

Identification and Enzymatic Characterization of Two Diverging Murine Counterparts of Human Interstitial Collagenase (MMP-1) Expressed at Sites of Embryo Implantation*

Received for publication, August 23, 2000, and in revised form, December 4, 2000
Published, JBC Papers in Press, December 12, 2000, DOI 10.1074/jbc.M009586200

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Remodeling of fibrillar collagen in mouse tissues has been widely attributed to the activity of collagenase-3 (matrix metalloproteinase-13 (MMP-13)), the main collagenase identified in this species. This proposal has been largely based on the repeatedly unproductive attempts to detect the presence in murine tissues of interstitial collagenase (MMP-1), a major collagenase in many species, including humans. In this work, we have performed an extensive screening of murine genomic and cDNA libraries using as probe the full-length cDNA for human MMP-1. We report the identification of two novel members of the MMP gene family which are contained within the cluster of MMP genes located at murine chromosome 9. The isolated cDNAs contain open reading frames of 464 and 463 amino acids and are 82% identical, displaying all structural features characteristic of archetypal MMPs. Comparison for sequence similarities revealed that the highest percentage of identities was found with human interstitial collagenase (MMP-1). The new proteins were tentatively called Mcol-A and Mcol-B (Murine collagenase-like A and B). Analysis of the enzymatic activity of the recombinant proteins revealed that both are catalytically autoactivable but only Mcol-A is able to degrade synthetic peptides and type I and II fibrillar collagen. Both *Mcol-A* and *Mcol-B* genes are located in the A1–A2 region of mouse chromosome 9, *Mcol-A* occupying a position syntenic to the human *MMP-1* locus at 11q22. Analysis of the expression of these novel MMPs in murine tissues revealed their predominant presence during mouse embryogenesis, particularly in mouse trophoblast giant cells. According to their structural and functional characteristics, we propose that at least one of these novel members of the MMP family, *Mcol-A*, may play roles as interstitial collagenase in murine tissues and could represent a true orthologue of human MMP-1.

* This work was supported in part by grants from the Plan Feder (1FD97-0214), EU-BIOMED II (BMH4-CT96-0017), the Arthritis and Rheumatism Council (to G. M.), and the Wellcome Trust (to V. K.). The Instituto Universitario de Oncología is supported by Obra Social Cajasur-Asturias. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ Recipient of a predoctoral fellowship from Ministerio de Educación, Spain.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ278461 and AJ278462.

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Controlled degradation of the extracellular matrix is an essential event in a variety of physiological conditions involving connective tissue remodeling such as embryonic growth and development, uterine involution, ovulation, bone growth and resorption, and wound healing (1, 2). In addition, excessive breakdown of connective tissue plays an important role in a number of pathological processes such as rheumatoid arthritis, atherosclerosis, pulmonary emphysema, and tumor invasion and metastasis (1, 2). Among the diverse proteolytic enzymes potentially involved in these physiological and pathological processes, many studies have focused on matrix metalloproteinases (MMPs),¹ a family of structurally related endopeptidases collectively capable of degrading the major protein components of the extracellular matrix and basement membranes. At present, 20 different human MMPs have been characterized at the amino acid sequence level (3). According to structural and functional characteristics, these human MMPs can be classified into at least six different subfamilies of closely related members: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs.

The collagenase subfamily of human MMPs consists of three distinct members: fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). An additional collagenase called collagenase-4 has been identified in *Xenopus laevis* (4), but to date the putative orthologues of this enzyme in other vertebrate species have not been described. Biochemical characterization of all these collagenases has revealed that they share the ability to cleave fibrillar collagens at a specific peptide bond, resulting in the generation of fragments of about three-fourths and one-fourth the size of the intact molecule. Then, the resulting fragments denature spontaneously to gelatin in physiological temperature and become susceptible to degradation by other MMPs (5–8). Interestingly, kinetic studies have revealed that each human collagenase shows distinct substrate preferences toward the diverse fibrillar collagens. Thus, MMP-1 degrades preferentially type III collagen (6), MMP-8 prefers type I collagen (7), and MMP-13 degrades type II collagen 6-fold more effectively than type I and type III collagens (8). It is also remarkable that MMP-13 displays about 40-fold stronger gelatinolytic activity than MMP-1 and MMP-8 (8). On the basis of these data, we have previously

¹ The abbreviations used are: MMP, matrix metalloproteinase; MT, membrane-type; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; TIMP, tissue inhibitor of metalloproteinases; PAC, P1 artificial chromosome; dpc, days post-coitum; bp, base pair(s); kbp, kilobase pair(s); Mca, (7-methoxycoumarin-4-yl)-acetic acid; Nva, norvaline; Dpa, L-dinitrophenyl-diamino propionic acid; Cha, cyclohexyl alanine.

A

Mcol-A

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1  AAGCTTCAGCATTCCGAGCCTTCCTTCTGCTGCTCTCTGGGCTGCTAGCTCATAACAGTTTCCTCTGTGTTCCACAAAGGACCCGCAAAATGTGGAG 100
   M P S L P L L L L L L W A A S S Y S F P V F H N G D R Q N V E
101 ACAGTCTGGAACTACCTGCGAACTACTCAACTTGGGCAAAACATGCAAGCAAAAAGCTGAAATGGCAAGAGATGGTGGTGAAGCTGAGCCGAA 200
   T V W K Y L E N Y Y N L G K N M Q A K N V N G K E M M A E K L R Q M
201 TGCAGCAGTATTGGGCTGAAAGTACTGGAATTCAGATCCTGAAACCCCTGAGAGCTATGAAGAAGCCAGGTGGGGTGCCTGATGTGGCCCCATA 300
   Q Q L F G L K V T G N S D P E T L R A M K K P R C G V P D V A P Y
301 TGCCATTACTCACAACAATCCTGTTGGACCAAAACACATCTGACATACAGCATTTTAAACTACACACCATATTGCCAAAGCAGTTGGGAAGATGCC 400
   A I T H N N P R W T R K T H L T Y S I L N Y T P Y L P K A V V E D A
401 ATCCGAGAGCTTTAGAGTCTGGAGTGTGACACACCTTACGTTCCAAAGAGTCTTTGAGGAGGAAGCCGATATTGCTCTCCCTCCACAGAGGAG 500
   I A R A F R V W S D V T P L T F Q R V F E E G D I V L S F H R G D
501 ACCATGGTGACAACAACCCATTTGATGGACCTAACTATAAGCTTGTCTACACTTTCAGCCAGCCGAGTTGGGGGTGATGTTCAATTGACCTTGA 600
   H G D N N P F D G P N Y K L A H T F Q P G P G L G G D V H Y D L D
601 TGAGACGTGGACCAACAGCAGTGAATAATTCACCTTGTCTATGTTACGGCTCATGAACCTGGGTCACCTCCCTTGGGCTCACTCATTCTAGTATATAGGA 700
   E T W T N S S E N F N L F Y V T A H E L G H S L G L T H S S D I G
701 GCACATAATGTTCCCGAGTTACAGCTGGTACACTGAAGCTTTGCTAAACAGGATGATTAATTCGCATCCAGGACTTATATGACCTTCCCAAACT 800
   A L M F P S Y T W Y T E D F V L N Q D D I N R I Q D L Y G P S P N P
801 CCATCCAGCCAAAGCTGCAACACACACCTCCATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 900
   I Q P T G A T T P H P C N G D L T F D A I T T F R G E V F F F K G
901 CAGGTTCTACATTCGGGTAATAGATTGATGCGAGACCTGAGCTCAATTTAATAGGATTTCTCGGCAAACTTCCAGTTAACTGAGCGCTGCTTAT 1000
   R F Y I R V N R F M P E P E L N L I G I L W P N L P V K L D A A Y
1001 GAAGCTAGTATGATAGATCAAGTCCGCTATTTCAAAGCAGCAAGTATGGGCTGTTCAAGAGGATGATGATGATGATGATGATGATGATGATGATGATGAT 1100
   E A S M I D Q V R Y F K G S K V W A V Q E Q S V L R G F P R D I H S
1101 GTTCTTGGGCTCCCTAGCAATGTGACACACATGATGCTGCTGTTTGTGAGGAAGAGACTGGAAAACTACTTCTTTTGGACACATGATGAGGAG 1200
   F F G F P S N V T H I D A A V C E E T G K T Y F F V D H H Y W R
1201 GTATGATGAAAATACAGCTTATGGATCCAGGTTCCAGATTAACAGCAGAGACTTCCCTGGAAATGATGATGATGATGATGATGATGATGATGATGATGAT 1300
   Y D E N T Q S M D P G Y P R L T A E D F P G I D D K V D D V F Q K
1301 GGAGAAAATTTCAATTTTCCACCAATCAGTCAACACAGATTTAACTCCAAATAAGAGAGTGTGATGATTCGGGTGATCTAGTATGATGATGATGATGAT 1400
   G E N F Y F F H Q S V Q H R F N L Q I R R V D D S R D S T T W F N C
1401 GCTAAAATATGGAACTCCTTATCAAGTAAATGAAATACATATTCATGATCTTAAGAAGTATTAACTGAAGCAAAATATTGTTATGTTTTAT 1500
   *
1501 TTACCTTCCGAGTCTGATACAT 1525
    
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FIG. 1. Nucleotide sequence and deduced amino acid sequences of mouse Mcol-A and Mcol-B. The deduced amino acid sequences for Mcol-A (A) and Mcol-B (B) are shown below the nucleotide sequences. Potential sites for N-glycosylation are underlined. C, comparison of the amino acid sequences of mouse Mcol-A and Mcol-B with mouse collagenases (MMP-8 and MMP-13), human MMP-1, and stromelysins. The multiple alignment was performed with the PILEUP program of the GCG package. Identical residues in all sequences are shadowed in gray. RGD residues exclusive of MMP-1 are underlined. Residues specific of collagenases are in bold and marked with an asterisk. Numbering refers to Mcol-A.

B

Mcol B

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1  GACATGCCAGCCTTCCTTGTCTGTGCTCTCTGGGCTGCGAGCTCATACAGTTTCCTGTGATGTCAGATGAGCTCCAGAAAAATGTGAAGACAGCT 100
   M P S L P L L L L R L W A A S S Y S F P V I O D G L Q K N V K T V W
101 GGAATACCTGGAACACTCTCAACTTGGGCAAAACATGCAAGCTAAACAGCTGAGGAGGATGGTGGTGAAGCTGAGCCAAATGAGCCACA 200
   K Y L E N Y Y N L G K N M Q A K N V N G K E V M A E K L R Q M O Q
201 GTTATTTGGGCTGAAAGTACTGAAATTCAGATCCTGAAACCCCTGAGAGCTATGAAGAAGCCAGGTGGGGTGCCTGATGGGCCCATATGCCATT 300
   L F G L K V T G N S D P E T L R A M K K P R C G V P D V A P Y A I
301 ACTCACAACAATCCTGTTGGACCAAAACACATCTGACTTACAGCATTTTAAACTACACACCATTTTGTCAAAGCAGTTTGGGAAGATGCCATCGGA 400
   T H N N P R W T R K T H L T Y S I L N Y T P Y L S K A A V E D A I A R
401 GAGCCTTGAAGTCTGGAGTGTGACACACCTTACGTTCCAAAGAGTCTTTGAGGAGGAGCCGATATTGCTCTCTCCACAGAGGAGACCAATGG 500
   A F R V W S D V T P L T F Q R V F E E G D I V L S F H R G D H C
501 TGACCTTACACATTTGATGATCTAATATCACTTGTCTCATGCTTTCTGCGAGCCTAGTTTGGGGGCAAGTGTGATGATGATGATGATGATGATGAT 600
   D L Y T T F D G S K Y H F A H A F L P G L G L G G N V H Y D L D Q K
601 TGACGGAACAATGAGGATTTCACTTGTCTATGTTACGGCTCATGAACCTGGGTCACCTCCCTTGGACTCTCCCATCTAATGATGAGAGGACCACTA 700
   W T D N N E D F N L F Y V T A H E L G H S L G L T H S S D I G
701 TGTTCCCGACTACACATGAGCAATAAAGACTTTGTCTAAACCCAGGATGATTAATCGCATCCAGGCTTATATGGAACCTTCCCAAAATCCCATCCA 800
   F P S Y T W S N K D F V L N Q D D I N R I Q A L Y G P S P N P I Q
801 GTTAAAGATGCAACACTGATCCATTAATAGTGTCTAACTTGTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 900
   L T D A T L D P C N S G L T F D A I I T Y R G E V I F F K D R F Y
901 ATTCCGGTAATAGCTTCTGCGAAGCCTTTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1000
   I R V I S F L P E P L I D V I D L T W P N L P G K P D A A Y E V S G
1001 GGATAGATGAATCCGCTTTTCAAAGCAGCAAGTATGGGCTGTTCAAGAAACAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1100
   V D E L R F F K G S K V W A V Q E Q N V L E G F P M D I Q S F F G
1101 CTCCTAGCAATGTGCAAAACATGATGCTGCTGTTTGTGAAGAAGAGACTGAAAAACATACTTCTTGTGACCACTGATCTGAGATATGATGATGAT 1200
   F P S N V T N I D A A V C E E T G K T Y F F V D H H Y W R Y D E
1201 AATACAGCTCCAGTCCAGGTTACCCGATTAATAGCAAGACTTTCCTGGAATCAAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1300
   N T R S M D E G Y P R L I A E D F P G I D Y K A G D D V I Q K E D N F
1301 TCTATTTCTTCCCAATCAATCAACAGATTTAACTCAAACAAGAAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1400
   Y F F H Q S I Q Y R F N L K T R R I D D S S D I N T W F N C
    
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proposed that the different human collagenases have evolved as specialized enzymes to participate in the remodeling of tissues with different collagen composition (8). The observation that the three human collagenases exhibit distinct tissue distribution and are subjected to different regulatory mechanisms (9, 10) is also consistent with the idea that they may play different functional roles in both physiological and pathological processes. To provide further experimental support to this proposal, it is essential that animal models be available in which the activity of the different enzymes can be selectively manipulated. However, these studies have been seriously hampered by the inability to detect the murine orthologue of MMP-1. In fact, to date only murine MMP-8 and MMP-13 have been identified and characterized at the amino acid sequence level (11–

13), whereas all attempts from many different groups to isolate murine MMP-1 have been repeatedly unsuccessful. These data have suggested that MMP-1 may be functionally substituted in murine tissues by other enzymes with collagenolytic activity such as MMP-8 and MMP-13. Nevertheless, the possibility that additional as yet unidentified murine enzymes could be structurally or functionally related to human MMP-1 cannot be definitively ruled out. To evaluate this possibility, we have performed an extensive screening of murine genomic and cDNA libraries using as probe the full-length cDNA for human MMP-1. As a direct result of this work, we report herein the identification of two novel members of the MMP gene family originally selected by their positive hybridization with the human MMP-1 probe, and contained within the cluster of MMP

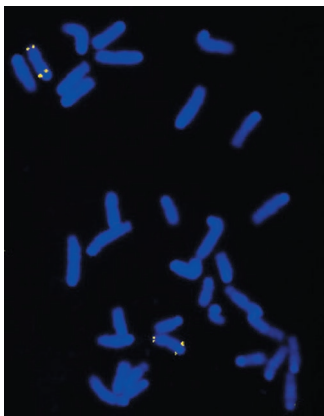


FIG. 2. **Chromosomal mapping of mouse *Mcol-A*.** Fluorescent *in situ* hybridization of mouse metaphase spreads was performed with the biotinylated PAC 528 C11 and BAC 55J6. Telomeric hybridization signal on chromosome 9 was obtained with the specific probe from BAC 55J6. PAC 528 C11 hybridized to the A1–A2 region of chromosome 9. Metaphase cells were counterstained with diamidine-2-phenylindole dihydrochloride.

Dpa-NH₂ (QF-41) (provided by C. G. Knight, University of Cambridge, UK). Routine assays were performed at 37 °C at substrate concentrations of 1 μM in an assay buffer of 50 mM Tris/HCl, 5 mM CaCl₂, 150 mM NaCl, 0.05% (v/v) Brij-35, pH 7.6, with a final concentration of Me₂SO of 1% (17). The fluorometric measurements were performed using an LS50B PerkinElmer Life Sciences spectrofluorometer. Enzyme concentrations were determined by active site titration using a standard TIMP-1 solution following 4-h preincubation to allow complex formation (17). Collagenolytic activity was determined by incubating soluble rat type I collagen (18) or acid-soluble bovine type II collagen (Biogenesis Inc., Poole, UK) with the recombinant enzymes at 25 °C, and the degradation products were demonstrated by SDS-PAGE. Additionally, we determined the specific collagenolytic activity of Mcol-A using ¹⁴C-labeled rat type I collagen in a fibrillar assay at 35 °C essentially as described (18). The activity of both murine orthologues against ¹⁴C-labeled gelatin and casein was determined by overnight incubation at 37 °C.

Homology Modeling—Three-dimensional models of catalytic domains of Mcol-A and Mcol-B were calculated using a semiautomated modeling server (19) and analyzed with the Swiss-PdbViewer. Briefly, the amino acid sequences of the respective catalytic domains were compared with the sequences of the macromolecules deposited in the Protein Data Bank to identify suitable templates. We chose nonredundant proteins that had the highest structural quality, and high similarity with Mcol-A and Mcol-B. The pdb files corresponding to these proteins are 2TCL (human MMP-1), 1JAN (human MMP-8), the B chain of file 830C (human MMP-13), and 1SLM (human MMP-3). The templates were superimposed and aligned structurally. Then, the target sequences were automatically threaded over the structure, built with ProMod II, and energy-minimized with Gromos96. The models were analyzed with Swiss-Pdb Viewer, whereas the electrostatic calculations were performed with MolMol (20). Charges of conserved ions were also included in the calculations: two Zn²⁺ and two Ca²⁺ for the catalytic domain, as present in 2TCL. The figures were modeled with MolMol and rendered with Megapov and POV-Ray (from the POV-Ray site on the Web).

RESULTS

Identification, Cloning, and Sequence Analysis of Two Novel MMPs—To identify putative murine MMPs structurally related to human interstitial collagenase (MMP-1), we screened a mouse PAC genomic library using as a probe a full-length cDNA coding for this human protease. After hybridization under low stringency conditions, several PAC clones were selected on the basis of positive hybridization to the probe. The inserts contained in these clones were characterized by endonuclease restriction analysis and selected fragments showing hybridization with the MMP-1 cDNA probe were cloned and subjected to nucleotide sequencing. This analysis allowed the identification of two DNA fragments, derived from PAC 528 C11, whose

nucleotide sequences were similar to those previously determined for other murine MMPs. Further sequence analysis of these fragments and comparison with the exon-intron distribution of other MMP genes led us to identify several putative exons of a presumably novel MMP gene. To try to determine the complete structure of this MMP, studies were undertaken to isolate a full-length cDNA encoding this enzyme. To do that, two primers covering the start and stop codons identified in the putative first and last exons of the cloned MMP gene were synthesized and used for RT-PCR amplification of total RNA obtained from mouse embryos. The PCR-amplified product was cloned, and its identity was confirmed by nucleotide sequencing. Computer analysis of the obtained sequence (Fig. 1A) revealed an open reading frame coding for a protein of 464 amino acids with a predicted molecular mass of 53.5 kDa, which was tentatively called Mcol-A (Murine collagenase-like A).

Further analysis, of additional clones obtained by RT-PCR amplification of murine embryos RNA with oligonucleotides derived from the sequence determined for Mcol-A, revealed the presence of sequences highly related to but distinct from that determined for this novel MMP. A full-length cDNA for this apparently distinct MMP was isolated following the same strategy as above and then characterized by nucleotide sequencing. Analysis of the resulting sequence (Fig. 1B) allowed the finding of an open reading frame encoding a protein of 463 residues, with a calculated molecular mass of 53.5 kDa, and tentatively called Mcol-B. Genomic clones for this second MMP gene were also identified from DNA fragments obtained from PAC 519 F1 and allowed to confirm the sequence determined by analysis of the cDNA amplified by RT-PCR of murine embryos RNA. A comparison of the deduced amino acid sequences determined for Mcol-A and Mcol-B showed that they were closely related, exhibiting about 82% identities between them. Pairwise comparisons for sequence similarities between the identified amino acid sequences (Fig. 1C) and those determined for other murine MMPs showed that the maximum percentage of identities was found with mouse neutrophil collagenase (MMP-8) (48% and 45% with Mcol-A and Mcol-B, respectively). Interestingly, a higher percentage of identities (58% in amino acids and 74% in nucleotides) was found with human interstitial collagenase (MMP-1). This comparative sequence analysis also revealed that both Mcol-A and Mcol-B display all structural features characteristic of archetypal MMPs, including signal sequences, prodomain regions with the conserved Cys residues in the conserved PRCGVPD motif (at positions 87–93), catalytic, and hemopexin domains (Fig. 1C). The percentage of identities of each domain of the murine proteins with human MMP-1 is 53% (prodomain), 63% (catalytic), and 59% (hemopexin) in the case of Mcol-A, and 53% (prodomain), 58% (catalytic), and 61% (hemopexin) in the case of Mcol-B. The amino acid sequence deduced for Mcol-A and McolB contains three and two potential sites of *N*-glycosylation, respectively, including the one at position 117 absolutely conserved in the catalytic domain of collagenases, macrophage metalloelastases, stromelysin-1 and -2, gelatinase B, and MT-MMPs.

To further explore the structural relationship between human MMP-1 and murine Mcol-A and Mcol-B, we next performed a more detailed sequence analysis with special emphasis aimed at comparing a series of residues conserved in all collagenases described to date and proposed as essential determinants of collagenase specificity. These residues include Tyr-210, Asp-231, and Gly-233 according to human MMP-1 numbering (5, 21). The equivalent residues at these three positions in Mcol-A are Phe-208, Asp-229, and Gly-231, whereas in Mcol-B these residues are Phe-208, Asp-229, and Glu-231, respectively (Fig. 1C). Therefore, it seems that Mcol-A is more

related to collagenases than Mcol-B at least in terms of occurrence of residues important for this activity. This structural analysis also revealed that both Mcol-A and Mcol-B contain an RGD (Arg-Gly-Asp) motif in the catalytic domain. This motif is present at equivalent position in the MMP-1 sequence from all species in which this protein has been characterized, but not in other MMPs, providing additional evidence on the structural relationship between MMP-1 and the newly identified family members Mcol-A and Mcol-B. By contrast, both enzymes lack the nine-residue insertion present in the hinge region of all stromelysins. They also lack the fibronectin-like domain present in gelatinases, the C-terminal extension rich in hydrophobic residues characteristic of MT-MMPs, and the furin activation motif (RX(R/K)R) mediating the intracellular activation of MT-MMPs and stromelysin-3 (22, 23). In summary, and taking collectively all these structural comparisons, most data point to the inclusion of Mcol-A and Mcol-B as members of the collagenase subfamily, although they cannot be unequivocally classified within this group on the exclusive basis of their amino acid sequence characteristics.

Physical Mapping of Mcol-A and Mcol-B Genes—To determine the chromosomal location of murine genes encoding Mcol-A and Mcol-B, metaphase spreads from a male mouse were hybridized with the biotinylated PACs 528 C11 and 519 F1 enclosing these genes and with the telomeric marker of chromosome 9, BAC 55J6. After single- and double-fluorescent in situ hybridization experiments with both probes, fluorescent signal corresponding to Mcol-A and Mcol-B genes was located to the A1–A2 region of chromosome 9 (Fig. 2). Other murine MMP genes (*MMP-7*, *-12*, *-13*, and *-20*) have been already mapped to this region (24–27), which is syntenic to human chromosome 11q22–23 in which at least eight human MMPs are clustered in a relatively small region (28, 29). To establish the relative order of Mcol-A and Mcol-B loci within the cluster of MMP genes in mouse chromosome 9, DNA was isolated from the YAC clone I139A1, which contains murine *MMP-8* and *MMP-13* (11) as well as the new Mcol-A and Mcol-B, as tested by PCR. DNA from this YAC was digested with different rare-cutting restriction endonucleases (*ClaI*, *MluI*, *NarI*, *NruI*, *SacI*, *SalI*, *SfiI*, *SpeI*), and the resulting fragments were separated by pulsed-field gel electrophoresis. After blotting, DNA was successively hybridized with probes specific for Mcol-A and Mcol-B, as well as with probes for other MMP genes potentially contained within the analyzed YAC (*MMP-3*, *MMP-7*, *MMP-8*, *MMP-10*, *MMP-13*). In addition, hybridization with those probes was performed on the DNA of the seven isolated PAC clones immobilized on a nylon filter (Table I, and data not shown). The results obtained after hybridization of the filters containing the YAC DNA separated by pulsed-field gel electrophoresis and of those containing the DNA from the different positive PACs were combined. Thus, a common *MluI* band of about 200 kbp was detected by Mcol-A, Mcol-B, MMP-3, MMP-8, and MMP-10 probes. MMP-10 and Mcol-A but not MMP-8 probes shared 125-kbp *NarI* and 50-kbp *ClaI* bands. MMP-3 and Mcol-B but not Mcol-A probes detected a common *NruI* band of 80 kbp. Taken collectively, these data show that the order of the novel genes within the cluster would be compatible with the following: *MMP-8/MMP-10/Mcol-A/MMP-3/Mcol-B/MMP-12/MMP-13*. The order and orientation of seven human MMP genes clustered on the long arm of chromosome 11 is: centromere/*MMP-8/MMP-10/MMP-1/MMP-3/MMP-12/MMP-7/MMP-13*/telomere (28). Therefore, we can conclude that Mcol-A is located in a position equivalent to that of *MMP-1* in the human genome. Nevertheless, further studies derived from the current human and mouse genome projects will be required to confirm this possibility. In this regard, it should be

TABLE I
Results of the dot blot hybridization of the indicated PACs with different MMP probes

PAC	519 F1	528 C11	365 N22	360 L22	636 I22
Mcol-A	–	+	+	+	+
Mcol-B	+	–	–	+	+
MMP-8	–	+	+	–	+
MMP-3	–	+	+	+	+
MMP-10	–	+	+	–	+
MMP-12	+	–	–	+	–
MMP-13	–	–	–	–	–

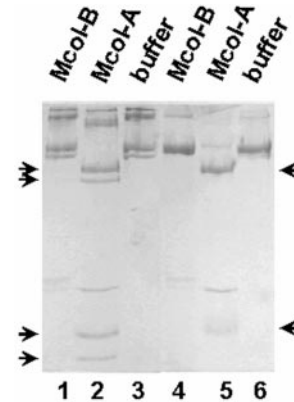


FIG. 3. Degradation of fibrillar type I and II collagen by Mcol-A and Mcol-B. Lane 1, Mcol-B was incubated with type I collagen for 24 h at 25 °C; lane 2, Mcol-A was incubated with type I collagen for 24 h at 25 °C; lane 3, type I collagen buffer control; lane 4, Mcol-B was incubated with type II collagen for 24 h at 25 °C; lane 5, Mcol-A was incubated with type II collagen for 24 h at 25 °C; lane 6, type II collagen buffer control. The reaction products were analyzed by SDS-PAGE and Coomassie Blue staining. The positions of the respective three-fourths and one-fourth fragments are indicated by arrows on the left and right.

TABLE II
Determination of k_{cat}/K_M for three quenched fluorescent peptide substrates for Mcol-A and Mcol-B

Substrate	Mcol-A	Mcol-B	Human MMP-1 ^a
	$M^{-1}s^{-1}$		$M^{-1}s^{-1}$
QF24	104	0	17000
QF35	58	0	69000
QF41	142	0	45000

^a M. Patterson, personal communication.

TABLE III
Determination of the specific activity of Mcol-A and Mcol-B versus macromolecular substrates (units) expressed as micrograms of collagen fibril solubilized/min

Substrate	Mcol-A	Mcol-B	Human MMP-1 ^a
	$units/nmol$		$units/nmol$
Type I collagen	1.44	0	120
Gelatin	0	0	2.2
Casein	0.07	0	Not determined

^a Data were taken from Knäuper *et al* (8).

mentioned that after submission of this manuscript two short genomic sequences (AZ349151 and AZ443051) containing partial information for Mcol-A and Mcol-B have been released to the Genome Survey Sequence data bank. According to our gene structure analysis,² AZ349151 would contain the exon 2 of Mcol-A, whereas AZ443051 would contain information for exon 7 of Mcol-B.

Production of Recombinant Mcol-A and Mcol-B in *E. coli* and Analysis of Their Enzyme Activity—According to the above

² M. Balbín and C. López-Otín, unpublished results.

described data, Mcol-A and Mcol-B have some structural features characteristic of members of the collagenase subfamily of MMPs, and we therefore expressed them in *E. coli*, refolded, and purified them as described under "Experimental Procedures." Our protocol was originally established to successfully refold procollagenase-3 and a transmembrane deletion mutant of MT1-MMP and allows the correct folding of collagenolytic MMPs (16). Therefore, we used this strategy to carry out a preliminary analysis of the ability of both refolded Mcol-A and Mcol-B to cleave triple-helical collagens. In addition, it's remarkable that SDS-PAGE analysis of the refolded proteins under reducing and nonreducing conditions revealed that they migrated faster under nonreducing conditions, indicating correct folding of the C-terminal domain, a prerequisite for our analysis of collagenolysis. Mcol-A was autoproteolytically converted to the active enzyme form (M_r 44,000) during the dialysis step employed to remove the imidazole from the enzyme preparation after purification using nickel-nitrilotriacetic acid-agarose (not shown). In contrast, a large proportion of the Mcol-B preparation was still in the proenzyme form (M_r 56,000 and M_r 54,000) following purification and dialysis, with minor bands at 46,000 and 43,000. It was, however, noted that Mcol-B underwent autoproteolytic conversion to these two lower molecular species (M_r 46,000 and M_r 43,000) when incubated for 24 h at 25 °C, a process that was inhibited by EDTA (not shown). When both enzyme preparations were analyzed for enzymatic activity, only Mcol-A was able to hydrolyze triple-helical type I and type II collagen into three-fourths and one-fourth fragments (Fig. 3), whereas Mcol-B remained inactive. These data indicate that, although we were unable to show enzymatic activity for Mcol-B *versus* macromolecular or quenched fluorescent peptide substrates, the propeptide domain was autoproteolytically removed, strongly suggesting that the enzyme is active. Nevertheless, the possibility that Mcol-B is incompletely folded cannot be ruled out. Activation trials for Mcol-B with either trypsin alone or in combination with stromelysin revealed no change in the ability of the enzyme to degrade macromolecular substrates. Thus Mcol-B might have a very restricted substrate specificity, which will need further investigation in the future.

The enzymatic activity of Mcol-A was analyzed in more detail. The enzyme was shown to hydrolyze three quenched fluorescent peptide substrates with distinct k_{cat}/K_m values, summarized in Table II. The Mcol-A activity *versus* these quenched fluorescent substrates was inhibited by TIMP-1, thereby indicating that this enzyme is a typical MMP (not shown). Comparison of the k_{cat}/K_m values of Mcol-A with human MMP-1 revealed that they were considerably reduced, indicating that, although the active site may be different, it still allows collagenolysis. Active site titrations were performed using the quenched fluorescent substrates and a standard TIMP-1 solution of known concentration that allowed us to determine the specific activity of Mcol-A against ^{14}C -labeled rat type I collagen, ^{14}C -labeled rat gelatin, and ^{14}C -labeled β -casein. The results obtained are shown in Table III, which also shows data for human MMP-1. Our data revealed that, although ^{14}C -labeled rat type I collagen represents a substrate for Mcol-A, the enzyme was unable to hydrolyze gelatin. This is a very surprising result, because all known human collagenases hydrolyze gelatin and thus Mcol-A may represent a more specific collagenase, needing the triple-helical conformation for activity. Additionally, ^{14}C -labeled β -casein was also cleaved by Mcol-A, but the specific activity determined was extremely low (Table III).

Homology Modeling of Mcol-A and Mcol-B—The homology models deduced for the catalytic domains of Mcol-A and Mcol-B show a clear superimposable pattern with the catalytic domain

of human MMP-1, consistent with the significant sequence similarity between them (Fig. 4, A–C). Likewise, the molecular surfaces of these domains are also very similar (Fig. 4D). Analysis of specificity determinants further strengthens the close relationship between MMP-1, Mcol-A, and Mcol-B. An essential factor for MMP specificity is the size of the S1' pocket (30–32). The depth of this hydrophobic pocket is largely determined by the side chain of the residue present at position 214 (MMP-1 numbering) (Fig. 4C). Most MMPs have a Leu residue at this position, and consequently their S1' sites are very deep and form a channel across the protein, allowing the digestion of substrates with large P1' side chains. Interestingly, MMP-1 as well as the novel murine enzymes have large residues at this position (Arg and Tyr, respectively), occluding the S1' channel and leaving a cavity that can only accept middle-sized substrates (Fig. 4C) (33, 34). The character of the S1 subsite depends mainly on residue 180, which is hydrophilic in MMP-1, Mcol-A, and Mcol-B (Asn, Lys, and His, respectively) and hydrophobic in the other MMPs. Taken together, these structural data support that the novel murine enzymes are more closely related to MMP-1 than to other MMPs. Furthermore, analysis of the molecular models depicted for Mcol-A and Mcol-B provide some clues to explain the observed differences in the catalytic activity of both enzymes. Thus, residue 181 is Leu in most MMPs, including Mcol-A and MMP-1, but Mcol-B is unique by possessing a Phe residue at this position. This bulky residue could hamper the access of the substrate to the active site cleft (Fig. 4, A and B). In addition, there is a series of residues in Mcol-B that could be important in terms of catalytic differences with Mcol-A. These include the Gly residue at position 233 (MMP-1 numbering) that is absolutely conserved in all collagenases but in Mcol-B is replaced by Glu, as well as the acidic residues 194 and 201 involved in calcium binding in MMP-1, which are changed to amide residues (Asn and Gln, respectively) in Mcol-B (Fig. 1C).

Analysis of Mcol-A and Mcol-B Expression during Murine Embryogenesis—To study Mcol-A and Mcol-B expression in murine tissues, we first performed RT-PCR amplification using specific oligonucleotides and RNA prepared from a variety of tissues (uterus, kidney, ovary, lung, and placenta) and embryos at different stages of development. These analyses revealed that Mcol-A and Mcol-B were expressed during fetal development. Mcol-A and Mcol-B expression during murine embryogenesis was also confirmed by Northern blot analysis (Fig. 5). Specific hybridization with Mcol-A probe was detected in the yolk sac and uterine tissue adjacent to mouse embryos at 9.5 and 10.5 dpc but not in tissue from embryos of 13.5, 15.5, 17.5, and 19.5 dpc. This specific hybridization signal was also observed in the rat. In this case, bands were observed in the yolk sac and uterine tissue adjacent to rat embryos of 11.5 and 12.5 dpc. These days of gestation correspond to the beginning of development of the chorioallantoic placenta. Hybridization was also detected in RNA obtained from placenta at 13.5, 15.5, and 17.5 dpc. To examine the identity of the cells responsible for the production of Mcol-A and Mcol-B in the murine tissue during embryogenesis, we carried out an *in situ* hybridization on tissue sections of rat and mouse embryos and adjacent tissue from 8.5 to 16.5 dpc. A clear expression for both Mcol-A and Mcol-B was found in a low number of extra-embryonic cells located at the maternal interface (Fig. 6). By contrast, no transcripts were detected in either the embryo or the maternal decidua. Hybridizations with the two probes in adjacent serial sections demonstrated that both enzymes were expressed in the same cells, although staining for Mcol-B was much weaker. Expression of both genes was restricted to a network of cells at the periphery of the embryo in contact with the adjacent decidual cells. Pos-

FIG. 4. Homology models of the catalytic domains of Mcol-A and Mcol-B. *A*, ribbon representations of the models of Mcol-A and Mcol-B superimposed to the catalytic domain of human MMP-1. The RO 31-4724 substrate analogue of MMP-1 and the histidine side chains that coordinate the catalytic Zn are also shown. *B*, detailed view of the interaction between RO 31-4724 and residues Leu-181 of Mcol-A, and Phe-181 of Mcol-B. *C*, cross-section of the catalytic domains of MMP-1, MMP-8, and modeled Mcol-A showing the different shapes of the S1' pocket. The residues in position 214, which determine the size of this pocket, are also shown. MMP-1 and MMP-8 substrate analogs are shown in *blue* and *green*, respectively. *D*, molecular surface of the catalytic domains of MMP-1, Mcol-A, and Mcol-B. Standard view showing the active site of the molecules. Electrostatic potentials lower than -1.8 V are in *red*, higher than 1.8 V are in *blue*, and neutral is in *white*. Intermediate values are interpolated.

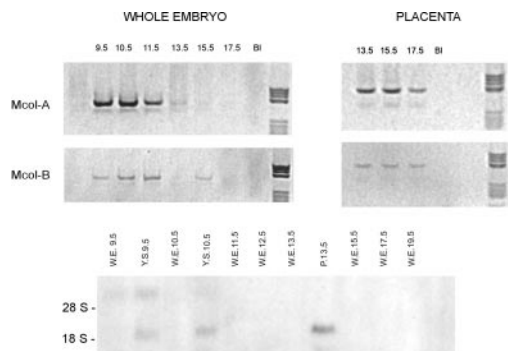
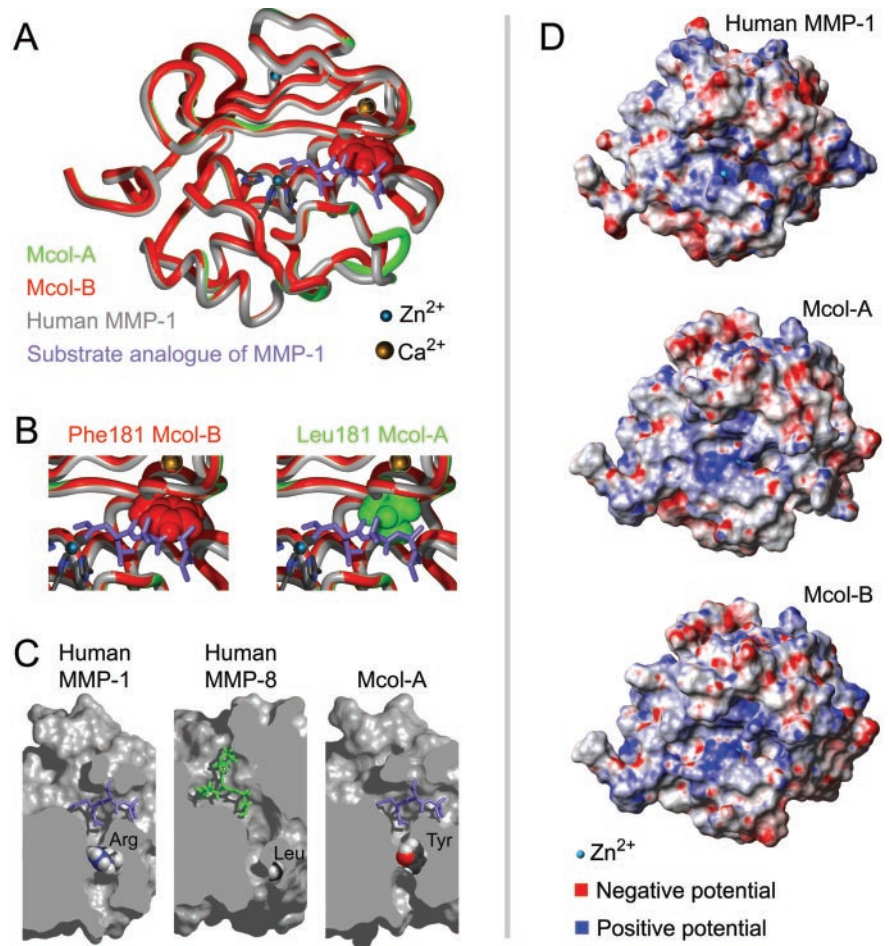


FIG. 5. Expression of Mcol-A and Mcol-B in mouse tissues. *A*, RT-PCR was performed on $1 \mu\text{g}$ of RNA from whole embryos or placenta at the indicated days of embryonic development, with specific oligonucleotides for Mcol-A and Mcol-B as primers. $20 \mu\text{l}$ of the final product were separated on a 2% agarose gel. *Bl* lane shows RT-PCR performed without added template. The *standard* lane is Marker V from Roche Molecular Biochemicals. *B*, samples of $20 \mu\text{g}$ of total RNA from whole embryo (*W.E.*), yolk sac (*Y.S.*), or placenta (*P.*) at the indicated days of development were separated by agarose gel electrophoresis under denaturing conditions, blotted onto nylon filters, and analyzed by hybridization with full-length cDNA for Mcol-A. Filters were exposed to autoradiography at -70°C for 7 days with Kodak BIOMAX MS films and screens. The positions of the 28 S and 18 S RNA are indicated.

itive cells were morphologically identified as trophoblast giant cells. To further identify these positive cells, adjacent serial sections were hybridized with antisense probes for MMP-9, which is considered as a typical marker for terminally differ-

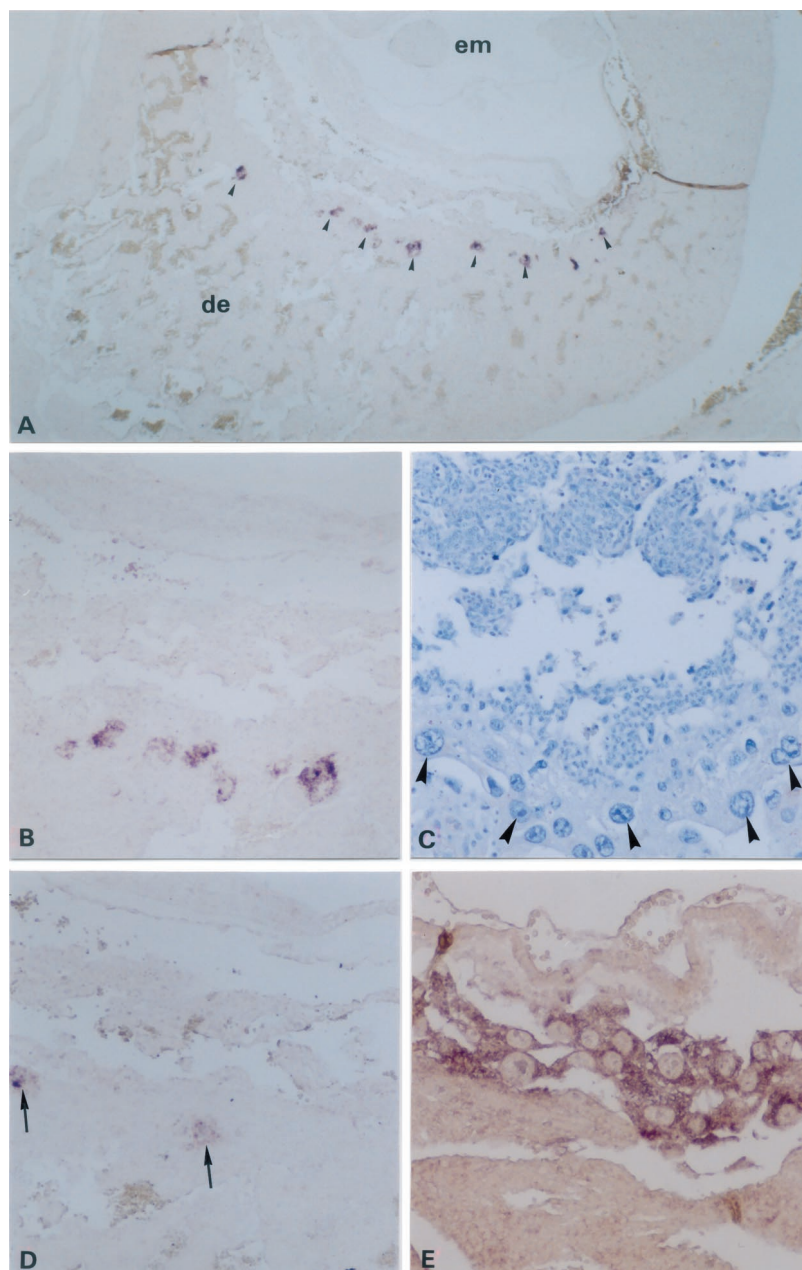
entiated trophoblast giant cells (35). Cells positive for Mcol-A and Mcol-B showed high expression levels of MMP-9, although cells expressing MMP-9 but not Mcol-A and Mcol-B were also found. Detection of Mcol-A and Mcol-B transcripts was restricted to sections of 9.5 and 10.5 dpc in mice and 10.5 and 11.5 dpc in rat embryos. In both cases the expression level significantly decreased with age of embryos and virtually no expression was found in mouse embryos older than 10.5 dpc.

DISCUSSION

This work describes the identification of two murine metalloproteases Mcol-A and Mcol-B, cloned as a result of their positive hybridization with a probe for human MMP-1. According to their structural and functional characteristics, we propose that at least one of these novel members of the MMP family, Mcol-A, may play roles as interstitial collagenase in murine tissues and could represent a true orthologue of human MMP-1.

Over the last years, the number and identity of collagenolytic enzymes produced by murine tissues has been a debated question within the MMP field. Studies performed by several groups have demonstrated the existence in mouse and rat cells and tissues of the corresponding orthologues of neutrophil collagenase (MMP-8) (11) and collagenase-3 (12, 13). However, and somewhat surprisingly, to date no evidence of occurrence of murine interstitial collagenase (MMP-1) has been reported. This is specially puzzling if we consider that MMP-1 was likely responsible of at least part of the collagenolytic activity discovered by Gross and Lapière in 1962 from the tail of the metamorphosing tadpole (36), as well as the first human MMP cloned and characterized at the amino acid sequence level (21). Furthermore, MMP-1 orthologues have been identified and

FIG. 6. *In situ* hybridization for Mcol-A, Mcol-B, and gelatinase B in mouse uterus and feto-maternal placental tissues. Hybridization was performed on transverse serial sections of an 8.5-dpc mouse uterus, including feto-maternal placental tissues. As can be observed in *A* and *B*, signal for Mcol-A (*arrowheads*) is found in a low number of cells located at the periphery of the embryo (*em*) in contact with adjacent decidual cells (*de*). *B*, a higher magnification of cells positive for Mcol-A; *C*, the same region in a parallel section stained with Gill's hematoxylin showing that positive cells can be morphologically identified as trophoblast giant cells (*arrowheads*). *D*, *in situ* hybridization for Mcol-B showing a weaker but specific signal also in trophoblast giant cells. *E*, *in situ* hybridization with gelatinase B antisense probe showing intense labeling in a higher number of trophoblast giant cells. Original magnifications: *A*, $\times 40$; *B*, *C*, and *D*, $\times 100$; *E*, $\times 64$.



cloned in a number of species including *Homo sapiens*, *Bos taurus*, *Sus scrofa*, *Oryctolagus cuniculus*, and *Rana catesbeiana* (21, 37–39). A partial cDNA sequence presumably encoding a C-terminal fragment of the guinea pig MMP-1 has been also reported, although it has been proposed that the guinea pig is more closely allied with lagomorphs than with rodents (40). Our approach to the identification of a putative murine orthologue of MMP-1 involved a combined strategy based on screening of a mouse PAC genomic library with a human MMP-1 cDNA probe, followed by RT-PCR amplification of mouse embryo RNA with oligonucleotides derived from the sequence of genomic clones hybridizing with the MMP-1 probe. This strategy led us to identify two murine cDNAs coding for proteins with a series of structural features present in MMPs, and more specifically in members of the collagenase subfamily. Both Mcol-A and Mcol-B were most similar to human interstitial collagenase (MMP-1). The overall identities (74% in nucleotides and 58% in amino acids) are maintained throughout the different domains of these proteins and are similar to those found in the comparison of mouse and human MMP-12 (76% in

nucleotides and 61% in amino acids) but are lower than those shared by mouse and human orthologues of most MMPs. Nevertheless, both Mcol-A and Mcol-B contain a characteristic RGD motif present in MMP-1 from all species in which this collagenase has been characterized but not in other MMPs. Conversely, both enzymes are devoid of any structural features defining members of other MMP subfamilies such as stromelysins, gelatinases, and MT-MMPs. An analysis of a phylogenetic tree constructed to evaluate the evolutionary relationships of mouse Mcol-A and Mcol-B to other MMPs also revealed that human MMP-1 was the most closely related to these novel proteases (Fig. 7). Finally, molecular modeling experiments based on the crystal structures known for diverse MMPs have confirmed that the overall fold of the catalytic domains of Mcol-A and Mcol-B is topologically very similar to that of MMP-1, including the small size of the S1' specificity pocket. Nevertheless, there are considerable differences at the amino acid sequence level between Mcol-A and Mcol-B, which might be involved in structural features that determine different enzymatic activities.

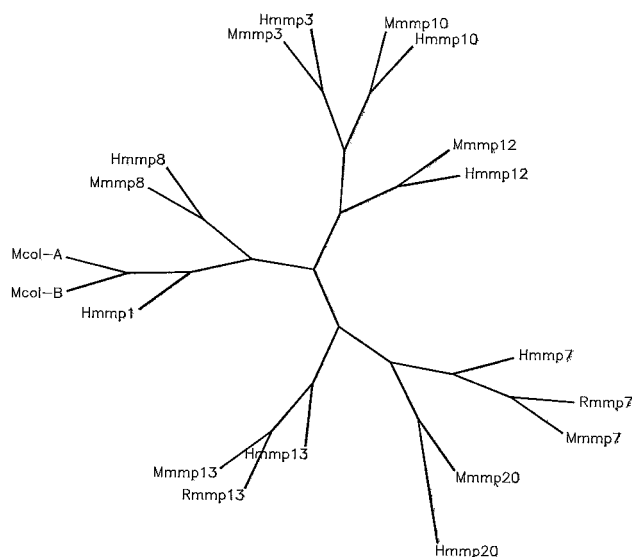


FIG. 7. Schematic illustration of evolutionary relationships between human and mouse MMPs. The phylogenetic tree includes the diverse MMPs clustered in human chromosome 11 and mouse chromosome 9 and was constructed on-line at the United Kingdom Human Genome Mapping Project Resource Center, using PIE, which provides a *www* interface to programs included in the PHYLIP software package.

In addition to the above features that suggest that both murine enzymes are structurally related to MMP-1, we have also provided functional evidence that at least Mcol-A exhibits the ability to act as a collagenolytic enzyme. In fact, recombinant Mcol-A displays proteolytic activity against type I and type II fibrillar collagens, although its specific activity *versus* fibrillar type I collagen is much lower than that described for human MMP-1 or MMP-13. Mcol-B is apparently devoid of collagenolytic activity, although it can autoactivate when incubated for 24 h at 25 °C or stored for prolonged periods of time at 4 °C. On the other hand, genomic studies have indicated that both *Mcol-A* and *Mcol-B* exhibit the same exon-intron distribution as human *MMP-1*, and their proximal promoter region is significantly similar to that of human *MMP-1*.² Furthermore, fine chromosomal mapping of the region containing these murine genes has revealed that *Mcol-A* seems to be located at a position syntenic to the *MMP-1* locus in the human genome. On these bases, together with the above enzymatic analysis, we can conclude that Mcol-A is closer to MMP-1 than Mcol-B in both structural and functional terms. Thus, Mcol-A could be a homologue of MMP-1 in murine tissues, Mcol-B being the result of a specific gene duplication event that has retained a number of MMP-1 features but that has also accumulated some changes resulting in an impairment of its ability to act as a collagenolytic enzyme. As mentioned above, sequence comparisons and molecular modeling of the catalytic domains of these enzymes have suggested some specific features of Mcol-B that could contribute to the observed catalytic differences with Mcol-A and human MMP-1. Nevertheless, further studies involving site-directed mutagenesis experiments will be required to elucidate the molecular basis for the differential activities among all these closely related members of the MMP family.

To investigate the functional role of these novel MMPs, we have also examined the tissue distribution of both Mcol-A and Mcol-B in murine tissues. According to RT-PCR and Northern blot analysis, these enzymes are mainly produced in yolk sac and uterine tissue adjacent to mouse embryos at early times during implantation. *In situ* hybridization demonstrated that expression of both genes is restricted to trophoblast giant cells present at the embryo/maternal interface, although in all

cases, the expression level of Mcol-A is higher than that of Mcol-B. According to these expression analysis, it is tempting to speculate that these novel murine proteases may participate in embryo implantation. The implantation of the mammalian embryo into the uterine stroma is a highly controlled process of tissue invasion that involves extensive remodeling of extracellular matrix components to accommodate the growing embryo as well as to establish the vascular structures necessary for transplacental exchange (41, 42). This process is initiated by the attachment of the blastocyst to the uterine epithelium on day 4.5 of mouse development and ceases on day 10.5, with placental function beginning on day 11. The observation that both Mcol-A and Mcol-B are produced by trophoblast cells on days 9.5–10.5 suggests that these enzymes may contribute to the final stages of the invasive process. Similarly, the observation that gelatinase B, whose expression peaks at 7.5 days, colocalizes with Mcol-A and Mcol-B could be indicative of the occurrence of a putative proteolytic cascade involving these proteases. Nevertheless, the possibility that Mcol-A and Mcol-B are involved in other processes distinct from blastocyst invasion, including angiogenesis regulation or collagen turnover accompanying decidualization, cannot be ruled out. It is also noteworthy in the context of the putative relationships between these novel murine MMPs and human MMP-1, that this interstitial collagenase has been also found to be produced by trophoblastic cells during human pregnancy (43, 44). These data suggest that the parallels between human MMP-1, and murine Mcol-A and Mcol-B, could be extended to their respective expression patterns. However, it is still unclear if the murine enzymes will share its wide distribution in processes such as wound healing or tumor progression, in which the presence of human MMP-1 has been repeatedly described (45, 46).

In conclusion, our structural and functional analysis suggest that Mcol-A and Mcol-B can be considered as putative murine counterparts of MMP-1, although it seems that they have diverged much more rapidly than other mouse and human MMP orthologues. Furthermore, on the basis of differences between both enzymes, it is tempting to speculate that Mcol-A is functionally closer to MMP-1 than Mcol-B. Further studies will be required to provide definitive evidence on the proposal that the newly identified murine MMPs, and, more specifically, Mcol-A represent structural and functional counterparts of MMP-1. It will be also of interest to evaluate if the observed structural divergence between them may underlie diverging functional roles for these proteolytic enzymes.

Acknowledgments—We thank Drs. G. Velasco, A. M. Pendás, I. Santamaría, J. M. P. Freije, X. S. Puente, M. J. Jiménez, and J. A. Uría for helpful comments, and S. Álvarez for excellent technical assistance.

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