Tryptase 4, a New Member of the Chromosome 17 Family of Mouse Serine Proteases*

Guang W. Wong‡§, Lixin Li¶, Mallur S. Madhusudhan‖, Steven A. Krilis¶, Michael F. Gurish‡, Marc E. Rothenberg**, Andrej Šali‖‡‡, and Richard L. Stevens‡§§

*From the ‡Department of Medicine, Brigham and Women's Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, ¶Department of Medicine, University of New South Wales, and Department of Immunology, Allergy, and Infectious Disease, St. George Hospital, Kogarah, New South Wales 2217, Australia, ‖Rockefeller University, New York, New York 10021, and **Department of Pediatrics, Division of Pulmonary Medicine, Allergy, and Clinical Immunology, Children's Hospital Medical Center, University of Cincinnati Medical Center, Cincinnati, Ohio 45229*

Genomic blot analysis raised the possibility that uncharacterized tryptase genes reside on chromosome 17 at the complex containing the three genes that encode mouse mast cell protease (mMCP) 6, mMCP-7, and transmembrane tryptase (mTMT). Probing of GenBank's expressed sequence tag data base with these three tryptase cDNAs resulted in the identification of an expressed sequence tag that encodes a portion of a novel mouse serine protease (now designated mouse tryptase 4 (mT4) because it is the fourth member of this family). 5′- and 3′-rapid amplification of cDNA ends approaches were carried out to deduce the nucleotide sequence of the full-length mT4 transcript. This information was then used to clone its ~5.0-kilobase pair gene. Chromosome mapping analysis of its gene, sequence analysis of its transcript, and comparative protein structure modeling of its translated product revealed that mT4 is a new member of the chromosome 17 family of mouse tryptases. mT4 is 40–44% identical to mMCP-6, mMCP-7, and mTMT, and this new serine protease has all of the structural features of a functional tryptase. Moreover, mT4 is enzymatically active when expressed in insect cells. Due to its 17-mer hydrophobic domain at its C terminus, mT4 is a membrane-anchored tryptase more analogous to mTMT than the other members of its family. As assessed by RNA blot, reverse transcriptase-polymerase chain reaction, and/or *in situ* hybridization analysis, mT4 is expressed in interleukin-5-dependent mouse eosinophils, as well as in ovaries and testes. The observation that recombinant mT4 is preferentially retained in the endoplasmic reticulum of transiently transfected COS-7 cells suggests a convertase-like role for this integral membrane serine protease.

A complex of genes resides on human chromosome 16p13.3 that encodes the homologous tryptases α1, α2, βI, βII, βIII, transmembrane tryptase (TMT)[1]/tryptase γ, and eosinophil serine protease-1 (Esp-1)[2] (1–7). The corresponding complex of genes resides on the syntenic region of mouse chromosome 17 at the interface between bands 17A3.3 and 17B1 (7–11). Although our previous genomic blot analysis suggested the presence of additional mouse tryptase genes in the family (7), the only genes and/or transcripts cloned so far at the mouse complex are those that encode mouse mast cell protease (mMCP) 6, mMCP-7, and mouse TMT (mTMT).

The amino acid sequences of mMCP-6 and mMCP-7 are ~75% identical, and the mast cells (MCs) in the skin and skeletal muscle of BALB/c mice express both tryptases (12, 13). Nevertheless, *in vivo* and *in vitro* studies have established that these two homologous serine proteases are metabolized quite differently during allergic reactions (12). Moreover, they are functionally distinct. Because fibrinogen is a physiologic substrate of mMCP-7 (14), this tryptase appears to help prevent the deposition of fibrin/platelet clots in inflammatory sites so that the various granulocytes and lymphocytes in the blood are not physically prevented from entering an inflamed mouse tissue site. Recent data have suggested that mMCP-7 also regulates eosinophil extravasation in tissues.[3] MCs are essential for combating bacterial infections in the peritoneal cavity (15–17). Because peritoneal MCs express mMCP-6 (11, 18), because the complement factors C3a and C5a induce peritoneal MCs to degranulate (19), and because recombinant mMCP-6 is a potent and selective inducer of neutrophil extravasation into the peritoneal cavity (20), mMCP-6 appears to play an important and protective role in bacterial infections. The function of mTMT is not yet known, but its C-terminal hydrophobic domain (7) probably causes its prolonged retention at the plasma membrane when mTMT⁺ MCs are immunologically activated.

[1] The abbreviations used are: TMT, transmembrane tryptase; ER, endoplasmic reticulum; hEsp-1, human eosinophil serine protease-1; FISH, fluorescent *in situ* hybridization; MC, mast cell; mMCP, mouse MC protease; mT4, mouse tryptase 4; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; mTesp, mouse testicular serine protease; UTR, untranslated region; EST, expressed sequence tag; kb, kilobase pairs; bp, base pairs; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide *N*-glycosidase F.

[2] Genomic sequencing of the 35.7-kb cosmid clone 352F10 by D. O. Ricke and co-workers (GenBank™ direct submission, accession number AC005361) resulted in the initial identification and chromosomal location of the 4.6-kb human gene in 1998 that others (4, 5, 44) designated as the *hEsp-1/testisin* gene in 1999.

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

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Because our previous genomic blot analyses suggested the presence of undiscovered tryptase-like genes in the mouse genome and because mouse chromosome 17A3.3 to 17B1 has not been sequenced in its entirety, we used a gene-hunt approach in the present study to identify a new member of this important family of serine proteases. We now describe the isolation, characterization, and expression of the fourth member of the mouse tryptase family of serine proteases.

EXPERIMENTAL PROCEDURES

Cloning of the mT4 cDNA and Gene—The nucleotide sequences of the mTMT, mMCP-6, and mMCP-7 transcripts were used as templates to search for novel, but related, mouse tryptase-like ESTs in the GenBank™ data base. Sequence analysis of the mouse testis-derived clone AI326140, obtained from the "Integrated Molecular Analysis of Gene Expression" consortium, revealed that its insert corresponded to a portion of what appeared to be a novel serine protease. Based on the deduced nucleotide sequence of this clone, 5′- and 3′-rapid amplification of cDNA ends (RACE) approaches were carried out on a pool of testis cDNAs (CLONTECH) to deduce the nucleotide and amino acid sequences of the full-length transcript. The 3′-RACE reaction was carried out with 5′-CACCTACAATAACTTCATCCAGC-3′ and the anchor oligonucleotide 5′-CCATCCTAATACGACTCACTATAGGGC-3′. The resulting DNA products were purified on a 1% agarose gel, subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and the inserts in two of the arbitrarily selected clones were sequenced. The 5′-RACE was carried out with the RLM-RACE kit (Ambion, Austen, TX). The mT4-specific primer 5′-GACTGGATCCAGTTGTAGTGATGACTG-3′ and the outer primer 5′-GCTGATGGCGATGAATGAACACTG-3′ were used in the first PCR. One microliter of the generated product was then used as a template in the second PCR step with the mT4-specific primer 5′-CCCAGCAGTCAGTTCGGTTCTC-3′ and the inner primer 5′-CGCGGATCCGACACTCGTTTGCTGGCTTTGATGAAA-3′. The resulting products were purified and subcloned, and the inserts in six of the arbitrarily selected clones were sequenced.

Because all mouse and human tryptase genes so far cloned in this family are <6 kb in size, a long range nested PCR approach was used to isolate and characterize the *mT4* gene from BALB/c mice. The oligonucleotides 5′-GCAAGACGTTGGTGCCACTGCTG-3′ and 5′-GTGACGTACACGTGTGGGCTCAGGCAG-3′ were used in the initial 30-cycle PCR, whereas the oligonucleotides 5′-ATGGCCTTACAGTCAACCTATTTGCAG-3′ and 5′-GCCTGAGCAGCCCATTGCGGATC-3′ were used in the subsequent 28-cycle reaction. Recombinant *Thermus thermophilus* DNA polymerase (PerkinElmer Life Sciences) was used in both PCRs.

Genomic Blot Analysis and Chromosomal Location of the mT4 Gene—Some of the human tryptases that have been cloned during the last decade are ~98% identical (3). Thus, to determine whether or not the mouse genome contains other genes that closely resemble the *mT4* gene, replicate 25-μg samples of BALB/c mouse genomic DNA were digested separately at 37 °C for ~15 h with *Eco*RI, *Dra*I, *Bam*HI, *Avr*II, *Eco*RV, *Bgl*II, or *Hin*dIII (New England Biolabs, Beverly, MA). The digests were fractionated on a 1% agarose gel, and the separated fragments were blotted onto a MagnaGraph nylon membrane (Micron Separations Inc., Westborough, MA). The resulting DNA blot was incubated for 2 h at 65 °C in QuikHyb solution (Stratagene, La Jolla, CA) containing a ^{32}P-labeled 536-bp probe corresponding to the 5′ end of the mT4 cDNA. The blot was washed twice at room temperature for 20 min each in 2× SSC containing 0.1% SDS and then twice at 65 °C for 20 min each in 0.2× SSC containing 0.1% SDS before being exposed to BIOMAX film.

A fluorescent *in situ* hybridization (FISH) technique was used by Human Genome Systems (St. Louis, MO) to determine the chromosomal location of the *mT4* gene. Preliminary studies revealed that the *mT4* gene resided in the company's BAC mouse genomic clone F1062. Slides containing normal metaphase chromosomes derived from mouse embryonic fibroblasts were therefore incubated with the digoxigenin dUTP-labeled clone F1062 in the presence of 50% formamide, 10% dextran sulfate, 2× SSC, and sheared mouse DNA. After this hybridization step, the slides were incubated with fluoresceinated anti-digoxigenin antibody and counterstained with 4,6-diamidino-2-phenylindole.

Expression of mT4 at the mRNA Level—To determine which tissues in the mouse contain abundant levels of mT4 mRNA, a blot (CLONTECH) containing ~2 μg of poly(A)$^+$ RNA from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis was probed under conditions of high stringency with a radiolabeled 536-bp probe that

corresponded to the 5′ end of the mT4 transcript. After the probe was random primed with [α-^{32}P]dCTP using the *Redi*prime kit (Amersham Pharmacia Biotech), it was hybridized to the RNA blot at 65 °C for 2 h in Quickhyb solution. The blot was washed twice at room temperature for 20 min each in 2× SSC containing 0.1% SDS and then twice at 65 °C for 20 min each in 0.2× SSC containing 0.1% SDS before being exposed to BIOMAX film for 1–2 days. After the bound mT4 probe was removed, the blot was reprobed with a β-actin cDNA (CLONTECH) to evaluate the amount of RNA loaded in each lane. In a similar manner, total RNA from normal mouse bone marrow cells, IL-5 transgenic mouse bone marrow cells, and eosinophils purified from IL-5 transgenic mice (21) were evaluated for the presence of the mT4 transcript. Day-7, -11, -15, and -17 mouse embryos also were evaluated for their mT4 mRNA levels.

BALB/c mice exhibit a substantial, but transient, T cell-dependent increase in the number of eosinophils and MCs in their jejunum 7 and 14 days, respectively, after the animals are infected with *Trichinella spiralis* (22). To determine if the eosinophils or MCs that develop in the jejunum also contain mT4 mRNA, total RNA was isolated from the small intestine, spleen, and liver of day-7 helminth-infected mice (n = 2) and day-14 helminth-infected mice (n = 2). RT-PCR was then used to determine whether or not any of the tissue specimens contained mT4 mRNA. In this assay, the RT step was carried out at 55 °C for 30 min. Forty five cycles of PCR were performed with the primers 5′-CAACAG-CATGTGTAACCATATG-3′ and 5′-GCCTGAGCAGCCCATTGCG-GATC-3′; each cycle consisted of a 5-s denaturing step at 94 °C, a 5-s annealing step at 60 °C, and a 15-s extension step at 72 °C. This RT-PCR approach also was used to evaluate mT4 expression in the ovaries and testis before and after sexual maturation. In these reactions, 32 cycles were carried out rather than 45.

Location of mT4 mRNA-expressing Cells in the Testis—For the non-radioactive *in situ* hybridization approach, the mT4-specific oligonucleotide 5′-CTATTTGGTAACGGTTGGAATAGGCCTGTAGGTTCCAG-AGAGATGGCCTGG-3′ was labeled with digoxigenin-alkaline phosphatase with a commercially available 3′ end labeling kit (Roche Molecular Biochemicals). BALB/c mouse testis was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4 °C. The preparation was washed twice with PBS containing 2% dimethyl sulfoxide and then dehydrated and embedded in JB4 glycomethacrylate according to the manufacturer's instructions (Polysciences Inc., Warrington, PA). Sections were cut on a Reichert-Jung Supracut microtome (Leica, Deerfield, IL) at 5-μm thickness and picked up on glass slides. The slides were incubated sequentially for 15 min at 37 °C in 0.025% trypsin containing 2 mM calcium chloride and then for 30 min in Target Retrieval solution (Dako, Carpinteria, CA) at 95 °C. After the slides were washed three times in distilled water, they were incubated in prehybridization solution containing 50% formamide, 4× SSC, 1× Denhardt's solution, sonicated salmon sperm DNA, and 10% dextran sulfate at 42 °C for 30 min. The prehybridization solution was removed; 50 μl of hybridization buffer containing the digoxigenin-labeled oligonucleotide was added, and each specimen was incubated overnight at 42 °C. The next morning, each slide was washed twice with 2× SSC, once with 1× SSC at room temperature, and then incubated with anti-digoxigenin-biotin complex for 30 min at room temperature. The slides were washed twice with Tris-buffered saline, incubated with streptavidin complex conjugated to horseradish peroxidase, and washed twice again with Tris-buffered saline. Color development was performed according to the manufacturer's instructions (Dako). In this analysis, cells that contained abundant levels of mT4 mRNA stain brown.

A radioactive *in situ* hybridization approach was used to confirm the obtained data with the above digoxigenin-alkaline phosphatase-labeled probe. For this second type of *in situ* hybridization analysis, nucleotides 1–536 of the mT4 cDNA (Fig. 1) were subcloned into pCR2.1 in both the sense and antisense directions. The resulting two plasmid DNA samples were linearized with *Spe*I (New England Biolabs) and transcribed with T7 RNA polymerase (Promega, Madison, WI) in the presence of [α-^{33}P]dUTP (PerkinElmer Life Sciences) to generate antisense and sense radiolabeled RNA probes. Tissue sections were placed on slides, deparaffinized, fixed in 4% paraformaldehyde in PBS, and treated with proteinase K. After washing in 0.5× SSC, the sections were covered with 50 μl of hybridization solution (50% deionized formamide, 0.3 M NaCl, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 10 mM dithiothreitol, and 20 mM Tris-HCl, pH 8.0) and incubated for 2 h at 55 °C. ^{33}P-Labeled antisense or sense RNA probes (3 million cpm/slide) were added to the hybridization solution, and the sections were incubated for an additional 12–18 h at 55 °C. The resulting sections were washed for 20 min in 2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA and then were exposed to RNase A (10 μg/ml) for 30 min at room temperature. To minimize the possibility of nonspecific binding of the

radiolabeled probe, each section was incubated for an additional 2 h at 60 °C in 0.1× SSC, β-mercaptoethanol, and 1 mM EDTA. The resulting sections were dehydrated, dipped in photographic emulsion NTB₃ (Kodak), and stored at 4 °C. After 7 days of exposure, the radiolabeled sections were developed, and cells in these sections were counterstained with hematoxylin and eosin. In this radioactive analysis, black silver granules appear over those cells that contain abundant levels of mT4 mRNA.

Expression of mT4 Protein in COS-7 Cells and Insect Cells and Protein Modeling of Its Translated Mature Product—To address whether or not the mT4 transcript encodes an enzymatically active, membrane-anchored protease, the entire coding region of mT4 cDNA was placed in the expression vector pcDNA3.1/V5-His-TOPO (Invitrogen). This expression vector was chosen because the resulting product will contain the 14-mer V5 peptide (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) at its C terminus, thereby allowing its detection with anti-V5 antibody. Vector lacking an insert was used as a negative control in the transfection experiments. African green monkey, SV40-transformed kidney COS-7 cells (line CRL-1651, ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transient transfections were performed with SuperFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Cells were plated at a density of 2×10^5 cells/well in 6-well plates 24 h prior to transfection. After the cells were transfected for 2–3 h, they were trypsinized, and portions of the resulting cells were placed in 24-well plates containing 11-mm coverslips for immunofluorescence microscopy. The remainder of the cells were placed in 12-well plates for SDS-PAGE/immunoblot analysis. Conditioned media and cells were collected 24 and 48 h post-transfection.

For immunofluorescence microscopy, transfected COS-7 cells grown on coverslips were washed once with PBS, fixed in 4% paraformaldehyde for 10 min, and permeabilized in methanol for 10 min. The treated cells were washed three times with PBS. They were then exposed to 10% donkey serum in PBS for 1 h at room temperature to prevent nonspecific binding of the relevant mouse and rabbit antibodies. After this step, the cells were stained with mouse anti-V5 antibody (Invitrogen) in the absence or presence of a mixture of rabbit anti-calnexin and anti-calreticulin antibodies (StressGen, Victoria, British Columbia, Canada). Calnexin and calreticulin reside in the endoplasmic reticulum (ER). Thus, antibodies directed against these two proteins were used to confirm the ER location of recombinant mT4 protein in the transfectants. Cy®2-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect the mT4-V5 fusion protein, whereas Cy®3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) was used to detect calnexin and calreticulin. Stained cells were viewed with a Nikon Eclipse 800 microscope. Images were digitally captured using a CCD-SPOT RT camera and compiled using Adobe Photoshop software.

For SDS-PAGE/immunoblot analysis, samples of the transfected COS-7 cells and their conditioned media were boiled in SDS sample buffer containing β-mercaptoethanol. After electrophoresis, the resolved proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad). Each protein blot was exposed to Tris-buffered saline (15 ml) containing 5% non-fat milk, 0.1% Tween 20, 0.5% goat serum, and 3 μg of mouse anti-V5 antibody (Invitrogen) for 2 h at room temperature. After each blot was washed 3 times with Tris-buffered saline containing 0.1% Tween 20, it was exposed to Tris-buffered saline (15 ml) containing 5% non-fat milk, 0.1% Tween 20, 0.5% goat serum, and a 1:1000 dilution of a stock solution of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) for 1 h at room temperature. The immunoreactive proteins were then visualized using a chemiluminescence kit (Genotech, St. Louis, MO) and BioMax MR film (Kodak). In order to confirm that mT4 is a membrane-anchored protein, $\sim 4 \times 10^6$ mT4-expressing COS-7 cells were suspended in 750 μl of buffer (4 mM HEPES, pH 7.0, containing 50 mM sucrose, 0.4 mM EDTA, and 0.2 mM dithiothreitol) and lysed by subjecting the cell suspension to multiple freeze-thaw cycles using liquid nitrogen and a 45 °C water bath. After centrifugation for 1 h at $>100,000 \times g$, aliquots of the obtained supernatant representing the cytosolic fraction and aliquots of the pelleted microsomal fraction were boiled in Laemmli buffer in the presence of β-mercaptoethanol and then subjected to SDS-PAGE/immunoblot analysis as described above.

Other members of the tryptase family contain *N*-linked glycans (23, 24). Analysis of the predicted primary amino acid sequence of mT4 revealed four potential *N*-linked glycosylation sites. Thus, to determine if mT4 contains *N*-linked glycans, a sample of the COS-7 cell-derived recombinant material was incubated with PNGase F (New England Biolabs) using the manufacturer's suggested reaction conditions. The

resulting digest was then subjected to SDS-PAGE/immunoblot analysis.

As noted under "Results," mT4 can be generated in COS-7 cells using the above expression strategy. Unfortunately, because mT4 remains tightly associated with the ER membrane of the cell, contaminating ER proteins cannot be removed easily to evaluate the enzymatic activity of the generated recombinant protein. We previously generated pseudozymogen forms of mMCP-6 and mMCP-7 in High Five insect cells that in each instance contain an enterokinase-susceptibility sequence between the natural propeptide and catalytic domain (14, 20). Because each pseudozymogen also contains the FLAG peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys at its C terminus, it can be purified using an anti-FLAG antibody immunoaffinity column. Thus, to generate a bioengineered form of recombinant mT4 that can be purified to near homogeneity for functional studies, an expression construct was created in which the C-terminal membrane-spanning domain of mT4 (*i.e.* residues 249–270) was replaced by the 8-residue FLAG peptide. Insect cells might be unable to activate constitutively the generated product. To guard against that possibility, an enterokinase-susceptibility sequence also was created in the construct by inserting the 5-residue peptide Asp-Asp-Asp-Asp-Lys in between the natural propeptide of mT4 and the first Ile residue in its catalytic domain. In this way, the purified pseudozymogen could be activated by enterokinase if needed. High Five insects cells, cultured in serum-free media, were induced to express the bioengineered form of mT4 as described previously (14, 20, 25, 26) for other recombinant mouse and human tryptases. Insect cell lysis buffer from PharMingen was used to liberate the cell-associated recombinant mT4 from pelleted cells. After a 30-min incubation on ice, the resulting lysates were centrifuged at $\sim 20,000 \times g$ for 30 min at 4 °C. The supernatant was removed and passed through a 0.45-μm cellulose acetate filter. The cleared supernatant was then loaded at 4 °C onto an ~ 1-ml column containing anti-FLAG M1 antibody (Sigma). After the column was washed with 250 ml of Tris-buffered saline, pH 7.0, 0.1 M glycine, pH 3.5, buffer was used to elute bound mT4. Ten 1-ml fractions were collected into tubes containing 20 μl of 1 M Tris-HCl, pH 8.0. Samples of resulting fractions were analyzed by SDS-PAGE for the presence of Coomassie Blue⁺ proteins and for immunoreactive mT4.

Casein is a protease-sensitive protein. Thus, resorufin-labeled casein (Roche Molecular Biochemicals) was used to determine whether or not insect cell-derived mT4 is enzymatically active. In this assay, a 50-μl sample of the eluate from the above immunoaffinity column was added to 50 μl of 5 mM calcium chloride and 10 mM Tris-HCl, pH 5.0, containing or lacking 0.4 unit of enterokinase (New England Biolabs); the sample was then incubated at 37 °C for 2 h. To evaluate the enzymatic activity of the resulting recombinant material, 100 μl of 0.1 M Tris-HCl, pH 7.8, containing 10 mM calcium chloride and 200 μg of resorufin-labeled casein was added. After an 18-h incubation at 37 °C, each reaction was stopped by the addition of 480 μl of 5% trichloroacetic acid, followed by a 10-min centrifugation at $>8,000 \times g$. A 400-μl sample of the resulting supernatant was mixed with 600 μl of 0.5 M Tris-HCl, pH 8.8, and the absorbance at 574 nm was measured. Because low molecular weight resorufin-labeled peptides released from its substrate are not precipitated by the trichloroacetic acid step, the concentration of resorufin-labeled peptides in the supernatant is directly proportional to the general proteolytic activity present in the sample. Trypsin (Sigma) was used as a positive control in this enzymatic assay.

A three-dimensional model of the extracellular domain of mature mT4 (i.e. residues 1–256) was built by MODELLER (27, 28) as described for other MC proteases (7, 12, 29, 30). The primary template for comparative protein structure modeling was the crystal structure of human tryptase βII (31). Within this region, the amino acid sequences of the two tryptases are 39% identical.

RESULTS

Cloning and Sequence Analysis of mT4 cDNA—When the mTMT, mMCP-6, and mMCP-7 cDNAs were used as templates to screen GenBank's EST data base, an EST (accession number AI326140) was identified that was somewhat homologous to all three target tryptases. The relevant EST was obtained from the Integrated Molecular Analysis of Gene Expression consortium, and nucleotide sequence analysis of the entire insert in the clone revealed that it encoded a portion of a serine protease (designated mT4 because it is the fourth member of this family of serine proteases) not previously described. 5′- and 3′-RACE approaches (Fig. 1*A*) were therefore carried out to deduce the nucleotide sequence of the missing portion of the transcript
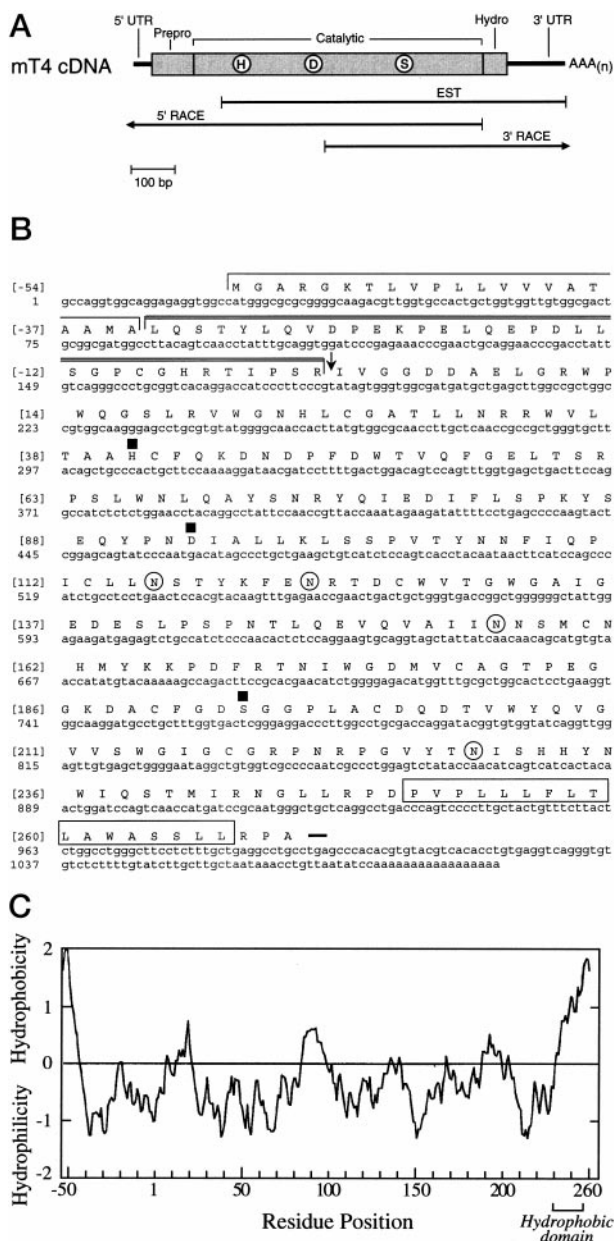
A

B

[-54]   M G A R G K T L V P L L V V V A T
1   gccaggtggcaggagaggtggccatgggcgcgcgggggcaagacgttggtgccactgctggtggttgtggcgact

[-37]   A A M A | L Q S T Y L Q V D P E K P E L Q E P D L L
75   gcggcgatggccttacagtcaacctatttgcaggtggatcccgagaaacccgaactgcaggaacccgacctatt

[-12]   S G P C G H R T I P S R I V G G D D A E L G R W P
149   gtcaggggccctgcggtcacaggaccatcccttcccgtatagtgggtggcgatgatgctgagcttggccgctggt

[14]   W Q G S L R V W G N H L C G A T L L N R R W V L
223   cgtggcaagggagcctgcgtgtatggggcaaccacttatgtggcgcaaccttgctcaaccgccgctgggtgctt

[38]   T A A ■ H C F Q K D N D P F D W T V Q F G E L T S R
297   acagctgcccactgcttccaaaaggataacgatcctttttgactggacagtccagtttggtgagctgacttccag

[63]   P S L W N L Q A Y S N R Y Q I E D I F L S P K Y S
371   gccatctctctggaacctacaggcctattccaaccgttaccaaatagaagatatttcctgagccccaagtact

[88]   E Q Y P N D I A L L K L S S P V T Y N N F I Q P
445   cggagcagtatcccaatgacatagccctgctgaagcctgtcatctccagtcacctacaataacttcatccagccc

[112]   I C L L (N) S T Y K F E (N) R T D C W V T G W G A I G
519   atctgcctcctgaactccacgtacaagtttgagaaccgaactgactgctgggtgaccggctggggggggctattgg

[137]   E D E S L P S P N T L Q E V Q V A I I (N) N S M C N
593   agaagatgagagtctgccatctcccaacactctccaggaagtgcaggtagctattatcaacaacacgcatgtgta

[162]   H M Y K K P D F R T N I W G D M V C A G T P E G
667   accatatgtacaaaaagccagacttccgcacgaacatctggggagacatggtttgcgctggcactcctgaaggt

[186]   G K D A C F G D S ■ G G P L A C D Q D T V W Y Q V G
741   ggcaaggatgcctgcttttggtgactcgggaggacccttggcctgcgaccaggatacggtgtggtatcaggttgg

[211]   V V S W G I G C G R P N R P G V Y T (N) I S H H Y N
815   agttgtgagctggggaataggctgtgggtgccaatcgccctggagtctatcaccaacatcagtcatcactaca

[236]   W I Q S T M I R N G L L R P D | P V P L L L F L T
889   actggatccagtcaaccatgatccgcaatgggctgctcaggcctgacccagtccccttgctactgtttcttact

[260] | L A W A S S L L | R P A —
963   ctgcctgggcttcctcctttgctgaggcctgcctgagcccacacgtgtacgtcacacctgtgaggtcagggtgt
1037   gtctcttttgtatcttgcttgctaataaacctgttaataatccaaaaaaaaaaaaaaaa

C

FIG. 1. **Cloning of the mT4 cDNA.** *A,* a three-step approach was used to isolate the full-length mT4 transcript. A computer search of GenBank's EST data base resulted in the identification of an EST that encodes a portion of a novel mouse serine protease. Based on its nucleotide sequence, 5'- and 3'-RACE approaches were then carried out on a pool of mouse testis-derived cDNAs to deduce the remaining portion of the expressed transcript. The diagram in *A* highlights the 5'-UTR, prepropeptide, the catalytic domain of the mature enzyme with its catalytic triad (*circled H, D,* and *S* amino acid residues), C-terminal hydrophobic segment, 3'-UTR, and poly(A) tail. *B,* the nucleotide and amino acid sequences of the full-length mT4 transcript were deduced. The four potential *N*-linked glycosylation sites in mT4 are *circled* in *B* and the C-terminal hydrophobic extension is *boxed*. Components of the catalytic triad (■), translation-initiation site (*), translation-termination site (−), leader peptide (*single bracket*), and propeptide (*double bracket*) are indicated. Nucleotide numbering begins at the 5'-UTR of the isolated transcript; amino acid numbering (*within brackets at left*) begins with the putative mature protein. *C,* a Kyte-Doolittle hydropathy plot of the translated product was generated. The individual residues in the coding region of mT4 are indicated on the *x* axis; the extent of their hydrophobicity and hydrophilicity are on the *y* axis.

(Fig. 1*B*). The full-length mT4 cDNA consists of ~1095 nucleotides, and the 5'- and 3'-untranslated regions (UTRs) of the transcript consist of 23 and >97 nucleotides, respectively. The
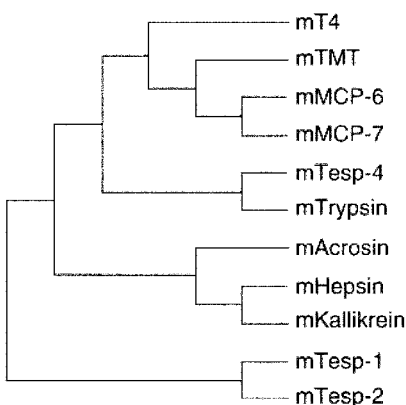
putative translation-initiation codon conforms to the sequence present in most eukaryotic transcripts (32).

Analysis of mT4 at the Protein Level—The deduced amino acid sequence of its cDNA suggested that mT4 could be initially translated as an ~36-kDa zymogen consisting of 324 amino acids (Fig. 1*B*). Hydropathy plot analysis (Fig. 1*C*) disclosed that mT4 possesses an unusual hydrophobic domain in its C terminus at residues 251–267. Phylogenetic analysis (Fig. 2*A*) of all known mouse proteins revealed that mT4 is most closely related to mTMT. However, the degree of identity is only 44%. Every tryptase in this family has an N-terminal sequence of Ile-Val-Gly-Gly when its propeptide is removed (Fig. 2*B*). Because the zymogen form of mT4 also possesses this sequence near its initially translated N terminus, it is predicted that the 54-residue prepropeptide is cleaved at the Arg-Ile site indicated in Fig. 1*B*. In its mature form, the protein portion of the catalytic domain of mT4 is predicted to have a molecular mass of ~30 kDa. However, because the mature protein contains four potential *N*-linked glycosylation sites at Asn^{116}, Asn^{123}, Asn^{156}, and Asn^{229}, post-translationally modified mT4 is expected to be somewhat larger in size *in vivo*.

When transfected into COS-7 cells, the level of immunoreactive mT4 in the conditioned media of the transfectants was below detection by SDS-PAGE/immunoblot analysis (Fig. 3*A*). Thus, very little, if any, recombinant mT4 was constitutively released from the transfectants. The fact that mT4 was preferentially recovered in the microsomal fraction of the cell lysates (Fig. 3*B*) confirmed that mT4 is a membrane-anchored protein. Immunohistochemical analysis of the transfectants revealed that mT4 was preferentially retained in the calexin/calreticulum-enriched ER (Fig. 3, *D–F*). The fact that immunoreactive mT4 was not released after trypsin treatment (data not shown) confirmed that very little, if any, mT4 targets to the plasma membrane in transfected COS-7 cells. As assessed by SDS-PAGE analysis, an immunoreactive protein of ~40 kDa was identified in COS-7 cell transfectants that shifted to ~35 kDa after PNGase F treatment (Fig. 3*C*). Based on the magnitude of this change in its molecular weight, mT4 contains more than one *N*-linked glycan.

By using resorufin-labeled casein as a substrate, the level of proteolytic activity in the lysates of the mT4-expressing COS-7 cells was significantly greater than that in the lysates of control non-transfectants (data not shown). However, the amounts of active enzyme were low, and recombinant mT4 remained tightly associated with the membrane of the ER (Fig. 3). Thus, a bioengineered form of mT4 possessing the FLAG peptide at its C terminus was expressed in High Five insect cells (Fig. 4). In contrast to what occurs when similar FLAG derivatives of mMCP-6 (20) and mMCP-7 (14) are expressed in insect cells, the FLAG derivative of mT4 was not constitutively secreted from the insect cells. Nevertheless, the recombinant protein could be purified from the lysates of its expressing cells using the immunoaffinity column (Fig. 4*A*). Even though the amount of protein in fractions 5 and 6 of the eluate of the column was below detection by Coomassie Blue staining of a duplicate gel (data not shown), the recombinant mT4 in these fractions exhibited substantial proteolytic activity *in vitro* (Fig. 4*B*). The fact that the enzymatic activity of the recombinant material did not increase dramatically following enterokinase treatment indicated that much of the purified mT4 was constitutively active. At present, we cannot ascertain whether activation of the mT4 pseudozymogen occurred inside the insect cells or during its purification. Nevertheless, because more trypsin was used on a weight basis in the depicted experiment, the broad enzymatic activity of recombinant mT4 is at least as good as that of trypsin in regard to its ability to degrade casein.

Mouse Tryptase 4

A

```
                                    mT4
                                    mTMT
                                    mMCP-6
                                    mMCP-7
                                    mTesp-4
                                    mTrypsin
                                    mAcrosin
                                    mHepsin
                                    mKallikrein
                                    mTesp-1
                                    mTesp-2
```

B

(Amino acid sequence alignment of mT4, hEsp-1, mTMT, mMCP-6, mMCP-7, mAcrosin, mTesp-1, mTesp-2, mTesp-4, mHepsin, mKallikrein, mTrypsin, hLeydin, and hProstasin. Loops labeled A, B, C, D, 3, 1, 2, and "Hydrophobic extension" are indicated.)
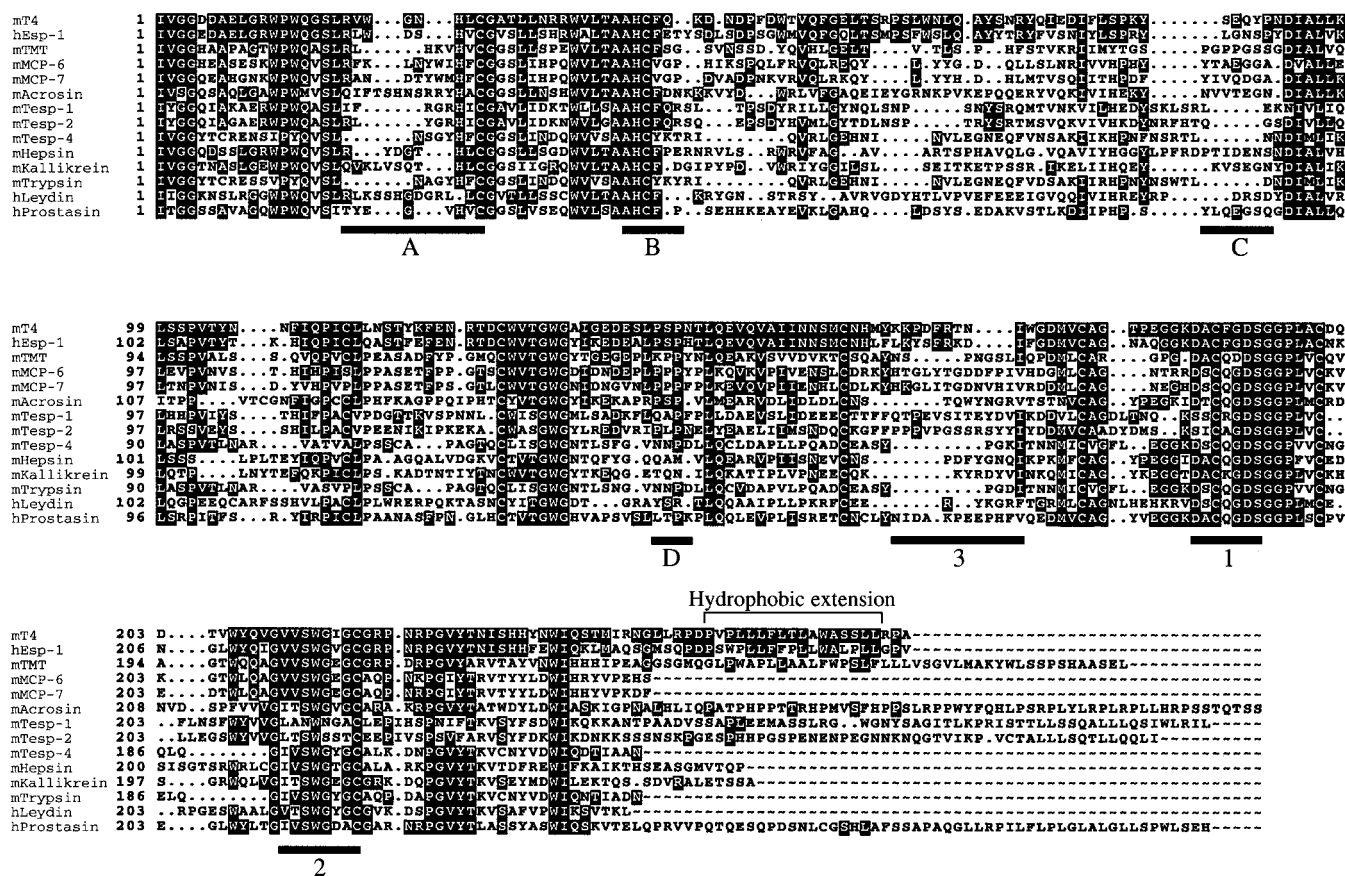
FIG. 2. **Comparison of the amino acid sequences of mT4 with the most closely related known mouse serine proteases.** *A,* dendrogram comparing mT4 with the closely related serine proteases in the mouse as generated by the GCG program "Distances" using the unweighted pair group with arithmetic mean (UPGMA) algorithm. (The *mTesp-3* gene, transcript, and translated product have not been described. Thus, the relationship of mT4 to this putative testis-specific serine protease cannot be evaluated at this time.) *B,* the amino acid sequences of hEsp-1, mTMT, mMCP-6, mMCP-7, mAcrosin, mTesp-1, mTesp-2, mTesp-4, mHepsin, mKallikrein, mTrypsin, hLeydin, and hProstasin were extracted from Swiss Protein Database and aligned with the PILEUP program of the Eugene "GCG" software package. Identical amino acids are *shaded*. Numbering (*left*) begins at the first residue in the mature portion of each protease. The C-terminal hydrophobic segment of mT4 is *bracketed*. The seven putative loops (designated *A–D* and *1–3*) that form the substrate-binding pocket of each of these proteases (also see Fig. *5B*) are *underlined*. The depicted amino acid sequence of mAcrosin is truncated at the C terminus.

The overall fold of mature mT4 is predicted to be similar to that of most serine proteases (Fig. 5A). For example, like all other functional serine proteases, mT4 possesses the conserved triad (*i.e.* His[41], Asp[93], and Ser[194]) in its putative catalytic site. Like mTMT, mT4 lacks a number of the Pro and Tyr residues (31) that are needed for human tryptase βII, mMCP-6, and mMCP-7 to form tetramers. However, mT4 possesses the functionally important surface Trp-rich domain found in all other tryptases in this family. The presence of Asp[188], Gly[215], and Gly[225] in mT4 also strongly suggests that it is a tryptase. Nevertheless, the seven loops that form its substrate-binding cleft are unique (Figs. 2*B* and 5*B*). For example, residue substitutions in loops 3 and A in mT4 are predicted to result in shape differences relative to human tryptase βII (Fig. 5*B*). Although mature mT4 has an overall net −5 charge at neutral pH, it is predicted to have two positively charged surface regions at diametrically opposite ends of the folded protein (Fig. 5*A*). Arg[33], Arg[34], and Arg[243] reside in one region, whereas

FIG. 3. **SDS-PAGE/immunoblot and immunohistochemical analysis of mT4-expressing COS-7 cells.** *A,* COS-7 cells were transfected with expression vector alone (*left lanes*) or expression vector containing an insert that encodes a bioengineered form of mT4 possessing the immunogenic V5 peptide at its C terminus (*right lanes*). Forty eight hours later, samples of the resulting conditioned media/supernatants (*S*) and lysates of the cell pellets (*P*)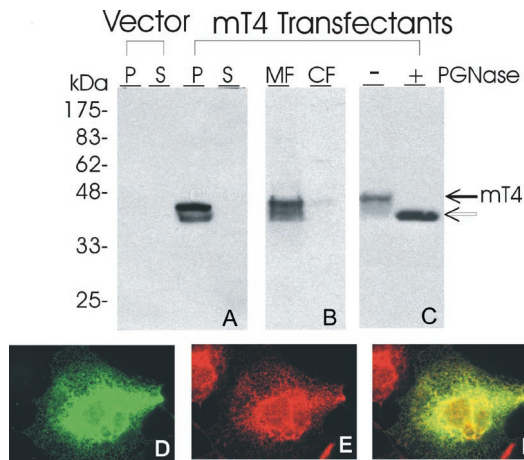 were analyzed for the presence of recombinant protein with anti-V5 antibody. *B,* lysates of mT4-expressing COS-7 cells were fractionated to determine whether or not mT4 is a membrane-anchored protein. Shown is the immunoblot analysis of the membrane- (*MF*) and cytosol (*CF*)-enriched fractions. *C,* a sample of the cell lysates of mT4-expressing COS-7 cells was incubated 1 h in the absence (−) or presence (+) of PNGase F prior to SDS-PAGE/immunoblot analysis to determine whether or not mT4 contains *N*-linked glycans. Molecular mass markers are shown on the *left.* The *arrow* and the *open arrowhead* on the *right* point to glycosylated and nonglycosylated forms of mT4, respectively. The deglycosylated product is ~35 kDa because it contains the additional V5 and His_6 peptides at its C terminus. *D–F,* mT4-expressing cells were stained with a mouse monoclonal antibody directed against the V5 peptide (*D*), a mixture of rabbit antibodies directed against calnexin and calreticulin (*E*), or antibodies directed against all three epitopes (*F*). *Yellow* color in *F* indicates co-localization of mT4 with calnexin and calreticulin. Based on this double staining approach, a substantial portion of the expressed mT4 is anchored in the ER membrane of the cell.

His^{162}, Lys^{165}, Lys^{166}, Arg^{170}, and Arg^{223} reside in the other region.

Nucleotide Sequence and Chromosomal Location of the mT4 Gene—Although two DNA fragments were detected when a blot containing *Avr*II-, *Hin*dIII-, or *Bgl*II-digested mouse genomic DNA was probed with the mT4 cDNA (Fig. 6*A*), subsequent nucleotide sequence analysis revealed that these findings were due to the presence of internal enzyme restriction sites within the gene. Thus, a single gene encodes mT4. The fact that no additional DNA fragment was observed when the genomic blot was probed at moderate stringency (data not shown) suggests that no closely related gene is present in the mouse genome.

The initial FISH analysis revealed that the *mT4* gene resides on the proximal region of a small-sized chromosome believed to be mouse chromosome 17. Based on that data, a second experiment was conducted in which a probe specific for the telomeric region of chromosome 17 was co-hybridized with the mT4-containing genomic clone. This experiment resulted in the specific labeling of the telomere and the proximal portion of mouse chromosome 17. Measurements of 10 specifically labeled chromosomes 17 demonstrated that the *mT4* gene is located at a position that is 23% of the distance from the heterochromatic-euchromatic boundary to the telomere of the chromosome. This area corresponds to the interface between bands 17A3.3 and 17 B1 (Fig. 6*B*) where the *mMCP-6, mMCP-7*, and *mTMT* genes reside. When 80 metaphase cells were analyzed, 77 exhibited specific labeling.

With a long range PCR approach, the entire *mT4* gene was isolated and sequenced. The *mT4* gene is ~5.0 kb in size and
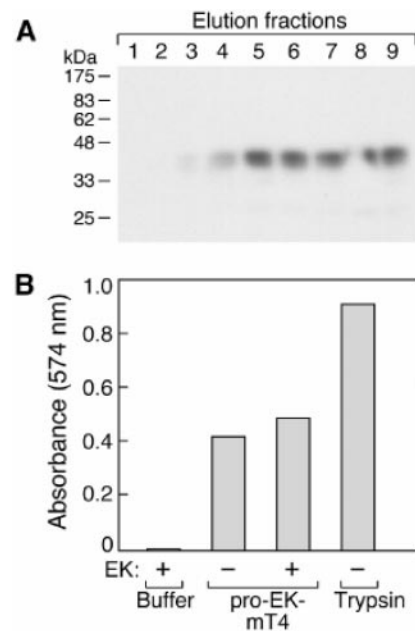
FIG. 4. **Evaluation of the enzymatic activity of insect cell-derived recombinant mT4.** *A,* recombinant mT4-FLAG was purified from the lysates of High Five insect cells using an immunoaffinity chromatography approach. The soluble proteins in lysates of mT4-FLAG-expressing cells were applied to an anti-FLAG antibody column. After the column was washed extensively, the pH of the buffer was changed to elute the bound recombinant protein. *B,* fractions 5 and 6 of the immunoaffinity column were pooled and evaluated for their enzymatic activity before (−) and after (+) enterokinase (*EK*) treatment. Trypsin and activating buffer containing enterokinase alone were used as positive and negative controls, respectively, in this casein-susceptibility assay. As assessed by Coomassie Blue staining of a duplicate gel, the amount of protein in fractions 5 and 6 was below detection. Thus, the amount of trypsin used in the depicted experiment exceeds that of recombinant mT4.

contains 6 exons (Fig. 7*A*). All exon/intron splice sites conformed to the GT/AG rule for other eukaryotic genes (33). Exons 1–6 consist of 126, 27, 166, 284, 155, and 237 bp, respectively, whereas introns 1–5 consist of 101, 291, 599, 2717, and 269 bp, respectively. The exon/intron organization of the *mT4* gene differs somewhat from that of the other three mouse genes in this family (Fig. 7*B*). For example, although the mT4 transcript is similar in size to that of the other three mouse MC tryptase transcripts, its gene is larger due primarily to intron 4. The *mT4* gene also has six exons, whereas the *mTMT* and *mMCP-7* genes have five exons.

Expression of mT4 in Immune and Non-immune Cells—As assessed by RNA blot analysis, the level of mT4 mRNA was below detection in normal mouse bone marrow (Fig. 8*A*). Transgenic mice that have been induced to express abnormally high levels of IL-5 exhibit a constitutive eosinophilia (21). Although barely detectable amounts of mT4 mRNA were found in the bone marrow of IL-5 transgenic mice, larger amounts of this transcript were present in the IL-5-dependent eosinophils purified from the transgenic animals (Fig. 8*A*). In confirmation of these data, mT4 mRNA also could be detected in the jejunum of a *T. spiralis*-infected BALB/c mouse (Fig. 8*B*) precisely when the number of eosinophils are maximal in this tissue (34).

mT4 mRNA could not be detected by RNA blot analysis in normal heart, brain, spleen, lung, liver, skeletal muscle, and kidney (Fig. 8*C*), as well as ear, tongue, stomach, and intestine (data not shown). Because these tissues contain substantial numbers of MCs, mT4 is the only member of its family that is not preferentially expressed in MCs. The level of mT4 mRNA also was below detection in day-7–17 mouse embryos (Fig. 8*C*). However, using an RT-PCR approach, mT4 mRNA was de-
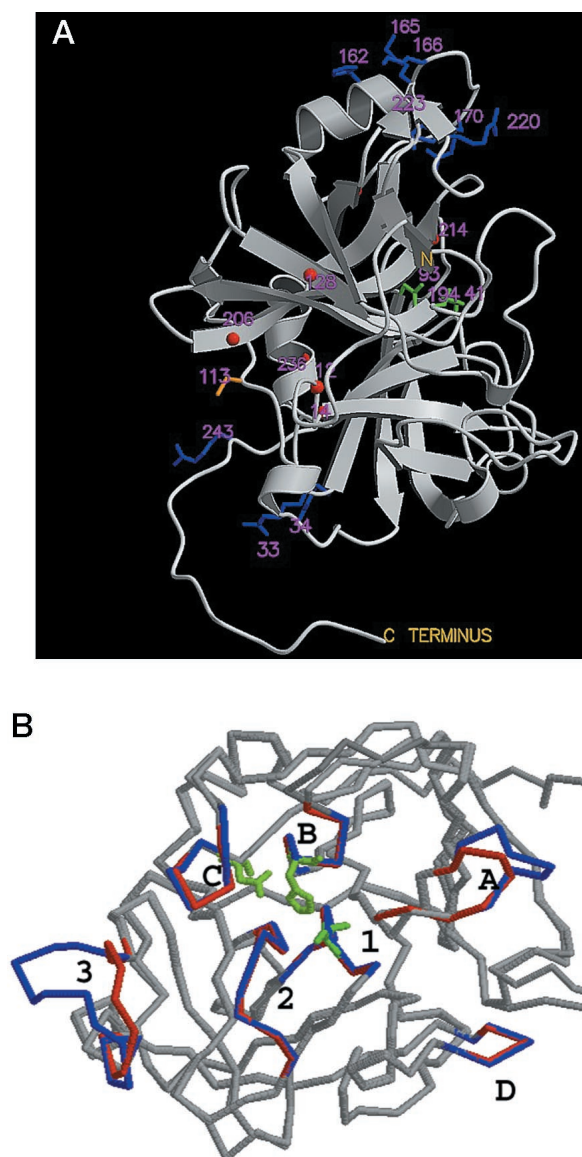
FIG. 5. **Three-dimensional model of the catalytic portion of mature mT4 based on the crystal structure of human tryptase βII.** *A,* a three-dimensional model of the catalytic domain of mature mT4 was created. Shown is the overall structure of residues 1–256 of mT4. Because human tryptase βII lacks a C-terminal hydrophobic domain, the short membrane-spanning domain in mT4 was not modeled. The active site residues (His^{41}, Asp^{93}, and Ser^{194}) are represented as *green sticks*. The free Cys residue 113 is shown in *orange*. The side chains of the residues (His^{162}, Lys^{165}, Lys^{166}, Arg^{170}, Arg^{220}, Arg^{223}, Arg^{33}, Arg^{34}, and Arg^{243}) that consist of the two positively charged surface regions are shown as *blue sticks*. The C-α atoms of the conserved residues (Trp^{12}, Trp^{14}, Trp^{128}, Trp^{206}, Trp^{214}, and Trp^{236}) that form the hydrophobic domain opposite the substrate-binding cleft are shown as *red spheres*. Two of the conserved residues (Trp^{35} and Trp^{174}) in the domain are hidden in this view. The figure was created with the programs Molscript (50) and Raster3D (51). The general orientation of the mT4 model is similar to that of the mMCP-6 (12), mMCP-7 (30), and mTMT (7) models in our previous publications. *B,* the putative substrate-binding cleft of mT4 was analyzed at a higher resolution using the modeling approach. The 7 loops that form the substrate-binding cleft of mT4 are marked *A–D* and *1–3* and are superimposed on the corresponding loops of human tryptase βII. The loops in mT4 and human tryptase βII are shown in *red* and *blue*, respectively; the active site residues are shown as *green sticks*.

tected in the testes and ovaries of adult mice (Fig. 8*D*). As assessed by two different *in situ* hybridization methods, mT4 is transiently expressed relatively late in spermatogenesis during the cap phase of acrosome formation (Fig. 9).
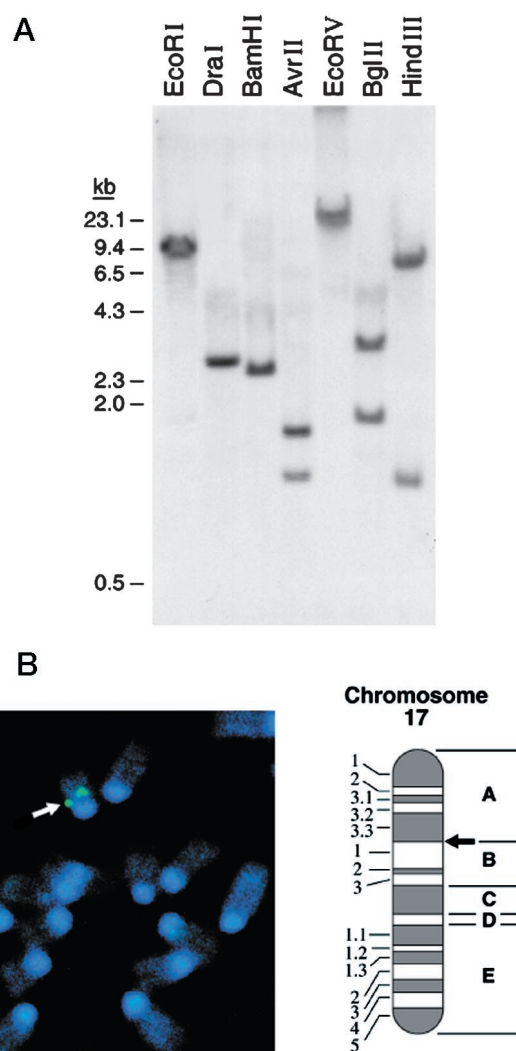
FIG. 6. **Genomic blot analysis and chromosomal location of the *mT4* gene.** *A,* a blot containing mouse genomic DNA digested with *Eco*RI, *Dra*I, *Bam*HI, *Avr*II, *Eco*RV, *Bgl*II, or *Hin*dIII was probed under conditions of high stringency with a radiolabeled 536-bp fragment derived from the 5′ end of the mT4 cDNA. DNA fragments of known molecular weight (*Hin*dIII-digested λ DNA) are indicated on the *left* of the blot. As noted in Fig. 7*A*, the *mT4* gene contains internal sites that are susceptible to *Avr*II, *Hin*dIII, and *Bgl*II. *B,* the chromosome location of the *mT4* gene was determined by FISH analysis. The fluorescent-labeled, mT4-containing BAC clone hybridized specifically to a small-sized chromosome (*arrow*, *left panel*) that was subsequently shown to be chromosome 17. The location (*arrow*) of the *mT4* gene on this chromosome is more clearly indicated in the *right panel*.

DISCUSSION

While at least six distinct tryptase genes reside at a complex on human chromosome 16 (1–3, 6, 7), only three corresponding genes have been identified so far on the syntenic region of mouse chromosome 17 (7–11). We now describe a new mouse gene in this family that is related to the genes that encode mTMT, mMCP-6, and mMCP-7.

The mT4 cDNA (Fig. 1) and gene (Fig. 7) encode a 324-residue polypeptide having a 54-residue prepropeptide and a 17-residue, C-terminal hydrophobic domain. When transiently expressed in COS-7 cells, mT4 remains cell-associated (Fig. 3). Thus, as predicted based on analysis of its cDNA and gene, translated mT4 is a membrane-anchored serine protease. Like the transmembrane protease angiotensin-converting enzyme (35), the C-terminal hydrophobic domain of mT4 is flanked by Asp and Arg residues. Because angiotensin-converting enzyme (35), prostasin (36), and acrosin (37) can be released from cells

A

```
1                        GCCAGGTGGCAGGAGAGGTGGCCATGGGCGCGCGGG
                                                    M G A R G        Exon 1
37   GCAAGACGTTGGTGCCACTGCTGGTGGTTGTGGCGACTGCGGCCATGGCCTTACAGTCAA
     K T L V P L L V V V A T A A M A L Q S T
97   CCTATTTGCAGGTGGATCCCGAGAAACCCCgtgagctgctctgggggtactgttgtgaga
     Y L Q V D P E K P E
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157  ctgggggcagtgacaggcagatgggggaagtgagggcgaagtgtcgcttattcttctctcc
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217  cttccacccagAACTGCAGGAACCCGACCTATTGTCAGgtaagatgctcagaacgtgttt
              L Q E P D L L S G                              Exon 2
277  tcctgcgtggtgggcctgcgtcgggggtgcacctcaagtattcctctccttttgccccagg
337  atcgagaactcaactgtgtttgataataacgcacacccctgccccggggtggtggtgttg
397  ggggcaggtgcacaccgatcatgtggagggtgtcccagcagaccctcaggaagtccctcc
457  ctttccttgcccgtggcttaaaaagtgtcagtggtggtgggtgggagggaaaaaaaaaa
517  tctctgctccggtctgatcctcctcacagGGCCCTGCGGTCACAGGACCATCCCTTCCGG
                                     P C G H R T I P S R
577  TATAGTGGGTGGCGATGATGCTGAGCTTGGCCGCTGGCCGTGGCAAGGGAGCCTGCGTGT   Exon 3
     I V G G D D A E L G R W P W Q G S L R V
637  ATGGGGCAACCACTTATGTGGCGCAACCTTGCTCAACCGCCGCTGGGTGCTTACAGCTGC
     W G N H L C G A T L L N R R W V L T A A
697  CCACTGCTTCCAAAAgtgagtctggagtcaagcctgggcagctgagggtgggaaggtta
     H C F Q K
757  gtaacctggccctgagtttgacacctgggtaagctttttagtgcagcctcaggagtagc
             BglII
817  tgaaatgggttagatctgtaagatttacattactgcccctgaaagggaatcctcttccag
877  ttcttctagaatgttctttcaggacattctcattcccttttgagtgctttgactgctcttt
937  ttttccttcaagtccttcccaaatatccctcacactagtcccccacacttgctcagctcctt
997  tggggggctccctaggctcactcaccttctgtcttttaggactactccaacagacacagga
             AvrII
1057 ccactgggg tagccactcttctgcttcttcactcagtgccc ggggagcaagggctcttcttg
1117 cccagcctctctgggttctgtggccccatagagggcttttggcccataaggactgggc
1177 agttctgctaaggatgaacgtgaggtagtcgtcgcctcagagagaggcccagctgagctc
1237 aggtggcttgaactgctctctcctctctgtcagGGATAACGATCCTTTGACTGGACAG
                                      D N D P F D W T V
1297 TCCAGTTTGGTGAGCTGACTTCCAGGCCATCTCTCTGGAACCTACAGGCCTATTCCAACC
     Q F G E L T S R P S L W N L Q A Y S N R
1357 GTTACCAAATAGAAGATATTTTCCTGAGCCCCAAGTACTCGGAGCAGTATCCCAATGACA   Exon 4
     Y Q I E D I F L S P K Y S E Q Y P N D I
1417 TAGCCCTTGCTGAAGCTGTCATCTCCAGTCACCTACAATAACTTCATCCAGCCCATCTGCC
     A L L K S V I S S H L Q N N F I Q P I C L
1477 TCCTGAACTCCACGTACAAGTTTGAGAACCGAACTGACTGCTGGGTGACCGGCTGGGGGG
     L N S T Y K F E N R T D C W V T G W G A
1537 CTATTGGAGAAGATGAGAGgtgagactgggtacagacgggtgggggagaggggcactaccc
     I G E D E S
1597 taacctttcacattagccctcctccctgggctctgaggccccacggagcccctctcgc
1657 tcactcaacgtttctttgtttattttcctttacttctgcttgcaagaaggacaggagctag
1717 tcctttcctcccagacaggaactcagaggtgccagggaggcctgaaggggtggggacctc
1777 atagggcctgtaggatggcttctacaacacagctttttatgatctacccactttggcaggc
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Right column:

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1837 accaatcaatcatcagtgagagacaggaaacacactcactctgcctgctccacccaccca
1897 aaattccgactgtgcacactccttcttggcgcacccctgaaatcccttttgggggtctaat
1957 cacgtgcctgcctgcagctcagcaggaaccctgcacaaagccccaccccaccccaag
2017 gagcactgagaacggtttccctctaggtgccctagctcttacatcctggagtgaccctgc
2077 tcagttctctctgacctggtccatctctgcaatgcccctaaccagcgtcatagtaatag
2137 attcggctccttccctgcaggtcacgtgcttggcttggcaggcttggggaccctggtgcat
2197 caaggcaacggcataattttttttgtaaaaaaaacttcattttattttatttttttatgtat
2257 atgagtgttttgcctgcaggtgtgtgtctcagattccctagagctggagttataggcat
2317 tagtgaactgccataccgggtgctggaaattgaacttgggtccttttagagccagctccta
2377 gcccaagagtgtaggtttaagaaagggatttgaagtatttgatttgatttgtatgtgtgc
2437 acgcatgtctcagtgatgtgcgtgtgtgtgtgcagatgcccacagaggacagaagagggtgt
             AvrII
2497 gggatccctaggagctgaagttagaggtgtttgtgaacctttcagtaataccgggatcct
2557 aattcgttctcatgatggagaaagtgttcttaacttctgaactatctctccagcctcctt
2617 tagagggattgcatctatctattttttgtttttaagaacatttatttaaaaagggggaaggga
2677 cagacagtggtggcaccttaatcccagctctcaggtggcagaagtgggcctatcttttt
2737 gagttcaagaccagcctggtttacagagtgagttccaggagagccagagctacagagaga
2797 aaccctgtcttgaaaaaaaacaaaacaacagcaaaaaatttatgtgtgtttgtgggcttg
2857 tgtgtgtgtgtgtgtgtgtgtatgtatgtatgtttatcgcatgatcgatgcaattcc
2917 tgcagaggctggtagagggcgttggctccctt ggaactggagctacaggtggttgtgagc
2977 tccctgtgggtaccgggaactgaacccatgctctctgaaagggctgtaagtgctcttcac
3037 tgctgagctgtctctccagcccatgctttgtaggtgatggggggtacaatcaatggccca
3097 tgtggaagagttgtcctctccttttaccatgcgggtcttgaggatcaaaattagacctaa
3157 tggcaagaaccgttacccactgaggaaattgagagtgctatggaaagtgtccaaggcccc
3217 agaactagtaaacggaggcaagaccaggttccatggctcagacaacataagggagttccc
3277 caaggtcagacagttggaactggcaggggagaagaggatgcaatggtcagtctgcctgttt
3337 ggtaggagaccatttcctcctcctcctctagaaagggtgagcccagagacccttccaggcctctttt
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3457 cctggcttcattcttaatttttggttcacacgagacaggtagcacatctctggtgtgaata
3517 tacttctgtttaccctggaactggggcaacgttttctttgctgtgtgctgactgggacac
3577 tttgaacaagacacttggctgaactgtgcctcagtttcctcttctgtgaagtagataaca
             BglII
3637 atcttgctggcttggtagaacacttctgagtactgagatctcagaggacaggaaacagcc
3697 actgtcgctcctgtcatcctctgcagagttgacaatgcctgaagcctcgacttagggatc
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3817 tgggccagaactgttctttaaattaatatgttctgatactgttgtattctcagtgctgctt
3877 tagggccttggctcaggcctaaggacatcccagaaggaaagggcaccatagagctgatt
3937 tctctgtgtccctggatgtgtctctgcaggacttggcttgctgcatccactccagctcctttcc
3997 cctgtacataacttggttgccagtccagcagtgtcttcatgtacattctacacacacagcct
4057 cagtgaatggcattgacaatgcctccatccactgaatcctgtgatactgttgattgcctg
4117 tgatgttagcatggggctgcagctgggacgtgtcttcatgtgggtgtctcagggctaggagtagga
4177 tgccccaggacaccctgttcccacaccctatgccattcctcccaggagaggaggatcca
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```
4237 ggggtctgtctctctctgtcttccatccccccacacagGTCTGCCATCTCCCAACACTCTCCA
                                         L P S P N T L Q
4297 GGAAGTGCAGGTAGCTATTATCAACAACAGCATGTGTAACCATATGTACAAAAAAGCCGACT   Exon 5
     E V Q V A I I N N S M C N H M Y K K P D
4357 CTTCCGCACGAACATCTGGGGAGACATGGTTTGCGCTGGCACTCCTGAAGGTGGCAAGGA
     F R T N I W G D M V C A G T P E G G K D
4417 TGCCTGCTTGgtaagtgtcacctcataccaccatgctcttctgggcccagtaccctagct
     A C F
4477 ccaactttcacccaactgagttttcatatagggcctcatttcatctcaggatttagacca
4537 tggcccacctttgcagatgaagaaattgagggcccactgctgtcagggctaggagtagga
4597 tcctcacccagtctgtcagcccccacagcacctccttctcccctagccccctctgctggtcc
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4657 tacttgtcatctcccaggcttacctatgttgcttctcagGGTGACTCGGGAGGACCCTTG
                                           G D S G G P L
4717 GCCTGCGACCAGGATACGGTGTGGTATCAGGTTGGAGTTGTGAGCTGGGGAATAGGCTGT   Exon 6
     A C D Q D T V W Y Q V G V V S W G I G C
4777 GGTCGCCCCAATCGCCCTGGAGTCTATACCAACATCAGTCATCACTACAACTGGATCCAG
     G R P N R P G V Y T N I S H H Y N W I Q
4837 TCAACCATGATCCGCAATGGGCTGCTCCGACCCGATCCCCCTTGCTACTGTTTCTT
     S T M I R N G L L R P D P V P L L L F L
4897 ACTCTGGCCTGGGCTTCCTCTTTGCTGAGGCCTGCCTGAGCCCCACACGTGTACGTCACAC
     T L A W A S S L L R P A *
4957 CTGTGAGGTCAGGGTGTGTCTCTTTTGTATCTTGCTTGCTAATAAACCTGTTAATATCC
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B

FIG. 7. **Structure of the *mT4* gene.** *A,* the nucleotides that consist of the six exons and five introns of the *mT4* gene were deduced and are shown in *upper* and *lowercase letters,* respectively. The exons are *boxed,* and the deduced amino acid sequence of the translated product is indicated, as well as the components of the catalytic triad (■). The putative polyadenylation signal site in exon 6 is *underlined.* The *Bgl*II and *Avr*II restriction sites in introns 3 are *italicized. B,* the exon/intron organization of the *mT4* gene was compared with that of the *mTMT, mMCP-6,* and *mMCP-7* genes. *Boxes* (■) indicate exons. The size of each gene is indicated on the *right. H, D,* and *S* refer to the catalytic triad amino acids in each tryptase.

by a proteolytic processing mechanism, the possibility has not been ruled out that under certain situations mT4 undergoes a similar post-translational processing event *in vivo* to cause its release from cells.

The zymogen form of mT4 contains 10 Cys residues. Based on the crystal structure of human tryptase βII (31), Cys^{26}, Cys^{42}, Cys^{127}, Cys^{160}, Cys^{179}, Cys^{190}, Cys^{200}, and Cys^{218} are predicted to form 4 disulfide bonds in the catalytic portion of the mature, properly folded protease (Fig. 5*A*). One of the additional Cys resides at residue −9 in the propeptide; the

other resides at residue 113. Although Cys^{113} is not present in mMCP-6 or mMCP-7 (Fig. 2*B*), a corresponding Cys is present in the two-chained proteases factor XI (38), plasma kallikrein (39), and acrosin (40). Because this Cys forms a disulfide bond with a Cys residue in the propeptide of each zymogen, mature mT4 could be a two-chain serine protease consisting of a 33-residue, non-catalytic N-terminal chain covalently linked to the larger sized catalytic C-terminal chain.

Although its physiologic substrate(s) was not deduced in this initial study, mT4 is enzymatically active when expressed in

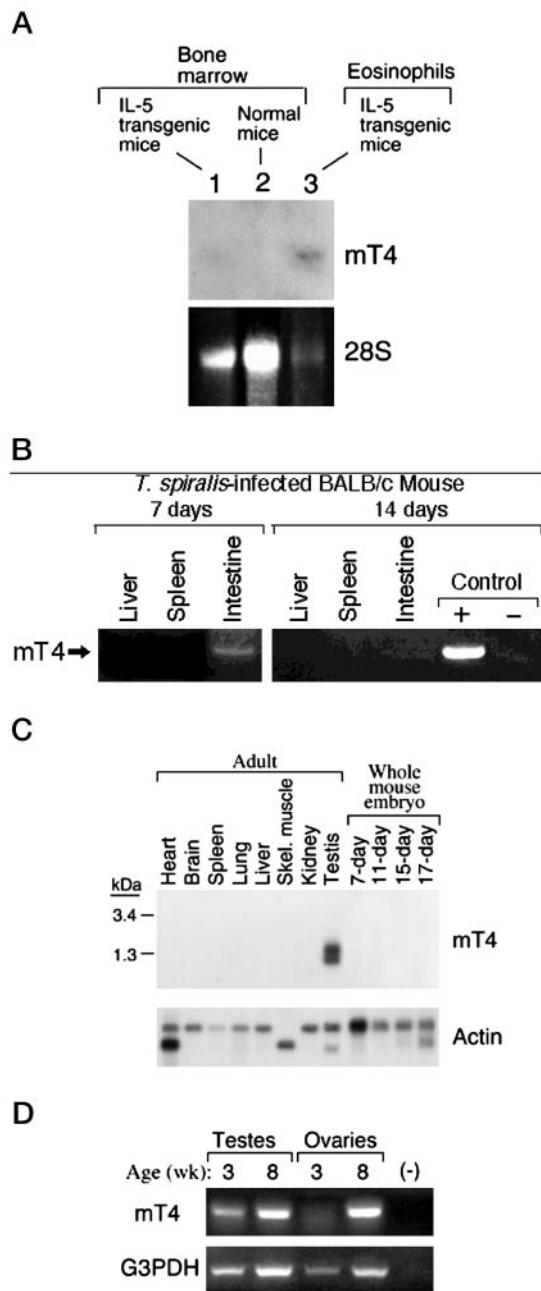FIG. 8. **Distribution of the mT4 transcript in tissues and cells.** *A,* a blot containing total RNA from normal mouse bone marrow (*lane 2*), bone marrow from IL-5 transgenic mice (*lane 1*), and eosinophils purified from IL-5 transgenic mice (*lane 3*) was probed under conditions of high stringency with the mT4 cDNA (*upper panel*). The amount of 28 S ribosomal RNA in each lane is shown in the *lower panel*. Much less total RNA was intentionally loaded in *lane 3* in order to show that the eosinophils are the major cell type in the bone marrow of the IL-5 transgenic mice that expresses mT4. *B,* RT-PCR was used to evaluate the levels of mT4 mRNA in the liver, spleen, and intestine of a day-7-infected mouse and a day-14-infected mouse. The *arrow* (*left*) points to the ~280-bp product that was generated only from the eosinophil-enriched intestine of the day-7-infected animal. The positive (testis RNA) and negative (no RNA) controls used in this RT-PCR are shown. Control RT-PCRs also were carried out with β-actin-specific primers to confirm that each sample contained non-degraded mRNA (data not shown). *C,* a blot containing poly(A)$^+$ RNA from various mouse tissues was probed under conditions of high stringency with the mT4 cDNA (*upper panel*). The blot was then reprobed with the β-actin cDNA (*lower panel*) to demonstrate that comparable amounts of RNA are present in each lane. Molecular mass markers are indicated on the *left. D,* RT-PCR analysis was carried out on RNA samples isolated from the testis and ovaries of 3- and 8-week-old mice. RNA blot analysis (data not shown) confirmed the presence of mT4 in the testes and ovaries of 8-week-old mice.
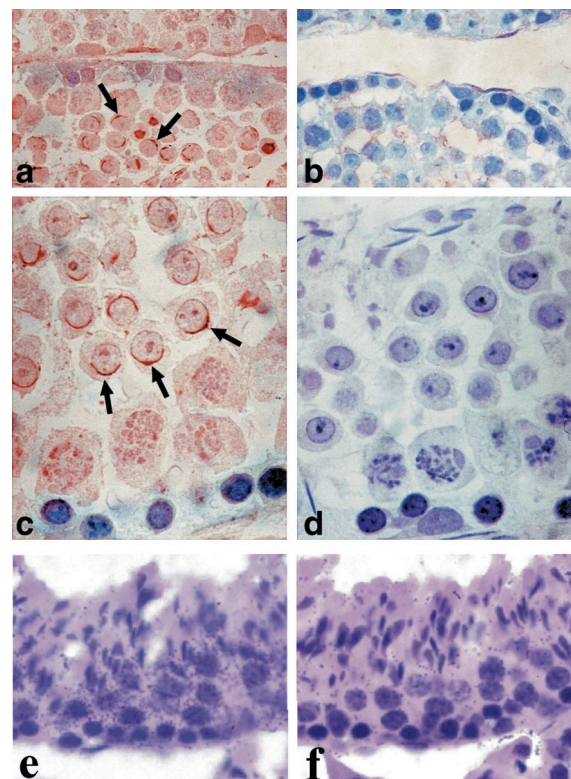
FIG. 9. **Location of mT4-expressing cells in the testis by *in situ* hybridization.** To identify the cell type(s) in the testis that contains abundant levels of mT4 mRNA, a JB4 glycomethacrylate-embedded mouse testis was sectioned and probed with an mT4-specific, digoxigenin-labeled oligonucleotide (*A* and *C*) using an *in situ* hybridization approach. For a negative control (*B* and *D*), the reaction was carried out on a replicate slide in the absence of the labeled oligonucleotide. Shown are different magnifications of the resulting tissue sections. Based on this analysis, the mT4 transcript (*arrows, A* and *C*) is transiently expressed relatively late in spermatogenesis. This conclusion was confirmed using a radioactive *in situ* hybridization approach with antisense (*E*) and sense (*F*) mT4 RNA probes.

insect cells (Fig. 4). mT4 has several features that indicate that it probably exhibits tryptic-like activity *in vivo*. For example, all tryptic serine proteases possess an Asp six residues N-terminal of the catalytic Ser residue. This Asp is needed for interaction of the serine protease with the P1 Lys or Arg residue in the susceptible substrate (41). Not only does mT4 have the conserved Asp at residue 188 (Figs. 1, 2*A*, 5, and 6), but also this residue is predicted to reside at the base of the substrate-binding cleft. As in other tryptic proteases, Gly^{215} and Gly^{225} are present in mT4. Based on the crystal structures of rat trypsin (42) and human tryptase βII (31), Gly^{225} is conserved because it contacts Asp^{188} at the base of the substrate-binding cleft. Gly^{215} resides in loop 2 (Fig. 5), and this surface loop helps define the substrate specificity of the serine protease (42). Although human tryptase αI is an exception (25), tryptases have a Gly at the corresponding site presumably because this small-sized amino acid residue facilitates entry of the bulky P1 residue of the substrate into the pocket of the enzyme.

When properly folded, a hydrophobic domain consisting of eight Trp residues forms on the surface of every MC tryptase opposite that of the substrate-binding cleft (31, 43). Expression/site-directed mutagenesis studies revealed that this domain is of functional importance in the maturation of mMCP-7 (26). The observation that mT4 contains these conserved residues (Trp^{12}, Trp^{14}, Trp^{35}, Trp^{128}, Trp^{132}, Trp^{206}, Trp^{214}, and Trp^{236}) (Fig. 2*B*) and that they reside on the appropriate surface region (Fig. 5*A*) is further evidence that mT4 is a functional tryptase *in vivo*.

Genomic blot analysis (Fig. 6*A*) revealed that there is only one *mT4*-like gene in the mouse genome, and FISH analysis (Fig. 6*B*) revealed that this new gene resides on mouse chromosome 17 quite close to the *mMCP-6, mMCP-7,* and *mTMT* genes. Two transcripts that differ slightly in their size were seen in the testis (Fig. 8*C*). Although the possibility has not been ruled out that the mT4 transcript can undergo alternative spicing in the testis, this seems unlikely because analysis of its gene (Fig. 7*A*) predicts that a functional enzyme would not be generated if any one of the 6 exons is deleted. Because sequence analysis of the eight RACE products failed to reveal a differentially spliced transcript, a more likely explanation for the RNA blot data is that different transcription-initiation sites are used to generate transcripts with variable sized 5′-untranslated regions.

The deduced amino acid sequence of mT4 is <45% identical to that of mMCP-6, mMCP-7, and mTMT (Fig. 2). Although mT4 is most homologous to human Esp-1 (hEsp-1), the sequence identity is still only 68%, and mT4 and hEsp-1 differ in a number of ways. At the protein level, hEsp-1 lacks the 3-residue cytoplasmic tail found in mT4 (Fig. 1*B*). Cytoplasmic tails often regulate intracellular routing of membrane proteins. hEsp-1 is a plasma membrane-anchored protease (4, 44). The fact that recombinant mT4 is unable to reach the plasma membrane in transfected COS-7 cells implies a regulatory role for the three cytoplasmic residues in the ER retention of mT4. The membrane-spanning domains of mT4 and hEsp-1 also differ substantially in their length and primary amino acid sequences, as do their prepropeptides. More important, the amino acid sequences that consist of 6 of the 7 loops that form the substrate-binding clefts of mT4 and hEsp-1 are very different (Figs. 2*B* and 5*B*). Thus, the preferred substrate specificities of these two tryptases are most certainly distinct *in vivo*. Although the mT4 (Figs. 8*C* and 9) and hEsp-1 (44) transcripts are present in abundance in the testis, their precise location in this tissue also differs. hEsp-1 has been reported to be expressed exclusively by primary spermatocytes before their first meiotic division (44). In contrast, as assessed by two different *in situ* hybridization methods, mT4 is transiently expressed in secondary spermatocytes (Fig. 9). Although RNA blot (Fig. 8*A*) and RT-PCR (Fig. 8*B*) data indicate that IL-5-dependent mouse eosinophils express mT4, the level of the mT4 transcript in those mouse eosinophils also appears to be considerably less than the level of hEsp-1 mRNA in human eosinophils (4) and human K562 leukemia cells.[4] Several tissues (*e.g.* lung, pancreas, spleen, and bone marrow) that lack mT4 mRNA in mice (Fig. 8, *B* and *C*) contain hEsp-1 mRNA in humans (4, 44). Moreover, mT4 is expressed in both the testis and ovaries (Fig. 8*D*). Finally, at the genome level, intron 4 is notably larger in the *mT4* gene (2717 *versus* 1985 bp), whereas introns 2 and 3 are notably larger in the *hEsp-1* gene (291 *versus* 344 bp and 599 *versus* 710 bp, respectively) (Fig. 7*B*). The regions of the mouse and human chromosomes where the tryptase complexes of genes reside have not yet been sequenced in their entirety. Nevertheless, based on all of the above differences, it is unlikely that mT4 is the mouse ortholog of hEsp-1/testisin. If it is the mouse ortholog, substantial divergence occurred in this gene, its translated product, its expression pattern, and presumably its substrate preference during the last 40–100 million years of evolution.

Penetration of the egg zona pellucida by sperm is essential for fertilization. Because various trypsin inhibitors can block sperm penetration of the zona pellucida (45), one or more tryptic proteases appears to play an essential role in fertilization. Acrosin is a major tryptic protease in the testis. However, no fertilization defects were observed in mice when the acrosin gene was disrupted (46). Thus, an unidentified tryptase must play a more critical role in this biologic process. Although mouse testicular serine protease (mTesp)-1 (47), mTesp-2 (47), and mTesp-4 (48) were recently cloned from mouse testis and found to reside in the acrosomal compartment of sperm, nothing is known about their *in vivo* functions and substrate specificities. The observation that each of these proteases possess an Arg residue in its propeptide adjacent to the N-terminal Ile in the mature enzyme suggests that an undefined tryptic-like enzyme is required for the proteolytic processing of their propeptides. Membrane-anchored, tryptic-like convertases such as human furin/PACE and yeast Kex2 play important roles in the post-translational processing of a diverse array of biologically active proteins (49). Because mT4 is a membrane-anchored serine protease that resides in the ER of transfected COS-7 cells and because this serine protease is predicted to possess tryptic-like enzymatic activity *in vivo*, this protease probably plays an important convertase-like role in the maturation of certain families of proteins.

REFERENCES

1. Miller, J. S., Westin, E. H., and Schwartz, L. B. (1989) *J. Clin. Invest.* **84,** 1188–1195
2. Miller, J. S., Moxley, G., and Schwartz, L. B. (1990) *J. Clin. Invest.* **86,** 864–870
3. Vanderslice, P., Ballinger, S. M., Tam, E. K., Goldstein, S. M., Craik, C. S., and Caughey, G. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3811–3815
4. Inoue, M., Kanbe, N., Kurosawa, M., and Kido, H. (1998) *Biochem. Biophys. Res. Commun.* **252,** 307–312
5. Inoue, M., Isobe, M., Itoyama, T., and Kido, H. (1999) *Biochem. Biophys. Res. Commun.* **266,** 564–568
6. Pallaoro, M., Fejzo, M. S., Shayesteh, L., Blount, J. L., and Caughey, G. H. (1999) *J. Biol. Chem.* **274,** 3355–3362
7. Wong, G. W., Tang, Y., Feyfant, E., Šali, A., Li, L., Li, Y., Huang, C., Friend, D. S., Krilis, S. A., and Stevens, R. L. (1999) *J. Biol. Chem.* **274,** 30784–30793
8. Gurish, M. F., Nadeau, J. H., Johnson, K. R., McNeil, H. P., Grattan, K. M., Austen, K. F., and Stevens, R. L. (1993) *J. Biol. Chem.* **268,** 11372–11379
9. Gurish, M. F., Johnson, K. R., Webster, M. J., Stevens, R. L., and Nadeau, J. H. (1994) *Mamm. Genome* **5,** 656–657
10. McNeil, H. P., Reynolds, D. S., Schiller, V., Ghildyal, N., Gurley, D. S., Austen, K. F., and Stevens, R. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11174–11178
11. Reynolds, D. S., Gurley, D. S., Austen, K. F., and Serafin, W. E. (1991) *J. Biol. Chem.* **266,** 3847–3853
12. Ghildyal, N., Friend, D. S., Stevens, R. L., Austen, K. F., Huang, C., Penrose, J. F., Šali, A., and Gurish, M. F. (1996) *J. Exp. Med.* **184,** 1061–1073
13. Stevens, R. L., Friend, D. S., McNeil, H. P., Schiller, V., Ghildyal, N., and Austen, K. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 128–132
14. Huang, C., Wong, G. W., Ghildyal, N., Gurish, M. F., Šali, A., Matsumoto, R., Qiu, W. T., and Stevens, R. L. (1997) *J. Biol. Chem.* **272,** 31885–31893
15. Echtenacher, B., Männel, D. N., and Hültner, L. (1996) *Nature* **381,** 75–77
16. Malaviya, R., Ikeda, T., Ross, E., and Abraham, S. N. (1996) *Nature* **381,** 77–80
17. Prodeus, A. P., Zhou, X., Maurer, M., Galli, S. J., and Carroll, M. C. (1997) *Nature* **390,** 172–175
18. Reynolds, D. S., Stevens, R. L., Lane, W. S., Carr, M. H., Austen, K. F., and Serafin, W. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3230–3234
19. Johnson, A. R., Hugli, T. E., and Müller-Eberhard, H. J. (1975) *Immunology* **28,** 1067–1080
20. Huang, C., Friend, D. S., Qiu, W. T., Wong, G. W., Morales, G., Hunt, J., and Stevens, R. L. (1998) *J. Immunol.* **160,** 1910–1919
21. Dent, L. A., Strath, M., Mellor, A. L., and Sanderson, C. J. (1990) *J. Exp. Med.* **172,** 1425–1431
22. Friend, D. S., Ghildyal, N., Austen, K. F., Gurish, M. F., Matsumoto, R., and Stevens, R. L. (1996) *J. Cell Biol.* **135,** 279–290
23. Benyon, R. C., Enciso, J. A., and Befus, A. D. (1993) *J. Immunol.* **151,** 2699–2706
24. Ghildyal, N., Friend, D. S., Freelund, R., Austen, K. F., McNeil, H. P., Schiller, V., and Stevens, R. L. (1994) *J. Immunol.* **153,** 2624–2630
25. Huang, C., Li, L., Krilis, S. A., Chanasyk, K., Tang, Y., Li, Z., Hunt, J. E., and Stevens, R. L. (1999) *J. Biol. Chem.* **274,** 19670–19676
26. Huang, C., Morales, G., Vagi, A., Chanasyk, K., Ferrazzi, M., Burklow, C., Qiu, W. T., Feyfant, E., Šali, A., and Stevens, R. L. (2000) *J. Biol. Chem.* **275,** 351–358
27. Šali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234,** 779–815
28. Šali, A., and Overington, J. P. (1994) *Protein Sci.* **3,** 1582–1596

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[4] G. W. Wong and R. L. Stevens, unpublished data.

29. Šali, A., Matsumoto, R., McNeil, H. P., Karplus, M., and Stevens, R. L. (1993) *J. Biol. Chem.* **268,** 9023–9034
30. Matsumoto, R., Šali, A., Ghildyal, N., Karplus, M., and Stevens, R. L. (1995) *J. Biol. Chem.* **270,** 19524–19531
31. Pereira, P. J., Bergner, A., Macedo-Ribeiro, S., Huber, R., Matschiner, G., Fritz, H., Sommerhoff, C. P., and Bode, W. (1998) *Nature* **392,** 306–311
32. Kozak, M. (1989) *J. Cell Biol.* **108,** 229–241
33. Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* **50,** 349–383
34. Friend, D. S., Gurish, M. F., Austen, K. F., Hunt, J., and Stevens, R. L. (2000) *J. Immunol.* **165,** 344–352
35. Wei, L., Alhenc-Gelas, F., Soubrier, F., Michaud, A., Corvol, P., and Clauser, E. (1991) *J. Biol. Chem.* **266,** 5540–5546
36. Yu, J. X., Chao, L., and Chao, J. (1995) *J. Biol. Chem.* **270,** 13483–13489
37. Baba, T., Watanabe, K., Kashiwabara, S., and Arai, Y. (1989) *FEBS Lett.* **244,** 296–300
38. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) *Biochemistry* **30,** 2056–2060
39. McMullen, B. A., Fujikawa, K., and Davie, E. W. (1991) *Biochemistry* **30,** 2050–2056
40. Topfer-Petersen, E., Calvete, J., Schafer, W., and Henschen, A. (1990) *FEBS Lett.* **275,** 139–142
41. Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973) *J. Mol. Biol.* **77,** 417–436
42. Perona, J. J., Tsu, C. A., Craik, C. S., and Fletterick, R. J. (1993) *J. Mol. Biol.* **230,** 919–933
43. Johnson, D. A., and Barton, G. J. (1992) *Protein Sci.* **1,** 370–377
44. Hooper, J. D., Nicol, D. L., Dickinson, J. L., Eyre, H. J., Scarman, A. L., Normyle, J. F., Stuttgen, M. A., Douglas, M. L., Loveland, K. A., Sutherland, G. R., and Antalis, T. M. (1999) *Cancer Res.* **59,** 3199–3205
45. Saling, P. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6231–6235
46. Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994) *J. Biol. Chem.* **269,** 31845–31849
47. Kohno, N., Yamagata, K., Yamada, S., Kashiwabara, S., Sakai, Y., and Baba, T. (1998) *Biochem. Biophys. Res. Commun.* **245,** 658–665
48. Ohmura, K., Kohno, N., Kobayashi, Y., Yamagata, K., Sato, S., Kashiwabara, S., and Baba, T. (1999) *J. Biol. Chem.* **274,** 29426–29432
49. Steiner, D. F., Smeekens, S. P., Ohagi, S., and Chan, S. J. (1992) *J. Biol. Chem.* **267,** 23435–23438
50. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950
51. Merritt, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50,** 869–873