The Matrix Metalloproteinase-14 (MMP-14) Gene Is Structurally Distinct from Other MMP Genes and Is Co-expressed with the TIMP-2 Gene during Mouse Embryogenesis*

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Suneel S. Apte§§, Naomi Fukai‡, David R. Beier¶, and Bjorn R. Olsen¶

From the §Department of Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, Ohio, the ¶Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, and the ¶Department of Genetics, Brigham and Women’s Hospital, Boston Massachusetts 02115

The matrix metalloproteinases (MMPs) are a family of zinc-containing matrix degrading endopeptidases. A subfamily of membrane type (MT) -MMPs has been described recently. We have determined the structure of the gene (Mmp14) encoding the first MT-MMP to be described, MT1-MMP (MMP-14), and mapped it to mouse chromosome 14. The mouse MMP-14 protein is encoded by ten exons. The novel C-terminal peptide domains of MMP-14 are encoded by a single large exon that also encodes the 3′-untranslated region. The structure of the exons encoding the catalytic domain and pro-domain of MMP-14 is distinct from previously described MMP genes, whereas the exons encoding the hemopexin-like domains are similar to those of most other MMP genes. Mmp14 and the gene for tissue inhibitor of metalloproteinases-2 (Timp2) show a temporally and spatially co-regulated expression during mouse development. They are co-expressed during vascular and urogenital development and during the development of osteocartilaginous and musculotendinous structures. The stringent co-expression of these two genes suggests common regulatory pathways that may have important functional implications for the activation of pro-gelatinase A in health and disease.

Extracellular matrix (ECM) remodeling results from the orchestrated interplay between matrix degradation and synthesis. A number of enzyme groups with a variety of substrates, biochemical optima, and mechanisms of action participate in matrix degradation. One group, the matrix metalloproteinases, consists of a group of structurally related zinc endopeptidases with a neutral pH optimum and the cumulative ability to digest most components of ECM (1–3). MMPs are believed to play a major role in embryonic development as well as in the invasion and metastasis of cancer (3). In general and with few exceptions such as stromelysin-3 (4), MMPs are secreted in zymogen form requiring proteolytic processing at the N terminus before they become catalytically active (1–3). In contrast to most secreted pro-MMPs, which are efficiently activated in the ECM, the activation of pro-MMP-2 is known to occur most efficiently at the cell surface (5). Recent work from a number of laboratories has identified a subfamily of MMPs (the membrane-type or MT-MMPs, comprising MMP-14, MMP-15, MMP-16, and MMP-17) that are distinct from the secreted enzymes in being type I membrane proteins (the N terminus containing the catalytic domain is extracellular) with a single membrane-spanning region (6–9). In addition, and in common with stromelysin-3 (MMP-11), the MT-MMPs have the potential to be activated intracellularly by furin or furin-like enzymes (6–9). MT1-MMP or MMP-14 has been shown to participate in cell surface activation of MMP-2 (6, 10, 11). The activation complex is trimolecular and in addition to MMP-14 and pro-MMP-2, it contains tissue inhibitor of metalloproteinases-2 (Timp2) (12, 13), but the precise geometry of this complex is not fully understood. Our understanding of the biochemical and biological function of the MT-MMPs and of the genes encoding them is incomplete. The gene structure of an MT-MMP has not been previously described although the structures of the genes encoding a number of “secreted” MMPs is known (14–22).

Given the unique structural characteristics of the MMP-14 protein, we sought to examine how its gene structure may have evolved to include new structural features and to evaluate its relationship with the known MMP genes. We show here that the structure of Mmp14 represents a significant departure from the structure of the previously described MMP genes and may be representative of a genetically distinct subfamily. Based on these observations and the previous descriptions of a number of MMP gene structures, we have proposed here a genetic classification of MMPs based on structural and evolutionary relationships that is independent of substrate preferences. The human MT-MMP genes are located on separate chromosomes (23, 24) and are not part of the MMP gene cluster on chromosome 11q22.3 (25), suggesting an evolution distinct from this cluster of genes. In this paper we describe the mapping of the mouse MMP-14 gene (Mmp14) to mouse chromosome 14.

2 MMP-14 and TIMP-2 denote proteins, Mmp14 and Timp2 denote the human genes, and Mmp14 and Timp2 denote mouse genes. MT1-MMP is used as an alternative to MMP-14 (in preference to the term MT-MMP1, see Ref. 9).
We reasoned that the proposed molecular model for activation of pro-gelatinase A was likely to be most relevant in situ at locations where MMP-14 and TIMP-2 are produced by the same or adjacent cell populations. We demonstrate here that Mmp14 and Timp2 appear to be exquisitely co-regulated during development, suggesting a possible role in the activation of pro MMP-2 at the sites of expression. Like the recent results of Kinoh et al. (26) our data suggest an important role in musculoskeletal development. We find, however, that there are differences between the expression of these two genes in bone depending on the mechanism of ossification. We have also discovered a consistent relationship between perichondrial expression of these genes and chondrocyte differentiation, which may have implications for skeletal development. We present novel observations of significant co-expression of Mmp14 and Timp2 in other organ systems. We find coordinate expression in the media of large arteries and in specific regions of the urogenital tract. Finally, we have found very prominent expression in a number of dense connective tissues such as tendons, ligaments, fascial sheaths, and joint capsules. The MMP-14 gene is, therefore, not just distinct in its gene structure but appears to be the only MMP thus far that is stringently co-expressed with a TIMP. From our developmental studies we infer that a shared mechanism of regulation exists for Mmp14 and Timp2.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Mmp14 Genomic Clones—Mmp14 probes were amplified by PCR from a human placenta cDNA library (CLONTECH) using primers based on the published sequence of Sato et al. (6). A 420-base pair cDNA from the 5'-half of the cDNA (including the pro-domain encoding region, clone pHTM420) and a 450-base pair cDNA from the 3'-half (including the putative transmembrane domain encoding region, clone pHTM450) were amplified using primer pairs (forward primer 5'-AGGCTGCTACACGGAAT-3' and reverse primer 5'-CGGCCGCTTCATGGA-3' and forward primer 5'-CGGTTTACACAGGACAGC-3' and reverse primer 5'-TCAGACCTTGTCAGCAGC-3', respectively). An Mmp14 cDNA was amplified with the sense primer 5'-AGGGTCCGCTTCATGGA-3' and antisense primer 5'-ACAGCGGTGCGACCTACA-3' based on published sequences (27) and cloned in both sense (p1011S) and antisense (clone p1011AS) orientations into pT7Blue (Invitrogen Corp.)

The Mmp14 clones were random primer labeled with [32P]dCTP and were used to screen a mouse genomic library from the 129sv strain in the AFIXIV vector (Stratagene). 3 × 10^6 plaques were screened. Phage DNA was isolated for further analysis from two positive clones pTm6a and pTm6b. Restriction mapping and Southern analysis with the above probes was used to identify putative exon-containing fragments. Suitable fragments were subcloned into pBluescript SK+ (Stratagene) for further characterization, including partial sequence analysis. Sequencing of both strands in selected regions was carried out using the chain termination method with internal or flanking primers. Sequence data were analyzed using the Lasergene software package (DNASTAR, Inc.). Exon-intron boundaries were assigned based on a comparison of the sequences of the Mmp14 cDNA and genomic sequences. While exon sizes were obtained directly from nucleotide sequences, introns were only partially sequenced, and in most cases intron size was determined by a combination of restriction mapping and PCR.

Chromosomal Mapping of Mmp14—Oligonucleotides primers 5'-TTCCGCTCCAGCTCTCG-3' (forward) and 5'-TGACATTACAGGTGTGC-3' (reverse) were designed to amplify a dinucleotide microsatellite repeat sequence in Mmp14 to test for SSLPs between mouse strains. Oligonucleotides were radiolabeled with [32P]ATP using polynucleotide kinase and genomic DNA from a series of mouse strains and were amplified using standard protocols (anneal at 55 °C for 1 min, extend at 72 °C for 2 min, and denature at 94 °C for 1 min for 40 cycles, with a final extension at 72 °C). 2 μl of the amplified reaction products were mixed with 0.5 μl U. S. Biochemical Corp. stop solution, denatured at 94 °C for 5 min, and immediately placed on ice. Two μl of this mixture was loaded on a 6% denaturing acrylamide sequencing gel and electrophoresed. We identified a polymorphism between C57BL/6J and DBA/2J mouse strains; DNA from the BXD recombinant inbred mouse strains was obtained from the Jackson Laboratory and genotyped. The strain distribution pattern was analyzed using the Map Manager program (28), and a map position was determined. Strain distribution data and human homologies were determined using the Mouse Genome Data base.3

Northern Analysis—Northern blots containing mRNA from embryos and a variety of adult mouse tissues (CLONTECH Inc.) were hybridized to the 32P-end labeled probes for Mmp14 (p1011AS), Timp2 (pmouseTIMP-2PCR, provided by Drs. Kevin Leco and Dylan Edwards, University of Calgary; Ref. 29) and β-actin (CLONTECH).

In Situ Hybridization—Riboprobes labeled with digoxigenin-11-UTP were generated with T7, T3, or SP6 RNA polymerases using reagents from Boehringer Mannheim. Templates used for transcription were p1011AS and p1011S (for Mmp14), pmouseTimp2-2PCR (for Timp2), and pmF0471 (for col2a1, the mouse α1II collagen cDNA; this probe was used for quality control of tissue sections and to identify sites of cartilage formation). Cryostat sections of mouse embryos obtained by timed pregnancies were used for expression studies. The morning of observation of the vaginal plug was taken to be day 0.5 post-coitum (p.c.), and embryos were obtained at days 7.5, 9.5, 10.5, 11.5, 12.5, 14.5, and 17.5 days p.c. Briefly, embryos were fix ed in 4% paraformaldehyde at 4 °C overnight, then immersed in 20% sucrose overnight, and embedded in OCT compound. 8 μm thick sections (cut in the sagittal plane at all stages but also in the horizontal plane in 14.5 and 17.5 days p.c. embryos) were hydrolyzed with 0.2 × HCl, digested briefly with proteinase K (10 μg/ml), acetylated, and hybridized to riboprobes at 53 °C for 16 h. Stringency washes were carried out as follows at 56 °C. Sections were washed briefly in 5 × SSC, then in 2 × SSC + 50% formamide, followed by 2 × SSC and 0.2 × SSC. Unbound riboprobe was destroyed by treatment with RNase A (10 μg/ml). Hybridized probe was detected using an alkaline phosphatase-conjugated anti-digoxigenin monoclonal antibody (Boehringer Mannheim) and NBT-BCIP color substrate mix (Boehringer-Mannheim). Endogenous alkaline phosphatase was inhibited by inclusion of levamisole (Sigma) to a final concentration of 1 μM in the substrate mix. Sections were counter stained with 0.1% (w/v) methyl green (Sigma).

RESULTS

Structure of Mmp14

The structure and degree of overlap of two independent clones containing Mmp14 is illustrated in Fig. 1a. A 7.5-kb BamHI-SalI fragment (the SalI site is in the AFIXIV vector and flanks the cloning site) from αXM6a was selected for more detailed analysis, because it appeared to contain most of Mmp14, as well as a 4.5-kb BamHI fragment that was found to contain the 5'-end of the gene (Fig. 1a). The Mmp14 polypeptide was found to be encoded within 10 kb of DNA, the 7.5-kb BamHI-SalI fragment containing 9 of these (Fig. 1, a and b). We determined the sequences of these exons and their boundaries with the flanking introns (Table I). We have tentatively numbered the first protein coding exon as exon 1 because the 5'-end of the Mmp14 transcript has not been identified. The derivation of peptide domains from each exon is shown in Fig. 1c. On comparison with other Mmp genes, Mmp14 was found to have a distinct structure (Fig. 1d). The novel C-terminal peptide domains of Mmp14 are encoded by a single large exon that also encodes the 3'-untranslated region. The structure of the exons encoding the catalytic domain and pro-domain of Mmp14 is distinct from all previously described MMP genes. In particular, exon 4 is larger than the corresponding exon of other MMP genes and encodes most of the catalytic domain. The exons encoding the hemopexin-like domains comprise the most conserved part of this gene.

Chromosomal Mapping of Mmp14

Two simple sequence repeats were identified in Mmp14 (Fig. 1a). A (GAA)12 repeat was identified within αXM6a in a region corresponding to the 3'-untranslated region of the Mmp14 mRNA (27). A (CA)12 repeat was found in intron 5. Primers corresponding to the latter microsatellite sequence identified an SSLP between inbred mouse strains. The BXD recombinant inbred series (30) was genotyped, and the strain

3 The internet site is http://www.informatics.jax.org/mgd.html.
Expression of Mmp14 and Timp2 during Mouse Embryogenesis

Northern Analysis—RNA from embryos showed Mmp14 and Timp2 transcripts at 7, 11, 15, and 17 days p.c. (Fig. 2). While Mmp14 message increased over these time points, Timp2 transcript levels were constant except for a decrease at 11 days. The transcripts were also present in a variety of adult organs (not shown). Mmp14 and Timp2 generated transcripts of about 4.5 kb and 4.0 + 1.2 kb in size, respectively, both in adult and embryonic tissues in agreement with previous reports (26, 29).

In Situ Hybridization—Sense probes for Mmp14 and Timp2 did not hybridize to sections (Figs. 3C, 4B, and 5B). Sections in which either probe was omitted did not show the presence of endogenous alkaline phosphatase activity. The col2a1 probe hybridized specifically to chondrocytes (not shown).

At 7.5 (late implantation stage), 9.5, and 10.5 days p.c., localized expression of Mmp14 and Timp2 was not seen in embryos. However, there was distinct expression of both of these genes in the uterus, particularly in the endometrial glands and in myometrium at the periphery of the uterus (not shown).

At 12.5 days p.c., Mmp14 and Timp2 were prominently expressed in large arteries, e.g., the aorta, its branches, and the umbilical arteries (Figs. 3 and 7A). Low level labeling was visible in the myocardium, but the cardiac outflow tract, particularly the root of the aorta (including the prospective semilunar valves), was prominently labeled (Fig. 3, A and B). The expression appeared in the smooth muscle cells of the prospective arterial tunica media. The vascular endothelium was negative for expression of both genes (Figs. 3, D and E, and 7A). At this age, diffuse expression of Mmp14 and Timp2 was visible throughout the craniofacial mesenchyme and was higher in mesenchymal cells surrounding the cartilage condensations for Meckel's cartilage and the clavicle. Expression of Mmp14 and Timp2 was seen in the nasal epithelium and the liver capsule (not shown) and in differentiating mesenchyme in the vicinity of the prospective kidneys (Fig. 3D).

At 14.5 and 17.5 days p.c., endochondral ossification was established so that chondrocyte hypertrophy as well as the formation of primary centers of ossification is apparent. At these developmental stages, Mmp14 and Timp2 were expressed...
strongly in the musculoskeletal system (Figs. 4-6), with continued expression in the arterial tunica media (Fig. 7 A) and additional expression in the urogenital tract (Fig. 7, A and B). There was prominent expression in the perichondrium-investing hypertrophic chondrocytes but lower levels in perichondrium around immature (small) chondrocytes. Bones that were sectioned longitudinally showed that perichondrial labeling achieved a peak at the point of transition of chondrocytes from small to hypertrophic cells. (Figs. 4 A and 5 A). We found negligible labeling in chondrocytes at these developmental stages. In contrast, col2a1 expression was seen in cartilage alone at 12.5, 14.5, and 17.5 days of gestation (not shown).

TABLE I

The junctions of the Mmp14 exons and introns

| EXON | Intron-EXON-intron junctions | Exon size | Intron sizea |
|------|------------------------------|-----------|--------------|
| 1b   | ..............CCC GAaagtgaagctctcccg | >280      | 2.6          |
| 2    | TGG ............ATG ATgtaagttctggctccca | 149       | 0.4          |
| 3    | GCC ATG ............ACT TTC TGgtgatctctcag | 123       | 0.6          |
| 4    | ATT CAG ............CTA AAT Ggtgataagggagacc | 308       | 0.8          |
| 5    | AAT ............CTT TAT Ggcgagttaaccatcc | 162       | 0.8          |
| 6    | AGC AAG ............TTT AAGtgagaggaagaagt | 161       | 0.3          |
| 7    | TGG ............TTT AAA Ggtgaggaagaga | 139       | 0.25         |
| 8    | AGT AAG ............GGG AAT AAGtgaagcttcagct | 148       | 0.5          |
| 9    | TGG ............TTT GAA Ggtgaggaagacaag | 116       | 0.325        |
| 10c  | TCC ACA ............TTT ACA | >1000     |              |

a Except for intron 9, the introns have not been completely sequenced. Intron size has been determined by a combination of PCR and restriction enzyme analysis and is given in kilobase pairs.

b The 5'-end of exon 1 has not been determined. Therefore, the precise size of exon 1 is presently unknown.

c Since a complete MMP-14 cDNA has not been reported, the 3'-end of exon 10 and its size are presently unknown.

FIG. 2. Northern analysis of Mmp14 and Timp2 expression in the developing mouse embryo. Embryo age in days is indicated above each lane. RNA molecular weight markers are indicated to the right of each autoradiogram. The panel at the bottom of the figure shows hybridization to a β-actin probe as an indicator of RNA loading.

FIG. 3. Mmp14 (A, D, and E) and Timp2 (B) are expressed in the media of muscular arteries. A-C, show the cardiac outflow tract at 12.5 days p.c. Note hybridization of the aorta (a) but not of the heart (h) in panels A (Mmp14) and B (Timp2). C, shows the cardiac outflow region hybridized to the Mmp14 sense probe. D, abdominal aorta (a) at 12.5 days p.c. hybridized to Mmp14 antisense probe. Note labeling in the media (see enlarged box alongside at upper right) and the absence of labeled cells in the endothelial layer. v, vertebral column; m, posterior abdominal mesenchyme. E, umbilical artery at 17.5 days p.c. hybridized to Mmp14 antisense probe. Boxed area is enlarged alongside (shown at lower left of figure) showing that labeling is restricted to the media. Arrowheads in panels D and E indicate the endothelium.
MMP-14 Gene Structure and Expression

**DISCUSSION**

**Structure of Mmp14 and Relationship to Other MMP Genes—**

The discovery of the MT-MMPs revealed the existence of a distinct subfamily of proteins within the MMP family. These proteins have some distinct and novel features. First, like stromelysin-3, but unlike other MMPs, they have a sequence insertion between the pro and catalytic domains that may be cleaved by furin or furin-like enzymes and may lead to intracellular activation. Second, unlike any other MMPs previously described, they have a membrane-spanning domain near their C terminus followed by a short cytosolic domain. These novel domains are connected to the hemopexin domains by a linker.

**Fig. 4. Mmp14 expression in the developing humerus and shoulder joint at 17.5 days p.c.** A, antisense-probe. The metaphysis of the primary ossification center is indicated by m. Proliferating (small) chondrocytes and hypertrophic zones of cartilage are indicated as p and h, respectively. h, respectively. b indicates bone formation in the acromion process; note labeled cells around periphery of bone. Arrow indicates the hybridization in the perichondrial waist. The asterisk indicates the insertion of the rotator cuff tendon. Labeled cells are seen in the metaphysis adjacent to the hypertrophic zone. The metaphyseal-cartilage junction has been enlarged and is displayed alongside (upper right of figure). Note that labeled cells are in the metaphysis at the site of hypertrophic cartilage resorption. B, corresponding section hybridized to Mmp14 sense probe.

**Fig. 5. Timp2 expression in the developing leg at 17.5 days p.c.** A, hybridization of antisense Timp2 probe. Labeling is seen in the perichondrial waist (arrow) and musculotendinous structures in the leg (asterisks). Note that cells in the metaphysis (m) do not show labeling (compare with Fig. 4A). Note also labeling in skin at upper right of figure. B, corresponding section hybridized to sense Timp2 probe.

Mmp14, but not Mmp14, was highly expressed in the trigeminal (Fig. 1D), vestibulocochlear, and dorsal root ganglia (Fig. 7C).

Both Mmp14 and Timp2 were expressed in developing muscle but in very discrete regions of a given muscle that did not correlate with definite anatomical features. Strong expression, approximating that seen in the perichondrium was found in developing tendons, ligaments, fascial sheaths between muscle compartments, and joint capsules throughout the 14.5 and 17.5 days p.c. embryo (Figs. 4–6). Very prominent labeling was found in the cruciate ligaments of the knee (Fig. 6A and B) and tendons around the shoulder joint. (Fig. 4A). Strong labeling was noted in joint capsule of the hip (not shown) and in the superficial zone of articular cartilage in the knee, shoulder, and hip joints (Figs. 4 and 6).

At 14.5 and 17.5 days p.c., Mmp14 and Timp2 expression was noted in the wall of the developing urinary bladder, particularly in the mesenchyme (developing musculature) of its outer half. Mesenchymal cells around the bulbourethral gland and the urethra (Fig. 7B) and cells in the bulbospongious and corpora cavernosa of the developing penis (not shown) also showed prominent expression of both genes in similar locations. In placenta, at 12.5 and 14.5 days, the Timp2 and Mmp14 transcripts co-localized to the junctional region between uterine deciduum and placental spongiotrophoblast with minimal labeling in the labyrinth (vascular) zone of the placenta (Fig. 8).

**Fig. 6. Mmp14 and Timp2 are expressed in developing synovial joints.** Shown are knee joints of 17.5 days p.c. embryos hybridized to an Mmp14 (A) or Timp2 (B) antisense probe. Hybridization is seen with cells of the posterior cruciate ligament (PCL), anterior cruciate ligament (ACL), and patellar tendon (PT). Note labeling of cells in superficial layers of articular cartilage in both femur (f) and tibia (t) and the absence of labeled cells in cartilage elsewhere.

**Fig. 7. Hybridization of Mmp14 probe during urogenital development.** A, transverse section through the developing urinary bladder (b) at 14.5 days p.c. showing labeled cells in the muscular wall (the lumen of the urogenital sinus is not seen in this section). Note also labeling of umbilical arteries (a) B, labeled cells are seen in and around the bulbourethral gland (b) adjacent to the bulb urethra (u). C and D, expression of Timp2 in ganglia of the 17.5 day mouse embryo. C, dorsal root ganglia (g); D, trigeminal ganglion.

**Fig. 8. Expression of Mmp14 (A) and Timp2 (B) at the placental implantation site (12.5 days p.c.).** Similar regions at the periphery of the implantation site are shown. m, myometrium; p, placenta (labyrinthine portion). The labeling appears in the junctional area that includes the decidual reaction of the uterus and spongiotrophoblast of the placenta.
region of variable length and sequence in each MT-MMP. The linker region of MT1-MMP is homologous to the calcium-binding region of troponin C.

In view of these peculiarities, we felt that the evolutionary origin of the MT-MMPs was probably significantly different from the secreted enzymes and that determination of the gene structure would be critical in understanding the manner in which the new domains of this protein had been assembled. Previous studies have established the gene structure of many of the secreted MMPs (14–21), most recently that for collagenase-3 (20). Our data show that the mouse gene encoding MMP-14 varies from all previously described MMP genes in the structure of the 5′-half and that the new domains at the C terminus are not added by modular assembly of new exons. This is in contrast to the genes encoding the gelatinases (MMP-2 and MMP-9) (15, 17) and matrilysin (MMP-7) (16) where peptide domains are added or omitted by introducing or deleting exons, respectively. However, despite this variation, the MMP-14 gene retains the traditional number of protein-coding exons (ten) seen in most MMP genes.

The present data suggests that the MMP-14 gene diverged early in the evolution of the MMP genes because it has few of the distinctive features shared by the chromosome 11 cluster genes (20). The MMP-2 (15) and MMP-9 (17) genes bear a greater similarity to the chromosome 11 cluster genes (if one discounts the modular exons) than to the stromelysin-3 gene (19) or the MMP-14 gene. We propose that the MMP-14, stromelysin-3, and chromosome 11 cluster genes represent distinct evolutionary branches. This proposal on the origin of diversity in the MMP gene family is corroborated by recent analyses of MMP peptide and domain composition (31). The origin of the new protein domains of MMP-14 is probably not by exon shuffling as in the MMP-2 and MMP-9 genes. It may be the result of an insertion, a translocation, or the consequence of a frameshift mutation in an ancestral MMP gene. Our preliminary studies using PCR have suggested that the structure of the exon encoding C-terminal domains is similar in MMP-15 and MMP-16. It remains to be established whether truncated or soluble MMPs can be derived from the MT-MMP genes using alternative exons or splicing variants. Our studies have not addressed this possibility. Although the human and mouse MMP-14 genes (6, 26, and this paper) have been found to generate a single transcript, we have found that Mmp14 can generate a shorter transcript (1.2 kb)4 that cannot encode the full-length MMP-16 cDNA sequence previously published (8) and might encode a short form of MMP-16.

Location of Mmp14—The region of mouse chromosome 14 containing Mmp14 is a gene-rich region that also includes the phenotypic mutations gm (gunmetal, a coat color and platelet disorder), hph1 (hyperphenylalanemia, a metabolic disorder), and pn (pugnose, a skeletal abnormality). The human homolog, MMP14, has recently been shown to map to the corresponding region of the human genome (23, 24). pn (32) is a recessive mutation whose phenotype involves connective tissue and suggests Mmp14 as a possible candidate. Unfortunately, this mouse strain has not been maintained and may be extinct.

A Proposed Classification of MMP Genes—We have attempted to find similarities in structure of the various MMP genes to clarify the relationships between the different MMPs. We propose that the presently known MMP genes be divided into four distinct sub-families based on gene structure. Fig. 1d has a comparison of gene structures from each of the four proposed subfamilies. A classification system based on domain and gene structure is in use for the collagen superfamily of genes, and it is clear that genetically related members have structural and functional correlates (33). Given the increasing number of MMPs, mostly discovered by molecular cloning, this classification may be valuable in accommodating new MMPs on a genetic basis.

Group 1 consists of the collagenase subfamily/chromosome 11 sub-family (20), which includes MMP-1 (Fig. 1d), MMP-3, MMP-7, MMP-8, MMP-10, MMP-12, and MMP-13. These MMPs have a highly conserved gene structure with an identical number of exons and similar peptide domains derived from exons of similar size (Fig. 1d). The matrilysin gene (MMP-7) is an exception in that it lacks the exons encoding the hemopexin-like region but is otherwise similar to other group 1 genes. It is noteworthy that all these genes are located in the human chromosome 11q22.3 cluster (20, 25) and may have arisen by duplication and modification of an ancestral gene on this chromosome as previously proposed (20, 25).

Group 2 consists of the gelatinases/modular alterations subfamily, which includes MMP-2 (Fig. 1d) and MMP-9. The catalytic domains are encoded by exons similar to those of group 1. Three additional peptide domains with homology to fibronectin type II repeats are derived from new exons arranged in tandem in both MMP-2 (Fig. 1d) and MMP-9. In addition, exon 9 of MMP-9 (corresponding to exon 6 of the collagenase group) has an extension at its 5′-end encoding a collagen V-like peptide, which is part of the hinge region of MMP-9.

Group 3 consists of the variant hemopexin exon sub-family, which includes MMP-11 (Fig. 1d). The stromelysin-3 gene is similar to the first two groups in its 5′-half (encoding the catalytic domain) but is significantly different in the exons encoding the hemopexin-like domains.

Group 4 consists of the variant catalytic exon sub-family, which includes MMP-14 (Fig. 1d) possibly includes MMP-15, 16, and 17. The MMP-14 gene is similar to those of group 1 and group 2 in the exons encoding the hemopexin domains. However, the exons encoding the catalytic domain and pro-domain have a unique structure. Another unique feature of the gene is a single large exon at the 3′-end that encodes novel domains that are absent in proteins derived from genes of the first three groups.

Co-expression of the Mmp14 and Tmp2 Genes—We sought in situ corroboration of the physiological relevance of biochemical studies demonstrating that TIMP-2 and MMP-14 formed a complex for activation of gelatinase A. We rationalized that the two proteins must be synthesized by the same cells or in close proximity to each other to be able to form gelatinase A activating complexes with high efficiency. To identify the specific cell types or tissues expressing the Mmp14 and Tmp2 genes, we used non-isotopic in situ hybridization. While Northern analysis showed expression of both genes at days 7 and 11 of development, we were unable to discern localized expression by in situ hybridization of embryo sections at these stages. This discrepancy may have resulted from the relative lack of sensitivity of non-radioactive in situ hybridization as well as the possible existence of a more generalized expression at these stages.

Detailed previous studies have demonstrated that MMP-2 is widely and intensely expressed in mesenchymal tissues during mouse development, suggesting widespread presence of gelatinase A (34), and for this reason we have not repeated in situ hybridization with this probe. Kinoh et al. (26) have described prominent expression of Mmp14 and Tmp2 in bone and peri-chondrium during skeletogenesis. We recommend this article for a detailed description of expression in ossifying tissues. Our results provide novel information that supports an additional role for MMP-14 and TIMP-2 in development of the cardiovas-

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4 S. S. Apte, unpublished data.
cular and urogenital systems and in placental implantation. Our detailed analysis of gene expression during skeletal development suggests that both genes are regulated in the perichondrium in concert with differentiation of the underlying chondrocytes from a proliferating (small) to a hypertrophic population. At this transition point, long bones typically demonstrate a “waist” in which the perichondrium is thicker and more cellular. Recent data has demonstrated this region of the perichondrium to be critically important for the regulation of chondrocyte growth and differentiation by Indian hedgehog and parathyroid hormone-related peptide (35).

We noted differences in gene expression depending on the mechanism of ossification. In bones that are formed in membrane such as the mandible and maxilla, both Mmp14 and Timp2 were expressed in cells on the surface of newly formed bone as well as by mesenchymal cells in the immediate vicinity. In bones that form by endochondral ossification, however, such as long bones in the limb, we found a distinct difference between Mmp14 and Timp2 expression (compare Figs. 4 and 5). Timp2 was not expressed at the site of cartilage resorption in the metaphysis, while Mmp14 was very prominent at this location. Intriguingly, the expression of Mmp14 in this location coincided with that previously described for Mmp-9 (progelatinase B), which is expressed by osteoclasts (36). A recent report confirms that Mmp14 is expressed by rabbit osteoclasts as well as other cells in bone (37) and is not restricted to osteoclasts as previously suggested (26).

We report for the first time prominent co-expression of Mmp14 and Timp2 in tendons, ligaments, muscle, and joint capsules. The ECM of these tissues is principally composed of fibrillar collagens, in particular types I and III collagens. A recent report demonstrated that MMP-14 is a collagenase and gelatinase (38). Together, these observations suggest that MMP-14 may be involved in the remodeling of dense connective tissues on the manuscript.

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