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Identification of a New Member of the Tryptase Family of Mouse and Human Mast Cell Proteases Which Possesses a Novel COOH-terminal Hydrophobic Extension*

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Mapping of the tryptase locus on chromosome 17 revealed a novel gene 2.3 kilobase 3′ of the *mouse mast cell protease* (*mMCP*) *6* gene. This 3.7-kilobase gene encodes the first example of a protease in the tryptase family that contains a membrane-spanning segment located at its COOH terminus. Comparative structural studies indicated that the putative transmembrane tryptase (TMT) possesses a unique substrate-binding cleft. As assessed by RNA blot analyses, *mTMT* is expressed in mice in both strain- and tissue-dependent manners. Thus, different transcriptional and/or post-transcriptional mechanisms are used to control the expression of mTMT *in vivo*. Analysis of the corresponding tryptase locus in the human genome resulted in the isolation and characterization of the *hTMT* gene. The *hTMT* transcript is expressed in numerous tissues and is also translated. Analysis of the tryptase family of genes in mice and humans now indicates that a primordial serine protease gene duplicated early and often during the evolution of mammals to generate a panel of homologous tryptases in each species that differ in their tissue expression, substrate specificities, and physical properties.

Tryptases are stored in abundance in the secretory granules of mouse (1–4), rat (5–7), gerbil (8), dog (9), and human (10–15) mast cells (MCs).[1] In humans, the four homologous tryptases (designated *tryptases I, II/β, III*, and *α*) that have been cloned reside at a complex on chromosome 16 (16). Although only two tryptases (designated *mouse MC protease* (*mMCP*) *6* and *mMCP-7*) have been identified so far in the mouse, their genes reside ~1.2 centimorgans away from each other on the syntenic region of mouse chromosome 17 (17, 18). Despite the chromosomal clustering of their genes, these mouse tryptases are differentially regulated *in vivo* (1, 19–21) and *in vitro* (2, 3) at the levels of gene transcription (22) and mRNA stability.[2]

All known mouse and human tryptases in this family are initially translated as zymogens. They possess an ~20-residue hydrophobic signal peptide which is presumed to be removed in the endoplasmic reticulum immediately after the translated zymogen is translocated into the lumen. They also possess an ~10-residue propeptide preceding the mature portion of the enzyme which consists of ~245 amino acids. No mature tryptase with a membrane-spanning segment in its COOH terminus has been found in any species so far. Although tryptases undergo variable *N*-linked glycosylation during their biosynthesis (5, 12, 23, 24), the current members of the family appear to be targeted to the secretory granule by a serglycin proteoglycan-dependent mechanism (25, 26) rather than by a Man-PO_4-dependent mechanism as are classical lysosomal enzymes.

The amino acid sequences of mMCP-6 and mMCP-7 are 71% identical (1–4). Nevertheless, these homologous tryptases have different physicochemical properties (25, 26) and substrate preferences (27–29). Recent *in vivo* studies have suggested that mMCP-6 and mMCP-7 evolved to carry out different functions. In mice, mMCP-6 regulates neutrophil extravasation into tissues (28, 29), whereas mMCP-7 helps to minimize the deposition of fibrin/platelet thrombi during a MC-mediated inflammatory reaction (27).

The findings that recombinant mMCP-6 and mMCP-7 exhibit potent but different bioactivities *in vivo* highlight the need to identify and characterize all of the tryptase genes present in the mouse and human genomes. Because of the importance of mouse tryptases in inflammation (27–29), because more tryptases have been cloned from the human genome (13–16) than from the mouse genome (2, 3), and because adjacent serine protease genes in large superfamilies often reside within 7 kb of one another on their respective chromosomes (30, 31), a walk approach was carried out to identify the functional gene that resides on chromosome 17 immediately 3′ of the *mMCP-6* gene. We now describe a novel mouse gene, and its human ortholog, which encode an unusual transmembrane tryptase (TMT).

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The nucleotide and amino acid sequences reported in this paper for the human TMT gene, human TMT cDNA, mouse TMT gene, and mouse TMT cDNA have been submitted to the GenBank™/EBI Data Bank with accession numbers AF175759, AF175522, AF175760, and AF175523, respectively.

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[1] The abbreviations used are: MC, mast cell; bp, base pair; EST, expressed sequence tag; Ig, immunoglobulin; mBMMC, mouse bone marrow-derived MC; mMCP, mouse MC protease; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; TMT, transmembrane tryptase; UTR, untranslated region; kb, kilobase.

[2] R. L. Stevens, unpublished observations.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of the mTMT Gene—A λ bacteriophage clone (designated GW-1) was isolated by screening a 129/Sv mouse genomic library (Stratagene, La Jolla, CA) under conditions of high stringency with a radiolabeled probe specific for the *mMCP-6* gene (2). A 6-kb *Bam*HI-derived fragment liberated from GW-1 was subcloned into pBluescript (Stratagene) and its nucleotide sequence determined in both directions with standard dideoxy/cycle sequencing methodologies (32). Analysis of the obtained nucleotide sequence data revealed that this portion of chromosome 17 contained exon 6 of the *mMCP-6* gene, followed by 2.3 kb of flanking DNA, and then the 3.7-kb *mTMT* gene. The *mMCP-6* and *mTMT* genes were oriented in the same direction in GW-1. To confirm the position and spacing of the two genes in native chromosomal DNA, a long range polymerase chain reaction (PCR) approach was carried out in which each 50-μl sample contained 200 ng of BALB/c or 129/Sv mouse genomic DNA, 10 μmol of an oligonucleotide (5′-ACTCACTGCTTCCTGGTCAG-3′) that corresponds to a region in exon 6 of the *mMCP-6* gene, and 10 μmol of an oligonucleotide (5′-ACAGTGTGACCGTAAGCTC-3′) that corresponds to a region in exon 3 of the *mTMT* gene. Thirty cycles of PCR were performed with recombinant *Thermus thermophilius*-derived DNA polymerase (Perkin-Elmer); each cycle consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 58 °C, and a 4-min extension step at 72 °C. The amplified PCR products were subcloned into the TA vector pCR 2.1 (Invitrogen, San Diego, CA) and subjected to nucleotide sequence analysis.

To determine if homologous *mTMT* genes exist in the mouse genome, ~20-μg samples of BALB/c mouse genomic DNA were incubated separately at 37 °C for ~17 h with *Bam*HI, *Sca*I, *Hin*dIII, *Pst*I, *Bgl*II, *Sac*I, *Avr*II, or *Xba*I (New England Biolabs, Beverly, MA). The digests were fractionated on 1% agarose gels. The separated fragments were blotted onto MagnaGraph nylon membranes (Micron Separations Inc., Westborough, MA) (33), and the resulting DNA blots were incubated for 2 h at 65 °C in QuikHyb hybridization solution (Stratagene) containing a radiolabeled 224-base pair (bp) probe corresponding to a portion of exon 3 of the *mTMT* gene. This probe was chosen because it corresponds to a region in the *mTMT* transcript that is not present in the *mMCP-6* and *mMCP-7* transcripts. The DNA blots were washed twice for 15 min each at room temperature in 2 × SSC containing 0.1% SDS and then twice for 15 min each either at 65 °C or 50 °C in 0.2 × SSC containing 0.1% SDS. The blots were then exposed to BIOMAX film for ~3 days. In some instances, the DNA blots were stripped and reprobed with *mMCP-6* (2) and *mMCP-7* (3) gene-specific probes.

Isolation and Characterization of the mTMT Transcript, and Evaluation of Its Expression in Different Tissues and in mBMMCs Developed from Different Strains—Total RNA was isolated (34) from the v-*abl*-transformed V3 mouse MC line (21), non-transformed MCs (mBMMCs) developed with interleukin 3 (35) from the bone marrows of W/W^v (also know as Kit^W/Kit$^{W\text{-}v}$), BALB/c, C57BL/6, and 129/Sv mice, and from varied tissues of BALB/c and C57BL/6 mice. The RNA samples were applied to individual lanes of 1.2% agarose-formaldehyde gels (36). The gels were subjected to electrophoresis for 17 h, the separated RNA was transferred to nylon membranes (Schleicher and Schuell), and the resulting blots were analyzed with gene-specific probes for the *mTMT, mMCP-6* (2), *mMCP-7* (3), and β-*actin* (37) transcripts. The cDNA probes used in these analyses were random primed with [α-^{32}P]dCTP using the *redi*prime kit (Amersham Pharmacia Biotech) and hybridized to the RNA blots at 65 °C for 2 h in QuikHyb hybridization solution (Stratagene). The blots were washed twice for 15 min each at room temperature in 2 × SSC containing 0.1% SDS, and then twice for 10 min each at 60 °C in 0.2 × SSC containing 0.1% SDS before exposure to film.

Three different approaches were used to demonstrate that the newly identified mouse gene was transcribed *in vitro* and *in vivo* in different mouse strains. A search of the GenBank mouse expressed sequence tag (EST) data base revealed that clone AA266560 probably corresponded to a portion of the *mTMT* gene. This EST was derived from mixed organs of a FVB/N mouse. Sequence analysis of the EST, obtained from the "Integrated Molecular Analysis of Genomes and their Expression" (I.M.A.G.E.) consortium, revealed that it corresponded to residues 109 to 1112 in the 1.2-kb *mTMT* transcript. To obtain the 5′ portion of the *mTMT* transcript, 5′-Marathon RACE (rapid amplification of cDNA ends) was performed on a CLONTECH (Palo Alto, CA) preparation of BALB/c mouse liver-derived cDNAs, according to the manufacture's instructions. The first PCR was carried out using the anchor oligonucleotide 5′-CCATCCTAATACGACTCACTATAGGGC-3′ and the oligonucleotide 5′-ATCCACCACAGAGACTTTGGCCTCCTGAGG-3′ which corresponds to a region in exon 4 of the *mTMT* gene. The second nested PCR was carried out using the second anchor oligonucleotide 5′-ACTCACTATAGGGCTCGAGCGGC-3′ and the oligonucleotide 5′-CATCCCAGGGTAGAAGTCAGCTGAGGCCTC-3′ which corresponds to a region in exon 3 of the *mTMT* gene. Amplified products were subcloned into pCR 2.1 (Invitrogen) and their inserts sequenced. A reverse transcription (RT) PCR approach was then used to confirm that the *mTMT* transcript is expressed in W/W^v mBMMCs. Two sets of oligonucleotides were used in these latter reactions. The first (5′-CAGGCTAGCCTCCGTCTG-3′ and 5′-CATCCCAGGGTAGAAGTCAGC-3′) and second (5′-CTGTGAACTCGTCTGATTATC-3′ and 5′-ACACCTCATTCAGAGTTCCGAGGCCGCGTG-3′) sets of oligonucleotides cover exons 2 to 3 and exons 3 to 5, respectively, in the *mTMT* gene. The RT step was carried out at 55 °C for 30 min with a kit from Roche Molecular Biochemicals (Indianapolis, IN). Each of the 30 cycles of the PCR consisted of a 15-s denaturing step at 94 °C, a 30-s annealing step at 58 °C, and a 60-s extension step at 72 °C.

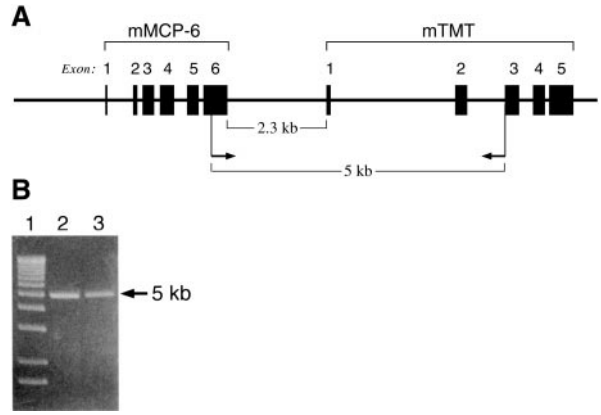


FIG. 1. **Organization of the *mMCP-6* and *mTMT* genes on chromosome 17.** *A,* the depicted map of the λ phage genomic clone GW-1 is not drawn to scale. Nevertheless, the six exons of the *mMCP-6* gene and the five exons of the *mTMT* gene are *boxed* and *numbered*. The two genes are separated by 2.3 kb of flanking DNA. *B,* long range PCRs were performed with genomic DNA from BALB/c (*lane 2*) and 129/Sv mice (*lane 3*). The *arrows* (→) in *A* indicate the locations of the two chromosome 17-derived oligonucleotides used in these reactions. The *arrow* on the *right* in *B*, indicates the generated 5-kb PCR product that spans from exon 6 of the *mMCP-6* gene to exon 3 of the *mTMT* gene. A 1-kb DNA ladder (Life Technologies, Inc., Grand Island, NY) was used in *lane 1* to determine the molecular weights of the generated PCR products. An identical sized fragment was generated when a similar long range PCR was performed on GW-1 (data not shown). The PCR products were subcloned and partially sequenced to confirm that they corresponded to the appropriate region in mouse chromosome 17.

Isolation and Characterization of the hTMT Transcript and Gene, and Chromosomal Location of the hTMT Gene—A PCR approach was used to determine whether or not there is a human ortholog of the *mTMT* transcript. A number of relatively conserved regions were found when the nucleotide sequence of the *mTMT* transcript was compared with those of varied mouse and human MC tryptase transcripts. Thus, an oligonucleotide (5′-TGCTGGGTCACTGGCTGG-3′) that corresponds to a relatively conserved region in all mouse and human MC tryptase transcripts and an oligonucleotide (5′-GATCCAGTTCACGTAGGC-3′) that corresponds to the 3′ end of the *mTMT* transcript were employed to amplify from a pool of human liver cDNAs (CLONTECH) a 326-bp fragment that corresponds to the middle portion of the *hTMT* transcript. Based on the nucleotide sequence of this PCR product, more specific oligonucleotides were used in 5′- and 3′-RACE approaches to obtain a more full-length *hTMT* transcript. In the case of 5′-RACE, the oligonucleotides 5′-CCAGCTCACAATGCCAGCCTGCACCCAG-3′ and 5′-GCATGTCGGGCTGAAGGATGCTGC-3′ were employed in the first and second nested PCRs, respectively, with the relevant anchor oligonucleotides. In the case of 3′-RACE, the oligonucleotide 5′-CGTACAGCCTGCGGGAGGTGAAAGTCTC-3′ was used with the anchor oligonucleotide. Reactions were performed with human liver and uterus Marathon cDNAs (CLONTECH) as templates. Using the primers 5′-AGGTGCACCTGGGGGAACTGGAGATCAC-3′ and 5′-AATGCACTTGGATTCCTGCCATCAGTCAG-3′, PCRs were also performed with human skin (Invitrogen) and cecum cDNA libraries. All amplified PCR products were subcloned into pCR 2.1 and their inserts sequenced.

A nested PCR approach was then used to elucidate which human

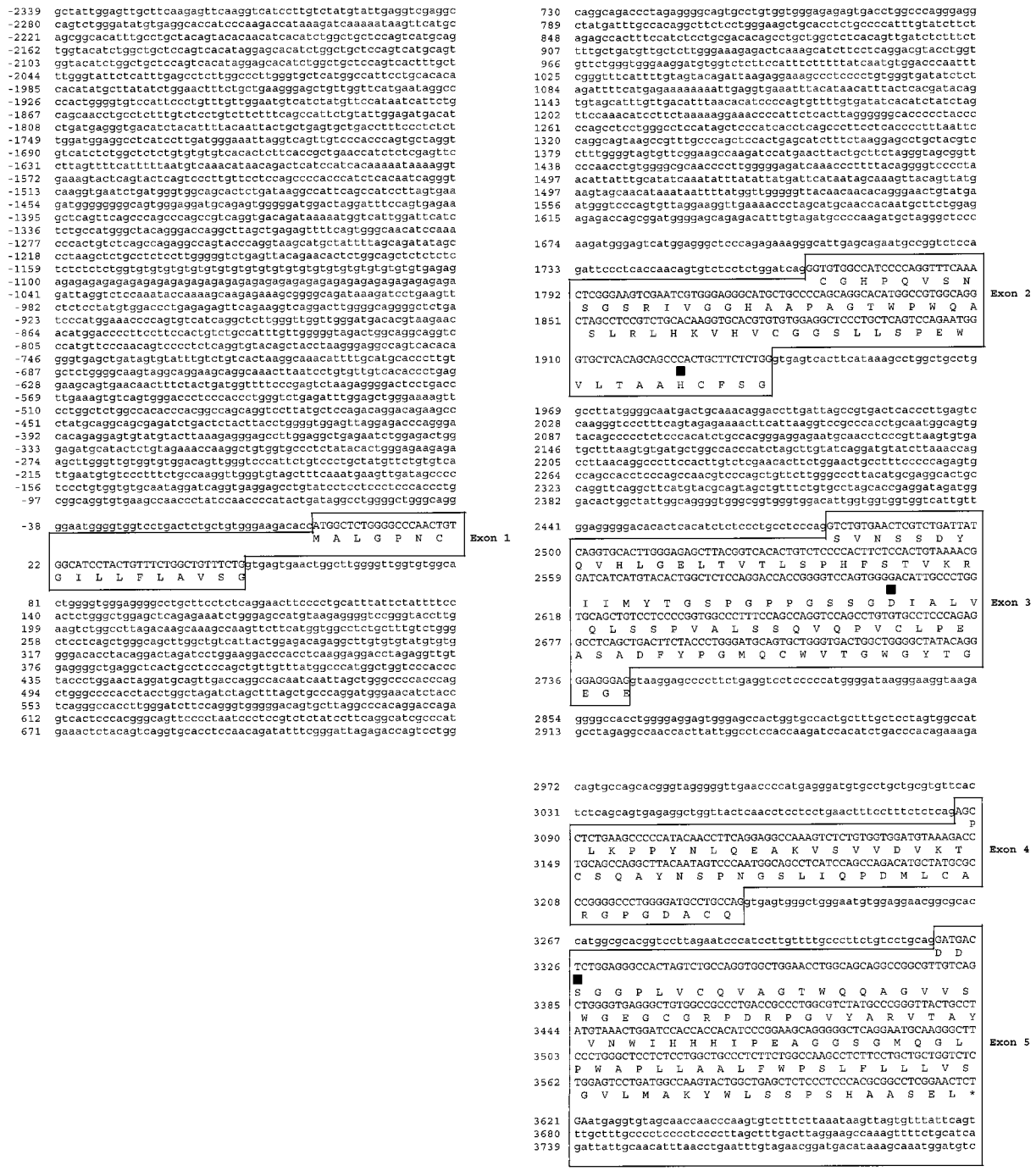


FIG. 2. **Structure of the *mTMT* gene.** The nucleotides that comprise the 5′-flanking region, four introns, and 3′-UTR of the *mTMT* gene are in *lowercase letters*, whereas those that comprise the five translated exons of the *mTMT* gene are in *uppercase letters*. Numbering of the nucleotides begins at the gene's translation-initiation site because the 5′-UTR in exon 1 has not been conclusively established. The exons are *boxed* and the deduced amino acids of the initially translated product are indicated, as well as the components of the catalytic triad (■). The last nucleotide in exon 6 of the *mMCP-6* gene resides immediately 5′ of the depicted sequence (*i.e.* residue −2340).

adult, fetal, and tumor tissues contained *hTMT* mRNA. The oligonucleotides 5′-AGGTGCACCTGGGGGAACTGGAGATCAC-3′ and 5′-AATGCACTTGGATTCCTGCCATCAGTCAG-3′ and then the oligonucleotides 5′-ACCGTGAGGCAGATCATCCTGCACTCCAG-3′ and 5′-CCAGCTCACAATGCCAGCCTGCACCCAG-3′ were used in this two-step process to generate the relevant 410-bp *hTMT* cDNA from four different human tissue cDNA panels (CLONTECH). The obtained products were analyzed by gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase oligonucleotides (CLONTECH) were used as a positive control in these mRNA analyses.

Based on the nucleotide sequence of the isolated *hTMT* transcript, four sets of oligonucleotides were exploited to amplify its gene in three overlapping fragments from human genomic DNA. The oligonucleotides 5′-CCGGTGTGTCCCTCAGGACTTTGCAG-3′ and 5′-CGCCGCACACGTGCATCCTCCGCAG-3′ and the oligonucleotides 5′-AGGTGCACCTGGGGGAACTGGAGATCAC-3′ and 5′-AATGCACTTGGATTCCTGCCATCAGTCAG-3′ were used to isolate the portions of the *hTMT* gene that span exon 1 to exon 2 and exon 3 to exon 5, respectively. A nested PCR approach with oligonucleotides 5′-GCGCATGGCCATGGCAG-3′ and 5′-CCAGCTCACAATGCCAGCCTGCACCCAG-3′, followed by the oligonucleotides 5′-GCAGGCCAGCCTCCGC-3′ and 5′-GCATGTCGGGCTGAAGGATGCTGC-3 were used to isolate the portion of the *hTMT* gene which spans exon 2 to exon 4.

A panel of 24 hamster/human somatic hybrid cell lines (Quantum Biotechnologies, Montreal, Canada) was probed to locate the *TMT* gene in the human genome. Each analyzed hybrid cell line contained a single, but different, human chromosome. Approximately 200 ng of genomic DNA from a cell line was utilized as the template in each 50-μl

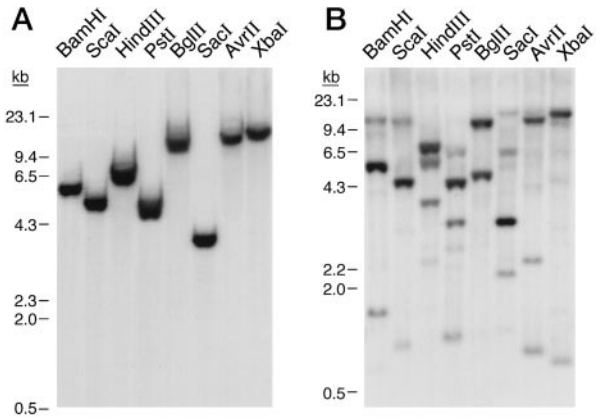
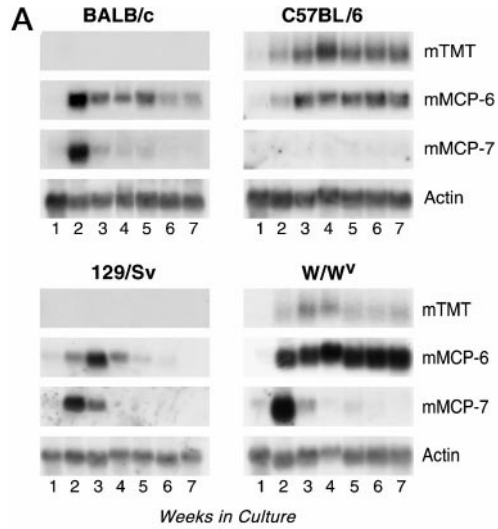


FIG. 3. **Genomic blot analysis.** Blots containing mouse genomic DNA that had been digested with *Bam*HI, *Sca*I, *Hin*dIII, *Pst*I, *Bgl*II, *Sac*I, *Avr*II, or *Xba*I were probed under conditions of high (*A*) or moderate (*B*) stringency with a radiolabeled 224-bp fragment derived from exon 3 of the *mTMT* gene. This probe corresponds to amino acid residues 75 to 149 in the mature tryptase. DNA fragments of known molecular weight (derived by Life Technologies, Inc. from a *Hin*dIII digest of λ DNA) are indicated on the *left* of each blot.



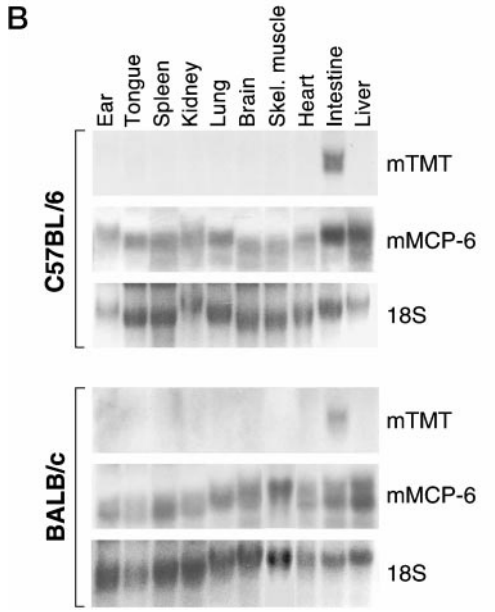


FIG. 4. **Kinetics of expression of the *mTMT* transcript in mBMMCs developed from four different mouse strains, and expression of the *mTMT* in various tissues of two adult mouse strains.** *A,* interleukin 3-dependent mBMMCs were developed from BALB/c, C57BL/6, 129/Sv, and W/W^v mice. Total RNA was isolated weekly from each culture. After 7 weeks of continuous culture, RNA blots were prepared and analyzed with gene-specific probes for *mTMT, mMCP-6, mMCP-7,* and *β-actin* mRNA to evaluate the kinetics of expression of the *mTMT* transcript in the mBMMCs developed from each strain. The level of *mMCP-7* mRNA is below detection in C57BL/6 mBMMCs because of a point mutation in the exon 2/intron 2 splice site of the gene in this strain (38). *B,* blots containing total RNA from C57BL/6 and BALB/c mouse ear, tongue, spleen, kidney, lung, brain, skeletal muscle (leg), heart, intestine, and liver were analyzed with gene-specific probes for *mTMT* and *mMCP-6* mRNA. The RNA gels used in these experiments were also stained with ethidium bromide to demonstrate that comparable amounts of 18 S rRNA were loaded into each lane.

PCR. Normal human genomic DNA and normal hamster genomic DNA served as positive and negative controls, respectively. The sense and antisense oligonucleotides in these PCRs were 5′-CGTACAGCCTGCGGGAGGTGAAAGTCTC-3′ and 5-TAATCTGATGCAGAAGACTCAGC-3′, respectively. The obtained products were analyzed by gel electrophoresis and then sequenced.

Immunohistochemistry—The anti-peptide approach used to obtain mMCP-6- (26) and mMCP-7- (24) specific antibodies in rabbits was employed to obtain hTMT-specific antibodies. The 16-mer peptide Val-Pro-Ala-Tyr-Val-Asn-Trp-Ile-Arg-Arg-His-Ile-Thr-Ala-Ser-Gly, which corresponds to residues 221 to 236 in mature hTMT, is not present in any protein in the GenBank protein data base. The models of the three-dimensional structures[3] of hTMT and mTMT predicted that this peptide would protrude from the surface of the folded tryptase. Thus, antibodies were raised in rabbits against the synthetic peptides by Quality Controlled Biochemicals (Hopkinton, MA); the synthetic peptide was then used to affinity purify the antibodies.

Immunohistochemistry was carried out essentially as described for other anti-tryptase antibodies (24, 26). The anti-hTMT antibodies were used to evaluate hTMT expression in human skin and large intestine. For these experiments, histologically normal skin ($n = 3$) or intestine ($n = 2$) specimens were snap frozen and placed in Tissue-Tek™ O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan). Cryostat sections (5 μm) were cut in a Reichert-Jung cryostat, mounted on gelatin-coated glass slides, and air-dried overnight at room temperature. Each section was fixed for 20 min at room temperature in 4% paraformaldehyde/phosphate-buffered saline. After the sections were incubated for 3 h at 37 °C with a 1:50 dilution of anti-hTMT immunoglobulin (Ig) in phosphate-buffered saline, they were sequentially washed in phosphate-buffered saline, incubated for 1 h with biotinylated anti-rabbit IgG $F(ab')_2$ (3.6 μg/ml) (Dakopatts, Glostrup, Denmark), for 1 h with alkaline phosphatase-conjugated streptavidin (Silinus, Hawthorn, Australia), and for 20 min with alkaline phosphatase substrate (0.2 mg/ml solution of naphthol 3-hydroxy-2-naphthonic acid 2,4-dimethylanilide phosphate (Sigma) containing 0.1 mg/ml Fast Red 4-chloro-2-methylbenzenediazonium (Sigma) in 0.1 M Tris-HCl, pH 8.2).

Human skin biopsies were also processed for immunoelectron microscopy in order to determine where TMT resides inside human cutaneous MCs. Tissue blocks (2 mm^3) were immersed for 4 h at room temperature in a 0.1 M cacodylate buffer, pH 7.4, containing 1.25% glutaraldehyde, 1% paraformaldehyde, and 0.025% $CaCl_2$. After an overnight incubation at 4 °C in 0.1 M cacodylate buffer, the blocks were postfixed for 2 h at room temperature in 2% osmium tetroxide. They were then stained for 2 h at room temperature with 2% of uranyl acetate, dehydrated in graded ethanol, and embedded in Spurr's low viscosity media (ProSciTech, Thuringowa, Australia). Sections (60–80 nm) were cut on an Reichert-Jung ultramicrotome and placed on grids. The grids were placed upon drops of reagents on the parafilm, etched for 15 min in 10% of hydrogen peroxide, and washed with water. Each section was equilibrated with Tris-HCl, 1% bovine serum albumin, pH 8.2, before the 4-h incubation at 37 °C with primary antibody diluted in this buffer. Antibody-treated sections were washed with the Tris-HCl/albumin buffer and then exposed for 1 h at 37 °C to gold-labeled anti-rabbit IgG (dilution of 1:50) (Ted Pella Inc., Redding, CA). The resulting sections were counterstained with lead citrate before being examined with a Hitachi 7000 electron microscope. For a negative control, sections were not exposed to the primary antibody.

[3] The three-dimensional models for mTMT and hTMT can be downloaded from the Web at http://guitar.rockefeller.edu/pub/sali/models.

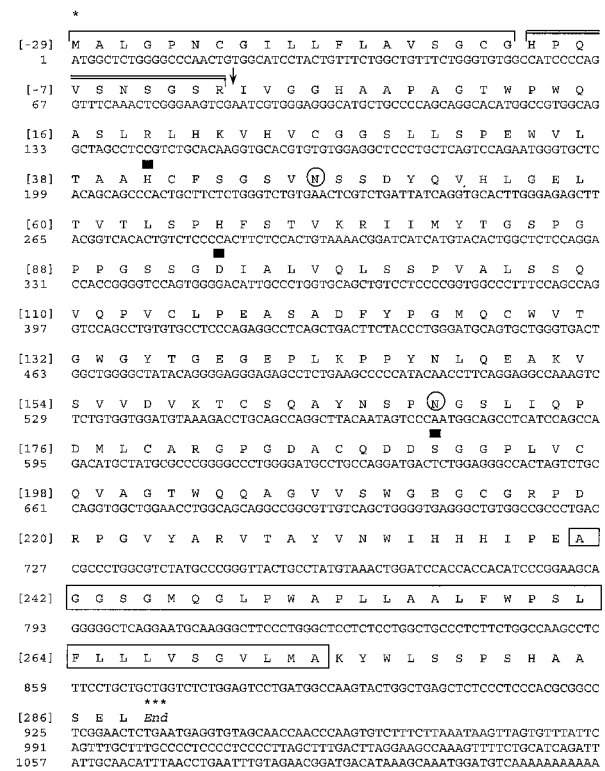


FIG. 5. **Nucleotide and amino acid sequences of the *mTMT* transcript.** Three different molecular approaches were used to deduce the nucleotide and amino acid sequences of the 1.2-kb *mTMT* transcript. As noted under "Results," an EST was identified, isolated, and sequenced from mixed mouse tissues that corresponds to 109 to 1112 of the *mTMT* transcript. A 5′-RACE approach was then carried out on a pool of liver cDNAs to isolate a cDNA that corresponds to residues 7 to 626 in the transcript. Finally a RT-PCR approach was used to isolate a cDNA from mBMMCs that corresponds to residues 1 to 937 in the *mTMT* transcript. The nucleotide and amino acid sequences of the cDNAs are depicted. The two potential *N*-linked glycosylation sites in mTMT are *circled* and its COOH-terminal transmembrane segment is *boxed*. Components of the catalytic triad (■), translation-initiation site (*), signal peptide (*single bracket*), and propeptide (*double bracket*) are indicated. Nucleotide numbering begins at the first nucleotide identified so far in the transcript; amino acid numbering (*within brackets* at *left*) begins with residue 1 of the mature protein.

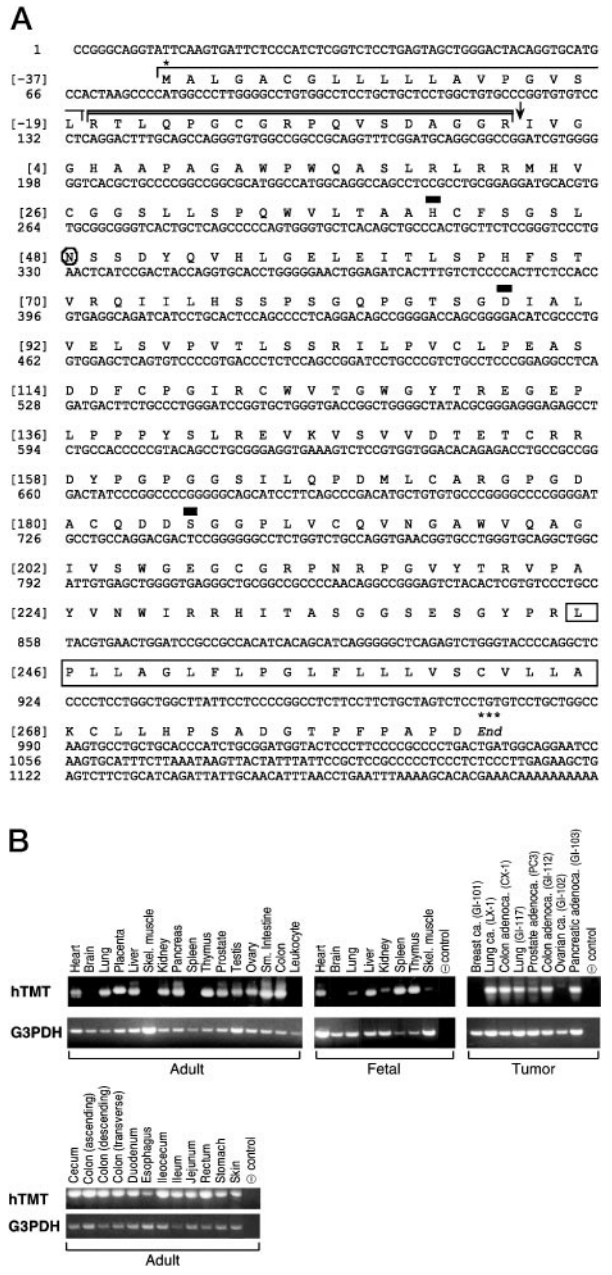


FIG. 6. **Nucleotide and amino acid sequences of the *hTMT* transcript, and evaluation of its expression at the mRNA level in varied adult, fetal, and tumor tissues.** *A,* a PCR approach was used to obtain a near full-length *hTMT* cDNA from human liver, cecum, uterus, and skin. The consensus nucleotide and amino acid sequences of the PCR products are depicted. The one potential *N*-linked glycosylation site in hTMT is *circled* and its COOH-terminal transmembrane segment is *boxed*. Components of the catalytic triad (■), translation-initiation site (*), signal peptide (*single bracket*), and propeptide (*double bracket*) are indicated. Nucleotide numbering begins in the 5′-UTR of the isolated transcript; amino acid numbering (*within brackets* at *left*) begins with residue 1 of the mature protein. *B,* cDNA panels from CLONTECH were used in a PCR approach to evaluate *hTMT* mRNA expression in the indicated normal adult and fetal tissues. The indicated transformed human cell lines from CLONTECH were also evaluated for their expression of *hTMT* and *glyceraldehyde-3-phosphate dehydrogenase* (*G3PDH*) mRNA. These latter cell lines were maintained as solid tumors in nude mice. The three indicated negative control PCRs (−) were carried out in the absence of template DNA.

RESULTS

Cloning and Analysis of the mTMT Gene—Mapping analysis with varied restriction enzymes revealed that the λ phage clone GW-1 contained two homologous but distinct genes in its ~13-kb insert (Fig. 1*A*). These genes were oriented in the same direction in GW-1 and were separated from one another by only 2.3 kb of flanking DNA. Nucleotide sequencing analysis revealed that one of the genes encoded mMCP-6; the other encoded a novel tryptase. A long-range PCR approach (Fig. 1*B*), carried out with both BALB/c and 129/Sv mouse genomic DNA, confirmed the closeness of the two genes on chromosome 17. The novel *mTMT* gene in GW-1 was 3.7 kb in size and consisted of 5 exons. The nucleotide sequence of the *mTMT* gene and its exon/intron organization are shown in Fig. 2, as well as the nucleotide sequence that separates the *mMCP-6* and *mTMT* genes.

Only one DNA fragment was detected when a mouse genomic DNA blot was probed under conditions of high stringency with a probe derived from exon 3 of the *mTMT* gene (Fig. 3*A*). Although this finding indicates that the probe used in the DNA and RNA blot analyses is relatively gene-specific, 3 to 5 genomic fragments were obtained when another blot was probed under less stringent conditions (Fig. 3B). Subsequent reprobing of these DNA blots indicated that the weaker hybridizing fragments were not derived from the *mMCP-6* or *mMCP-7* genes (data not shown). Thus, there appears to be at least three *mTMT*-like genes in the mouse genome.

Isolation and Characterization of the mTMT Transcript and

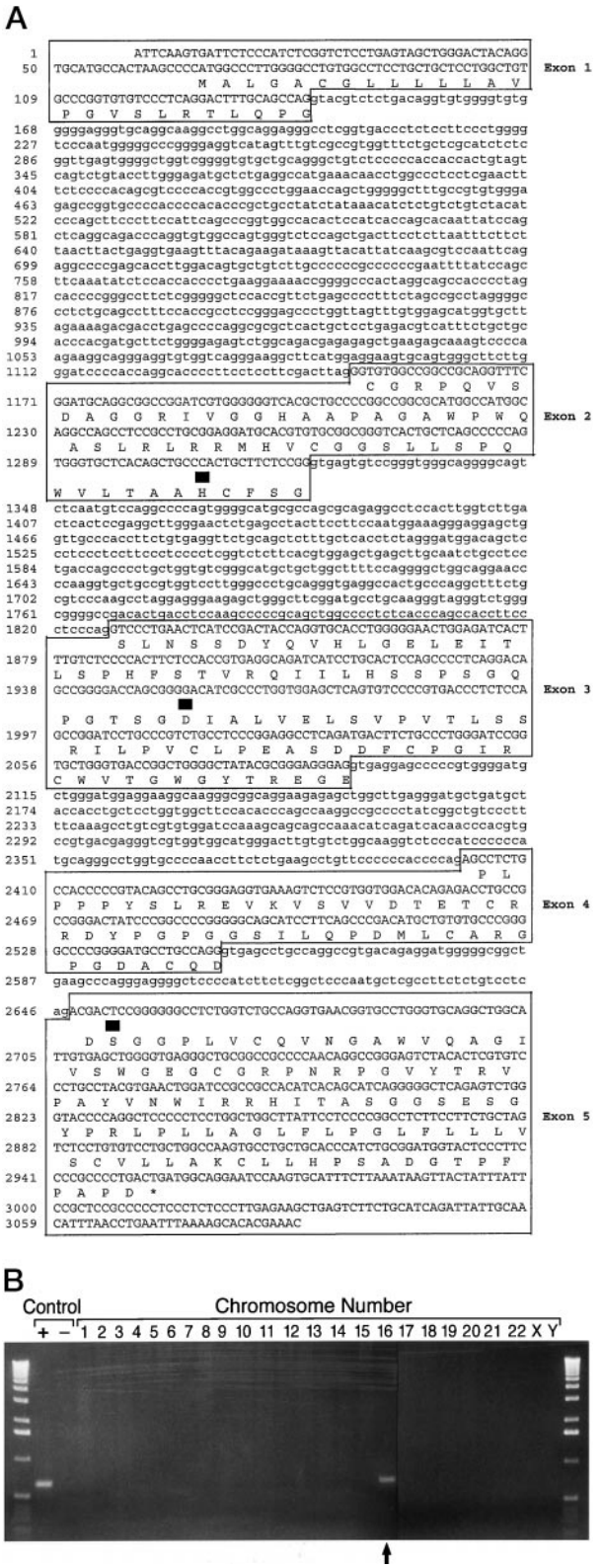


FIG. 7. **Structure and chromosomal location of the *hTMT* gene.** *A,* the nucleotides that comprise the exons and introns of the *hTMT* gene are in *upper* and *lower-case letters*, respectively. The exons are *boxed* and the deduced amino acids of the initially translated product are indicated, as well as the components of the catalytic triad (■). *B,* genomic DNA derived from 24 human/hamster somatic hybrid cell lines were used as templates in a PCR approach to determine the chromosomal location of the *hTMT* gene. Normal human and hamster genomic DNA were used as positive (+) and negative (−) controls, respectively. As noted (*arrow*), the relevant PCR product was only generated from the human/hamster cell line that contained human chromosome 16. The 1-kb molecular weight ladders are shown at both ends of the blot.

Evaluation of Its Expression—The steady-state level of the *mTMT* transcript was below detection in BALB/c mouse-derived V3 MCs (data not shown), as well as in BALB/c and 129/Sv mBMMCs (Fig. 4*A*). Nevertheless, the corresponding mBMMCs developed from W/W^v and C57BL/6 mice contained high levels of the *mTMT* transcript (Fig. 4*A*). Kinetic studies revealed that the *mTMT* transcript is expressed quite early in the differentiation process of uncommitted progenitors into immature MCs in the latter two mouse strains. While these data indicated that *mTMT* is expressed in mouse MCs in a strain-dependent manner, RNA blot analysis of varied tissues of the C57BL/6 mouse also indicated that *mTMT* is expressed in a tissue-dependent manner. Of those analyzed tissues in the C57BL/6 mouse (Fig. 4*B*), the intestine contained the highest level of *mTMT* mRNA. The level of *mTMT* mRNA in the intestine of the BALB/c mouse is substantially lower than that in the intestine of the C57BL/6 mouse (Fig. 4*B*). Nevertheless, its presence indicates that the *mTMT* gene can be transcribed *in vivo* in the BALB/c mouse. The *mTMT* transcript was not abundant in leg skeletal muscle which is rich in mMCP-6$^+$/mMCP-7$^+$ MCs in the BALB/c mouse and most other mouse strains.

Although most of the *mTMT* transcripts in C57BL/6 mBMMCs and intestine were ~1.2 kb in size, larger sized transcripts were occasionally detected. A search of the GenBank data base of ESTs was therefore carried out in the initial attempt to obtain a full-length mTMT cDNA. An EST clone generated from mixed mouse tissue (GenBank accession number AA266560) was identified in the data base. Nucleotide sequence analysis of this clone revealed that it possessed all but the 5′ portion of the putative ~1.2-kb *mTMT* transcript depicted in Fig. 5. Using a RACE approach, the remaining portion of the *mTMT* was isolated from mouse liver. To confirm the nucleotide sequence of the authentic mTMT transcript, a RT-PCR approach was then used to isolate a near full-length clone from W/W^v mBMMCs. The cumulated data (Fig. 5) revealed that the exon/intron boundaries of the 3.7-kb *mTMT* gene (Fig. 2) were correctly predicted.

Isolation and Characterization of the hTMT Gene and Transcript, and Chromosomal Location of the hTMT Gene—A search of the GenBank data base of ESTs was carried out to determine whether or not there is a human ortholog of the *mTMT* transcript. Two human EST clones (GenBank accession numbers AA327025 and AA503882) were identified in the data base which possessed short stretches of nucleotides that were quite similar to those of the query mouse sequence. Unfortunately, because neither clone was available from the I.M.A.G.E. consortium, it was not possible to determine the nucleotide sequences of their entire inserts. A PCR/RACE approach was therefore employed to isolate the *hTMT* transcript. An oligonucleotide that corresponds to a relatively conserved region in various MC tryptase transcripts and an oligonucleotide that corresponds to the 3′ end of the *mTMT* transcript was initially used to isolate a 326-bp fragment of the *hTMT* transcript from human liver. Based on the nucleotide sequence of this PCR product, more specific oligonucleotides were synthesized and used in subsequent 5′- and 3′-RACE approaches to obtain the near full-length *hTMT* cDNA from liver, uterus, cecum, and skin (Fig. 6*A*). Analysis of the resulting cDNAs revealed that the *hTMT* transcript has a 5′-untranslated region (UTR) which consists of at least 77 nucleotides and a 3′-UTR that consists of >225 nucleotides. Like the *mTMT* cDNA (Fig. 5), the *hTMT* cDNA (Fig. 6*A*) lacks a classical "AATAAA" polyadenylylation regulatory site in its 3′-UTR.

Analysis of varied human adult, fetal, and tumor tissues (Fig. 6*B*) suggests that the expression of *hTMT* at the mRNA

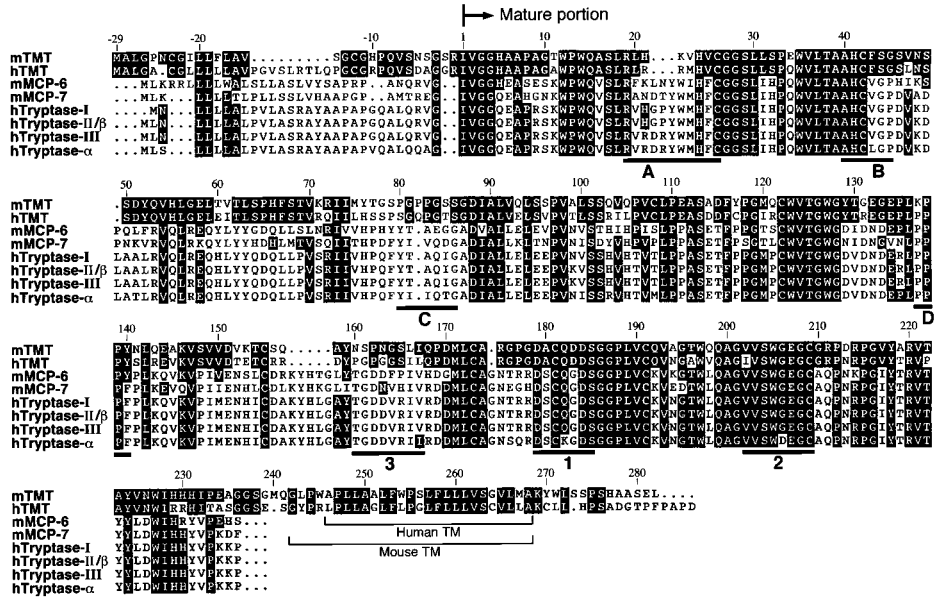


FIG. 8. **Comparison of the amino acid sequences of mTMT and hTMT with each other and with other mouse and human MC tryptases.** The amino acid sequences of mMCP-6 (1, 2), mMCP-7 (3, 4), hTryptase-I (15), hTryptase-II/β (14, 15), hTryptase-III (15), and hTryptase-α (13) were extracted from SwissProt data base. The depicted multiple sequence alignments were performed using the PILEUP program of the Eugene "GCG" software package. In each instance, identical amino acids in the sequences are *shaded*. Numbering begins at the first residue in the mature portion of the tryptase. The transmembrane segments of mTMT and hTMT are *bracketed*. The seven putative loops (designated *A-D* and *1–3*) that form the substrate-binding pockets of these tryptases are *underlined*.

level is not as restricted as its mouse ortholog. Although the *hTMT* transcript was found in many normal and tumor tissues, it was not detected in skeletal muscle. The failure to detect the *hTMT* transcript in adult spleen even though *hTMT* mRNA is present in fetal spleen (Fig. 6*B*) raises the possibility that the expression of this tryptase is developmentally regulated in certain human tissues.

Based on the *hTMT* mRNA data, a PCR approach was used to isolate and characterize the human gene (Fig. 7*A*). The exon/intron organizations of the *hTMT* and *mTMT* genes are similar. Except for intron 1, the exons and introns of this gene are comparable in size in the two species. Exon 1 encodes the hydrophobic signal peptide predicted to be removed during the maturation of the zymogen. Thus, as expected, exon 1 is the least conserved exon. Exons 2, 3, 4, and 5 of the *hTMT* and *mTMT* genes are 79, 79, 76, and 75% identical, respectively. Using the hamster/human hybrid cell lines that vary in which human chromosome they contain, it was discovered that the *hTMT* gene resides on chromosome 16 (Fig. 7*B*).

Amino Acid Sequence Analysis of mTMT and hTMT, and the Expression of these Proteases in MCs—mTMT is predicted to be translated as a zymogen which consists of a signal peptide of 19 residues, a propeptide of 10 residues, and a mature domain of 282 residues. The propeptides of mTMT and hTMT do not resemble those in any other MC tryptase in terms of their amino acid sequences (Fig. 8). hTMT consists of 321 amino acids and has 10 more residues than mTMT mainly due to an insertion of 9 amino acids in its prepropeptide. The overall amino acid sequences of mature mTMT and hTMT are 74% identical. When compared with other tryptases in the chromosome 17 family, mature mTMT is 45% identical to mMCP-6 and 46% identical to mMCP-7; mature hTMT is 48% identical to human tryptase I. However, if the dissimilar prepropeptide and COOH-terminal extension peptide of mTMT and hTMT are taken into account, the extent of homology of this new tryptase with the other members of its family is considerably lower. mTMT has two potential *N*-linked glycosylation sites but these sites are not conserved in other mouse and human tryptases in the family. At pH $>$ 6.5, mature mTMT and hTMT have overall charges of -6 and -3, respectively. However, at pH $<$ 6.5, mature mTMT and hTMT have overall charges of $+4$ and $+5$, respectively, and these positively charged residues are aligned predominately on one face of each tryptase.

Using an anti-peptide approach, hTMT-specific antibodies were generated to confirm that the isolated transcripts are translated in certain populations of human MCs. In control experiments, the antibodies recognized recombinant hTMT zymogen but failed to recognize recombinant mMCP-6, mMCP-7, mTMT, human tryptases α, or human tryptase II/β (Fig. 9*A*). Since the corresponding region in human tryptases I, II/β, and III are 100% identical, the antibodies also cannot recognize human tryptases I or III. Using this highly specific antibody, immunoreactive hTMT was found in the MCs that reside in human large intestine (Fig. 9*B*) and skin (Fig. 9*D*). At the ultrastructural level, most of the tryptase in the cutaneous MC resides in the secretory granules (Fig. 9*E*).

DISCUSSION

Complexes of tryptase genes reside on human chromosome 16 (16) and the syntenic region of mouse chromosome 17 (17, 18). While analyzing the mouse tryptase complex in greater detail, a novel 3.7-kb gene was discovered that encodes an unusual transmembrane tryptase. No serine protease has been discovered which possesses an overall structure that resembles mTMT or hTMT.

Adjacent genes on a mammalian chromosome tend to be separated by ~30 kb of flanking DNA. Nevertheless, a cluster of five ~3-kb trypsinogen genes that are separated by 7 kb or less of flanking DNA has been identified in the human T cell receptor locus (30). Another cluster of serine protease genes has been identified on mouse chromosome 14, some of which are also separated by 5 to 7 kb of flanking DNA (17, 31, 39). The observation that the chromosome 14 family of serine proteases has an extremely low recombination frequency suggested that the close spacing may be a common feature for the chromosomal organization of serine protease genes within an individual family. The fact that more tryptases have been cloned from humans (13–16) than mice (1–4), coupled with the fact that the

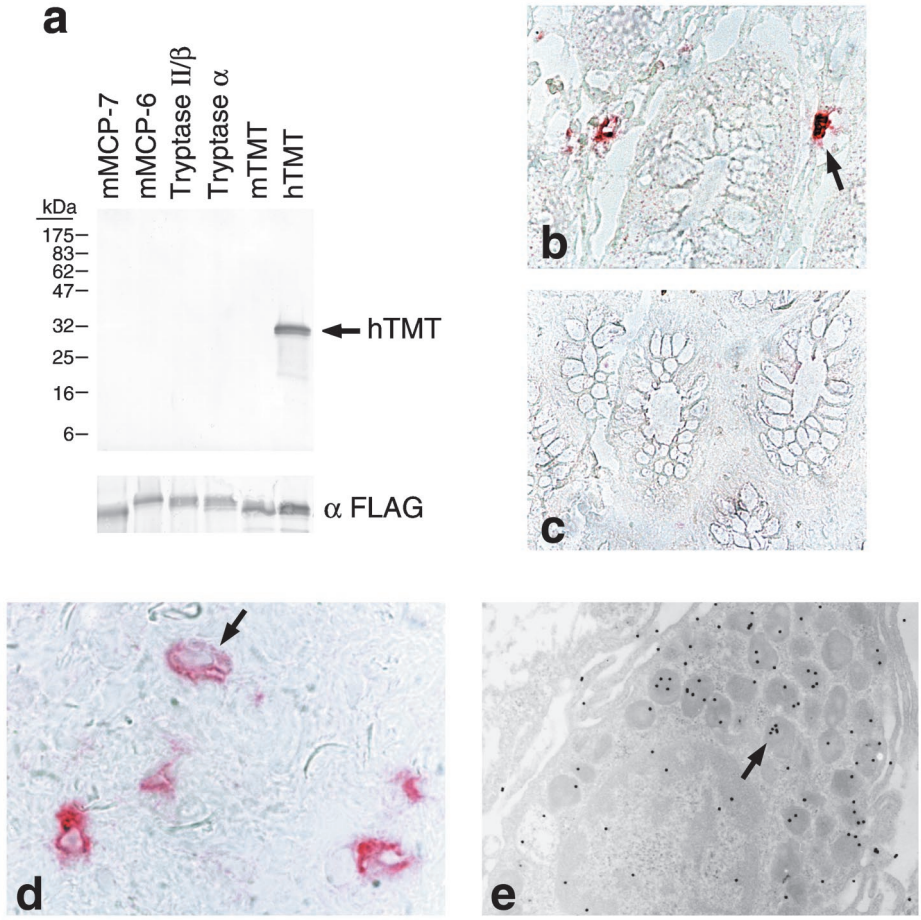


FIG. 9. **Immunohistochemistry.** Replicate SDS-PAGE/immunoblots containing similar amounts of the recombinant tryptases mMCP-7, mMCP-6, human tryptase II/β, human tryptase α, mTMT, and hTMT were probed in *A* with anti-hTMT Ig (*top blot*). Because each recombinant zymogen has the 8-residue FLAG peptide attached to its COOH terminus, the anti-FLAG antibody was also used in *A* to demonstrate that similar amounts of recombinant tryptase is present in each lane (*bottom blot*). Even though the overall amino acid sequences of hTMT and mTMT are 74% identical, anti-hTMT Ig does not recognize mTMT or any other recombinant mouse or human tryptase. Because anti-hTMT antibody is highly specific, human intestine (*B*) and skin (*D* and *E*) were stained with the antibody to evaluate the major cell types in these tissues which contain hTMT protein. For a negative control, human intestine was stained with an irrelevant anti-peptide rabbit antibody (*C).* Human skin was stained with gold-labeled anti-hTMT antibody to identify where hTMT resides in its expressing cell. As noted in *B* and *D*, cutaneous and intestinal MCs express hTMT protein (*arrows)* and most of this tryptase resides predominately in the secretory granules (*arrow*). To confirm that the immunoreactive cells in *B* are indeed MCs, the adjacent serial section (data not shown) was stained with a commercial antibody (Chemicon, Temecula, CA) that recognizes all human tryptases but hTMT.

mMCP-6 and *mMCP-7* genes are separated on chromosome 17 by ~1.2 centimorgans (17, 18), raised the possibility that undiscovered tryptase genes might reside on the chromosome between the *mMCP-6* and *mMCP-7* genes. Thus, a chromosome-walk approach was used to identify the functional gene that is adjacent to the *mMCP-6* gene. As noted in Figs. 1 and 2, a novel tryptase gene was identified 2.3 kb 3′ of the *mMCP-6* gene.

The isolation and characterization of the *mTMT* gene (Fig. 2) and its transcript (Fig. 5) eventually led to the isolation and characterization of a related gene (Fig. 7) and transcript (Fig. 6) in humans. Although genomic blot analysis revealed that the mouse genome contains at least three *mTMT*-like genes (Fig. 3), the isolated human gene (Fig. 7*A*) appears to be the ortholog of the *mTMT* gene because it possesses a high degree of sequence identity throughout nearly all of its exons. Like the four other human MC-restricted tryptase genes (16), the *hTMT* gene resides on chromosome 16 (Fig. 7*B*).

While the level of the *mTMT* transcript was below detection in BALB/c mBMMCs (Fig. 4*A*), the corresponding mBMMCs developed from C57BL/6 and W/W^v mice contained high levels of the transcript. Thus, *mTMT* mRNA is expressed in mice in a strain-dependent manner. W/W^v mBMMCs also differ from BALB/c mBMMCs in their expression of the chymases mMCP-2 and mMCP-4 (40). While it was initially thought that the *mMCP-2* and *mMCP-4* genes were not transcribed in BALB/c mBMMCs, subsequent studies revealed that these chymase transcripts are produced but often are rapidly degraded in this mouse strain by a novel cytokine-dependent, post-transcriptional mechanism (41). It was then discovered that the expression of *mMCP-7* mRNA in BALB/c mBMMCs is also regulated, in part, by a cytokine-dependent post-transcriptional mechanism.[4] Repetitive motifs residing in the 3′-UTR often regulate the stability of transcripts (42, 43). The finding that the 3′-UTR of the *mTMT* transcript has cytosine-rich motifs (Fig. 5) which resemble those in *mMCP-2* and *mMCP-4* transcripts (41) raises the possibility that the strain-dependent expression of *mTMT* mRNA is also regulated, in part, by a post-transcriptional mechanism.

Of those analyzed tissues, the intestine contained the highest level of the *mTMT* transcript (Fig. 4*B*). In most strains, the MCs in the jejunal submucosa express *mMCP-6* and *mMCP-7* (20). While the RNA data raised the possibility that *mTMT* is

[4] R. L. Stevens, unpublished data.

coordinately expressed *in vivo* with one or both tryptases, subsequent studies revealed that *mTMT* expression in the BALB/c mouse is regulated in a tissue-dependent manner independent of *mMCP-6* and *mMCP-7*. For example, the level of *mTMT* mRNA is below detection in skeletal muscle which contains large numbers of mMCP-6$^+$ MCs (Fig. 4*B*). While the expression of the *TMT* transcript may be less restricted in humans than in mice, it is of interest to note that *hTMT* mRNA also could not be detected in skeletal muscle (Fig. 6*B*). The latter RNA data support the nucleotide and protein sequence data which suggested that the isolated human gene is the ortholog of the *mTMT* gene.

mTMT and hTMT have all of the features of functional serine proteases (Fig. 8) and immunohistochemical data (Fig. 9) confirmed that the human transcripts are converted into protein. It is possible that hTMT is expressed by other cell types. However, at least in the skin and large intestine, its expression appears to be restricted to the MC. Both putative tryptases have the His-Asp-Ser catalytic triad and the NH_2-terminal Ile that becomes buried in the activation grove of a typical serine protease during its maturation (44, 45). Loops 1 and 2 of the substrate-binding cleft are the most conserved loops in the mouse tryptase family. Loop 1 is located at the base of the S1 pocket and therefore forms a critical portion of the substrate-binding cleft of serine proteases (44). Analogous to other tryptases, the conserved Asp residue that dictates tryptic specificity is present in loop 1 of mTMT and hTMT. These findings strongly suggest that mTMT and hTMT are tryptases. When comparing the three cloned mouse MC tryptases (Fig. 8), the other loops that form the substrate-binding clefts of mTMT, mMCP-6, and mMCP-7 differ substantially in their amino acid sequences. In addition, loops A and C differ in their length. Thus, the preferred substrate preference of mTMT almost certainly differs from that of mMCP-6 and mMCP-7.

One of the most distinctive features of TMT is the transmembrane segment located at its COOH terminus. Because this segment does not have a Tyr residue in either mouse or human TMT, the COOH terminus cannot undergo Tyr phosphorylation. However, because the cytosolic domain has a conserved Ser residue, it is possible that this residue undergoes phosphorylation during the metabolism of the tryptase. Other tryptases have conserved Tyr-, Pro-, and Trp-rich domains which are needed for tetramer formation (4, 45). Based on the crystallographic structure of human tryptase II/β, 6 surface loops are involved in the two different kinds of contacts within the tetramer. Because these loops are not conserved in sequence and length, it is unlikely that TMT is able to form a similar tetramer structure. Nevertheless, this putative tryptase has structural features that allow it to interact with other proteins. Comparative structural modeling analysis revealed that at pH < 6.5, mature mTMT and hTMT have a number of positive charged residues that are aligned predominately on one face of the tryptase. MC granule proteases use similarly positioned positively charged faces to ionically bind to negatively charged serglycin proteoglycans (25, 26, 46) and other granule constituents. Thus, hTMT and mTMT have the capacity of binding to certain negatively charged molecules during their biosynthesis and/or catabolism.

All mature human and mouse tryptases have 8 Cys residues that are needed for the proper formation of 4 disulfide bonds in the folded protease. These conserved Cys residues are found in mTMT and hTMT. mTMT has an additional Cys residue, whereas hTMT has 4 additional Cys residues. A tryptase has been cloned from dog mastocytoma (9) which has 4 more Cys residues than mMCP-6 and mMCP-7. The dog tryptase forms a tetramer that consists of two 66-kDa dimers with each dimer having disulfide-linked monomers (47). Thus, it is possible that the additional Cys residues in TMT allows this tryptase to form dimers with itself or another protein.

Those few membrane-associated serine proteases with a trypsin-like specificity that have been found so far regulate diverse processes. Whatever the function of TMT, the number of tryptase genes at the chromosome 17 complex in mice, and the corresponding chromosome 16 complex in humans has been underestimated. A primordial serine protease gene residing on chromosome 17 in mice (and the syntenic region of chromosome 16 in humans) duplicated early and often during the evolution of mammals to generate a panel of homologous tryptases in each species that differ in their tissue expression, substrate specificities, and physical properties.

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