at the MRC-MGU (Harwell, UK) and is characterized by a variegated pigmented phenotype in the coat, iris, and RPE cells, combined with a generalized reduced pigmentation, compared with either chinchilla mottled or YRT4 mice (Fig. 1; 34, 42). This enhanced hypopigmented phenotype, according to the differences observed between chinchilla mottled and YRT4 transgenic mice, suggested that at least two alternatives were possible to explain the molecular basis of the extreme dilution mottled mouse mutation: 1) the presence of an additional alteration in the LCR (or any regulatory element) within the Tyr<sup>c-em</sup> mutant allele, and/or 2) a mutation within the coding region of the Tyr<sup>c-em</sup> mutant allele eventually resulting in a severely reduced Tyr enzymatic activity. To understand the basis for the extreme dilution mottled mouse mutation, we decided to investigate these mutant mice with a variety of approaches, including detailed phenotypic characterization, biochemical properties of the mutant Tyr protein, and exhaustive genomic analysis of the Tyr<sup>c-em</sup> mutant locus.

First, we measured the observed decrease of pigmentation in extreme dilution mottled mice by quantifying the Tyr activity and melanin content in crude eye extracts from adult heterozygous mice (Fig. 2). Tyr enzymatic activities in crude eye extracts of extreme dilution mottled mice were low and intermediate between those of albino NMRI mice and wild-type pigmented YRT2 transgenic mice but comparable with those recorded in chinchilla mottled and YRT4 hypopigmented mice. However, the melanogenic activity of extracts from chinchilla
molted appeared somewhat higher than that of extreme dilution molted mice. In keeping with this trend, we observed a statistically significant decrease in the ocular melanin contents of extreme dilution molted mice compared with any other pigmented mice analyzed, and in particular with chinchilla molted mice. This slight discrepancy between the relative levels of Tyr activity measured in cell extracts and the melanin contents of the cells can be explained by several factors. Indeed, the enzymatic activities measured in cell extracts may not reflect perfectly the activity within the cell, or the actual melanin formation potential, likely as a result of altered subcellular location of the enzyme. This situation is not uncommon and is seen, for instance, in tyrosinase-positive albinisms. Alternatively, the lower melanin contents may reflect an actually reduced Tyr capacity to generate melanin in these mutant mice. In this respect, it is worth noting that Tyr<sup>−</sup>em carries a mutation in the CuB site and that mutations in the metal binding sites of Tyr are frequently associated with different effects on the various enzymatic activities of the protein (6, 56).

Next, we performed a systematic genomic analysis of the Tyr<sup>−</sup>em locus, aiming to discover any subsequent alterations present in the Tyr<sup>−</sup>em mutant allele (Fig. 3). First, the location of the LCR in the Tyr<sup>−</sup>em locus was found at a comparable genomic position with regard to the Tyr<sup>−</sup>em locus, from which it originated (Fig. 3, A and B). This rearrangement can mostly explain the variegated pigmentation pattern observed in extreme dilution molted mice compared with the similar reported phenotype in chinchilla molted mice (39–41). Second, we assessed the integrity of the Tyr gene in the Tyr<sup>−</sup>em locus and did not find obvious differences in the expected DNA banding pattern compared with either Tyr<sup>−</sup>em or Tyr<sup>+</sup> alleles. Third, we generated a full-length cDNA and entirely sequenced the coding region of the Tyr<sup>−</sup>em locus, thus detecting a single nucleotide substitution (C1220T) that would result in a predicted amino acid T373I change. This nonsynonymous substitution abolishes the sixth potential N-glycosylation site, starting at position 371, and located within the CuB binding motif (6, 47). Changes at this position have been associated with OCA1 cases and aberrant processing of human TYR (57, 58).

Correlations between Tyr glycosylation, processing, and intracellular traffic, and enzymatic activity have been demonstrated (4), although several exceptions to this rule have also been reported. For instance, Tyr protein variants with a similar extent of processing and glycosylation but displaying different enzymatic activities have been described previously (47). Therefore, we analyzed the effects of this mutation on the glycosylation pattern, processing, and enzymatic activity of the Tyr<sup>−</sup>em mutant compared with the wild-type enzyme and two other mutants, Tyr<sup>A6</sup> and Tyr<sup>Meb</sup>. In the Tyr<sup>−</sup>em mutant, the sixth glycosylation sequon is missing as a result of a N371Q substitution. The Tyr<sup>Meb</sup> quimeric construct is enzymatically inactive and underglycosylated in more than one site (47). For this study, HEK293T cells were selected as the most appropriate model system for the transient expression of the different tyrosinase forms. These cells share the same embryonic origin as melanocytes (54) and express wild-type tyrosinase and various enzymatically active mutants efficiently (47, 59). More importantly, the processing and glycosylation pattern of tyrosinase and other melanogenic proteins such as Trp1 seems identical in HEK293T cells and B16 mouse melanoma cells (47).

The electrophoretic mobility of the Tyr<sup>−</sup>em mutant protein was identical to the Tyr<sup>Meb</sup> mutant and lower than the Tyr<sup>Meb</sup> quimeric construct, underglycosylated in at least two sites. This is fully consistent with ablation of the sixth glycosylation sequon because of the observed T373I mutation, resulting in underglycosylation compared with wild-type. Moreover, digestion with Endo H followed by Western blot showed that a

![Image of three graphs: A, B, and C.](image-url)

**Fig. 4. Evaluation of the glycosylation pattern and subcellular distribution of Tyr from the Tyr<sup>−</sup>em mutant mice.** A, extracts from HEK293T cells transfected with the wild-type form or the indicated mutants cloned into pcDNA3 (3 μg of total protein/lane) were electrophoresed, blotted, and probed with αPEP7. The membranes were then stripped and reprobed with an anti-ERK2 antibody (Santa Cruz Biotechnology, Inc.), used as a loading control (lower blot). A representative blot of three independent experiments is shown in A, and the results of quantitative analysis of the band intensities are displayed in B (expressed as percent band intensity relative to wild-type; mean ± S.E., n = 3). C, extracts from HEK293T cells transfected with wild-type and extreme dilution molted construct pcDNA3 (6 μg of total protein/lane) were electrophoresed, blotted, and probed with αPEP7, with or without a previous deglycosylation treatment with Endo H or Endo F. The migration of molecular mass markers is shown on the left. C stands for undeglycosylated control extracts, and eH and eF for extracts digested with Endo H or Endo F, respectively. D, subcellular distribution of Tyr in the Tyr<sup>−</sup>em mutant mice. Colocalization analysis by confocal microscopy of wild-type Tyr<sup>+</sup> and mutant Tyr<sup>−</sup>em, Tyr<sup>A6</sup> (47) and Tyr<sup>Meb</sup> (47) tyrosinases in HEK293T-transfected cells with an ER marker is shown, αPEP7 (green) was used to identify the tyrosinase proteins and KDEL (red; 12) was used to detect ER. Colocalization of antibodies is shown in orange-yellow. Nuclei were counterstained with TO-PRO3 (blue). Scale bar = 5 μm.

**Table I**

| Protein | Tyrosine hydroxylase activity | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
|---------|-------------------------------|-------|-----------|---------------|
| Tyr<sup>+</sup> | 2,920 ± 20 (100%) | 79 ± 10 | 2.11 ± 0.07 | 0.027 |
| Tyr<sup>−</sup>em | 368 ± 13 (13%) | 139 ± 30 | 1.36 ± 0.19 | 0.010 |

* $V_{max}$ values are expressed in arbitrary units, as reported previously (47).
considerable fraction (52 ± 7%, n = 3) of the total Tyrc-em protein present in crude extracts is sensitive to the glycosidase, indicative of aberrant or incomplete processing and likely partial retention in the ER. This possibility was investigated further by immunocytochemistry, addressing the potential colocalization of Tyr and ER markers. In these experiments, no significant colocalization of wild-type Tyr and the ER marker was observed, further supporting the use of HEK293T cells as a suitable model for Tyr processing studies. On the other hand, the TyrMoB mutant, which provided a positive control for colocalization, displayed a clear ER retention phenotype consistent with its lack of enzymatic activity and its full sensitivity to Endo H (47). The Tyrc-em protein and the TyrMoB mutant showed a similar pattern, with limited but detectable colocalization with the ER marker in HEK293T-transfected cells. Notably, in HEK293T cells transiently expressing the various tyrosinase forms, the levels of the Tyrc-em protein were lower than those of wild-type and TyrMoB and comparable with TyrMeB. This suggests a decreased intracellular stability of the protein which cannot be explained exclusively by lack of occupancy of the sixth glycosylation sequon in Tyrc-em because the addition of N-glycan to this site is also abrogated in the TyrMoB mutant. Therefore, it would appear that the nonconservative substitution in Tyrrc-em induces a less stable configuration than the conservative mutation in TyrMoB, in addition to the common effect on glycosylation of both mutations.

The extreme dilution mutated Tyr retained some measurable enzymatic activity in contrast to a related and previously reported T373K mutation, which appears to be fully inactive. This partial activity in the Tyrc-em enzyme, together with the limited colocalization with ER markers, is consistent with the incomplete colocalization with ER markers, as opposed to the fully inactive TyrMoB protein. This partial activity of the Tyrc-em enzyme, together with the limited colocalization with ER markers and with the partial sensitivity to Endo H, agrees with our previous observations that glycosylation of the CuB acceptor sequon is not a sine qua non requirement for ER export, copper binding, and complete maturation of the enzyme. As opposed to this situation, the LCR translocation (responsible for most of the variegated pigmentation pattern observed) and a single bp mutation, C1229T, within the coding sequence of the Tyr gene (responsible for the production of a Tyr protein abnormally processed with severely reduced enzymatic activity). The mutant Tyrc-em locus encodes an altered Tyr protein that is glycosylated incorrectly and partially retained in the ER while conserving some detectable Tyr activity.

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52. Jeffery, G., Schutz, G., and Montoliu, L. (1994) *Dev. Biol.* 166, 460–464.
53. Jeffery, G., Brem, G., and Montoliu, L. (1997) *Brain Res. Dev. Brain Res.* 99, 95–102.
54. Shaw, G., Morse, S., Ararat, M., and Graham, F. L. (2002) *FASEB J.* 16, 869–871.
55. Porter, S. D., Hu, J., and Gilks, C. B. (1999) *Dev. Genet.* 25, 40–48.
56. Olivares, C., García-Borroño, J. C., and Solano, F. (2002) *Biochemistry* 41, 679–686.
57. Lee, S. T., Nicholls, R. D., Bundey, S., Laxova, R., Musarella, M., and Spritz, R. A. (1994) *N. Engl. J. Med.* 330, 529–534.
58. Park, K. C., Chintamaneni, C. D., Halaban, R., Witkop, C. J., and Kwon, B. S. (1993) *Am. J. Hum. Genet.* 52, 406–413.
59. Simonova, M., Wall, A., Weissleder, R., and Bogdanov, A., Jr. (2000) *Cancer Res.* 60, 6656–6662.
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