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Murine Matrix Metalloproteinase 9 Gene

5′-UPSTREAM REGION CONTAINS CIS-ACTING ELEMENTS FOR EXPRESSION IN OSTEOCLASTS AND MIGRATING KERATINOCYTES IN TRANSGENIC MICE*

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Carine Munaut‡§, Tuire Salonurmi§¶, Sirpa Kontusaari¶, Paula Reponen¶, Takako Morita‖, Jean-Michel Foidart‡, and Karl Tryggvason¶‖**

From the ‡Department of Biology, University of Liege, B-4000 Liege, Belgium, ¶Biocenter and Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland, and the ‖Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden

Knowledge about the regulation of cell lineage-specific expression of extracellular matrix metalloproteinases is limited. In the present work, the murine matrix metalloproteinase 9 (MMP-9) gene was shown to contain 13 exons, and the 2.8-kilobase pair upstream region was found to contain several common promoter elements including a TATA box-like motif, three GC boxes, four AP-1-like binding sites, an AP-2 site, and three PEA3 consensus sequences that may be important for basic activity of the gene. In order to identify cell-specific regulatory elements, constructs containing varying lengths of the upstream region in front of a *LacZ* reporter gene were made and studied for expression in transgenic mice generated by microinjection into fertilized oocytes. Analyses of the mice revealed that the presence of sequences between −2722 and −7745 allowed for expression in osteoclasts and migrating keratinocytes, *i.e.* cells that have been shown to normally express the enzyme *in vivo*. The results represent the first *in vivo* demonstration of the location of cell-specific control elements in a matrix metalloproteinase gene and show that element(s) regulating most cell-specific activities of 92-kDa type collagenase are located in the −2722 to −7745 base pair region.

Mammalian extracellular matrix metalloproteinases (MMPs)[1] form a family of related enzymes that are capable of degrading various components of the connective tissue (1–3). These proteases are either secreted or membrane-bound and are produced as latent enzymes. They have a conserved Zn^{2+} binding catalytic site and can be inhibited by specific tissue inhibitors of metalloproteinases (4–7). *In vitro* studies imply diverse substrates and functions for these enzymes *in vivo*. Several genetically distinct enzymes have been identified. Based on *in vitro* substrate specificities, they are placed in different categories: interstitial collagenases that degrade fibrillar collagens (8–10); the stromelysins (11–13) with activity against several noncollagenous proteins and collagens with interrupted triple helices; matrilysin that degrades fibronectin, laminin, casein, gelatin, and proteoglycans (2); macrophage metalloelastase, which degrades elastin (15, 16); MMP-2 and MMP-9, which cleave type IV collagen and gelatin (17–21), respectively; and finally a recently described group of the membrane type of matrix metalloproteinases that can activate other metalloproteinases and also degrade matrix proteins (22–24).

The metalloproteinases are believed to have an important role in normal turnover of extracellular matrix and in remodeling of tissues. Furthermore, they have been shown to be highly expressed in areas of inflammation and tumor invasion. However, the specific roles of the various metalloproteinases *in vivo* as well as regulation of their genes are still largely unexplored. MMPs 2 and 9, which form a distinct subgroup of MMPs, based on their primary structure and substrate specificity, have been shown to have high activity against gelatin, and they also degrade type IV, V, and VII collagens. However, they do not show high activity against type I collagen, proteoglycan, or laminin. Despite the apparently identical substrate specificity, their temporal and spatial expression *in vivo* varies extensively, indicating that the two enzymes are required for different purposes. This is also emphasized by the different expression patterns of the two genes (21). Thus, MMP-2 is primarily expressed in stromal fibroblast-like cells during mouse development (25), while its expression is insignificant in the stroma of adult mice. This suggests that MMP-2 has a major role in the remodeling of the stromal compartment, in addition to the proposed role in the turnover of basement membrane type IV collagen. In fact, it is possible that the enzyme mainly functions as a stromal gelatinase by removing gelatin derived from degraded fibrillar collagens. In invasive tumors, intense expression of MMP-2 is observed in stromal cells adjacent to the tumor front, not in the tumor cells themselves (26–29).

MMP-9 has a completely different expression pattern. By *in situ* hybridization analysis, its expression has been shown to be almost completely confined to osteoclasts at the site of bone formation during mouse development (30). This is the only known example of a highly osteoclast-specific proteinase, which indicates that the enzyme is important for the turnover of bone matrix, possibly as a gelatinase required for the removal of denatured collagen fragments (gelatin) generated by interstitial collagenases. MMP-9 expression has also been localized to keratinocytes of healing skin wound (31), macrophages (27, 28, 29, 32), and trophoblasts of the implanting embryo (33, 34).

We have previously shown that no cell-specific expression

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§ These two authors contributed equally to this work.

** To whom correspondence should be addressed: Division of Matrix Biology, Dept. of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden. Tel.: 46-8-728-7720; Fax: 46-8-31-61-65; E-mail: karl.tryggvason@mbb.ki.se.

[1] The abbreviations used are: MMP, matrix metalloproteinase; X-gal, 5-bromo-4-chloro-3-indolyl-β-galactopyronoside; kb, kilobase pair(s); bp, base pair(s).

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and regulation of the MMP-9 gene could be achieved through *in vitro* experiments using transiently transfected cells (35). Therefore, to examine the regulation of the MMP-9 gene and, in particular, the regulatory mechanisms of its tissue expression, we have cloned and characterized the mouse gene and studied its regulation using transgenic mice. In this study, we demonstrated that the minimum element(s) required for expression in osteoclasts and migrating keratinocytes reside in the 5′-flanking region of the gene between 2.7 and 7.7 kb upstream of the transcription start site.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of the Mouse MMP-9 Gene—Mouse genomic libraries cloned in the cosmid pWE15 (Stratagene, catalog no. 95303) and λ Fix phage (Stratagene, catalog no. 46309) were screened using a human MMP-9 cDNA probe (pHG1, Ref. 21) labeled with ^{32}P by random priming. Hybridization was performed in 5× SSC, 5× Denhardt's solution, and 0.1% SDS overnight at 42 °C, after which the filters were washed at a final concentration of 0.1× SSC and 0.1% SDS at 42 °C. The clones were isolated and purified utilizing standard procedures and mapped using restriction endonucleases.

DNA Sequencing—The nucleotide sequence was determined by the dideoxynucleotide chain termination procedure (36) using Sequenase or TAQuence DNA sequencing kits (U.S. Biochemical Corp.) and M13 universal primers or specific oligonucleotide primers. Both strands of the gene and its promoter were sequenced following subcloning into pBluescript II SK+/− (Stratagene).

Primer Extension—Total RNA from 7-day-old mouse skull was isolated by the acid guanidium thiocyanate/phenol chloroform extraction method (37). Primer extension was performed by hybridizing 20 μg of total RNA with an antisense nucleotide primer annealing at positions 117–144 in the cDNA (Ref. 30, Fig. 1). The primer was end-labeled by [γ-^{32}P]ATP using T4 polynucleotide kinase (38). The reverse transcription reaction was carried out under standard conditions, and the primer-extended products were run on a sequencing gel along with sequencing reactions from the mouse MMP-9 gene using the same oligonucleotide as in the primer extension assay.

Plasmid Constructs—Promoter-*LacZ* reporter gene constructs were created using the pKK2480 vector (kindly provided by Mikkel Rohde, University of Copenhagen, Denmark), which contains a multiple cloning site immediately upstream of the *LacZ* gene. Different length segments of the 5′-flanking region as well as the 5′-end of the MMP-9 gene containing the first exon and intron were excised with appropriate restriction enzymes and used for the construction of MMP-9/*LacZ* fusion genes.

Generation and Analysis of Transgenic Mice—Transgenic animals harboring promoter-reporter gene constructs were generated by injection of the linearized *LacZ* fusion constructs into pronuclei of fertilized mouse oocytes C57BL/6 × DBA/2 F1 (39). Microinjected eggs (15–20) were then transferred into the oviduct of pseudopregnant NMRI mice, and the mice were allowed to develop to term. At 3 weeks of age, tail DNA was isolated (40), and transgenic animals were identified by polymerase chain reaction analysis using two internal primers of the *LacZ* gene (41). Mouse embryos from positive mice were recovered at different time points and fixed for 2 h or overnight at 4 °C in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS. They were stained with 5-bromo-4-chloro-3-indolyl-β-galactopyronoside (X-gal) as described by Behringer *et al.* (42).

Histologic and Immunohistochemical Analyses—X-gal-stained mouse embryos and other tissues were rinsed several times in PBS, dehydrated, and embedded in paraffin. Sections of 5–8 μm were stained either by hematoxylin and eosin (43) or with safranin. Sections were stained for 2–5 min in 0.2% safranin, 1% acetic acid, differentiated for 1–5 s in 95% ethanol, 2–5 s in absolute ethanol, and mounted from xylene.

Immunohistochemical staining of paraffin sections (5–10 μm) from skin wounds was carried out by using either the ABComplex horseradish peroxidase kit (DAKO) or the TSA kit (NEN Life Science Products). Deparaffinized sections were treated 5 min. with 0.4% pepsin in 0.01 M HCl at 37 °C. Endogenous peroxidase activity was quenched by incubation for 20 min in 3% H_2O_2, and sections were then incubated with the antiserum raised against cytokeratin (rabbit anti-cytokeratin (Pan), Zymed Laboratories Inc.) for 1.5 h at room temperature. or MMP-9 (rabbit polyclonal antibody kindly provided by P. Carmeliet) overnight at room temperature. For the anti-cytokeratin immunostaining, the sections were washed in PBS, and subsequently, biotinylated Swine anti-rabbit IgG (Boehringer Mannheim) (1:400 dilution) was applied for 30 min at room temperature. After phosphate-buffered saline washes, a 20-min incubation with ABComplex/horseradish peroxidase was carried out (DAKO Code K355). Peroxidase activity was revealed by incubation with the chromogen substrate 3,3-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin and eosin. For the MMP-9 immunostaining, peroxidase swine anti-rabbit IgG (DAKO) was applied and followed by tyramide signal amplification (TSA kit; NEN Life Science Products). AEC chromogen substrate (DAKO) was used to detect peroxidase activity. Sections were counterstained with hematoxylin.

In Situ Hybridization—A mouse MMP-9 cDNA fragment of 324 bp containing *Sma*I and *Eco*RI restriction sites from the M92KD-2 cDNA clone (bases 1915–2239) (30) was subcloned into pSP64 ans pSP65 plasmid vectors (Promega). The pSP64 (sense) and pSP65 (antisense) plasmids vectors were linearized with *Eco*RI and *Bam*HI restriction enzymes, respectively, and the ^{35}S-uridine 5′-triphosphate (1000 mCi/nmol, Amersham Pharmacia Biotech) labeled RNA probes were transcribed using a transcription kit (Promega). The labeled probes were precipitated with ethanol, dissolved in hybridization buffer, and used at 50,000–60,000 cpm/μl. The *in situ* hybridization was carried out according to Wilkinson and Green (44). Prior to hybridization the mouse embryos were stained with X-gal, embedded in paraffin, and sectioned. The specimens were pretreated as described before (45) and hybridized with the probe at 5O °C for 16 h. After washing under high stringency conditions, the sections were dried and dipped in an autoradiographic emulsion (nitro blue tetrazolium-2; Eastman Kodak Co.), exposed for 14 days at 4 °C. After development of the sections, they were stained with hematoxylin and mounted.

RESULTS

Structure of the Gene and 5′-Flanking Region—Screening of the genomic libraries yielded several clones, one of which (cosmid MGC-1 (about 34 kilobases)), contained the entire 7.7-kilobase gene (Fig. 1), as well as 3 and 23 kilobases of the 5′- and 3′-end flanking regions, respectively. Another λ phage clone CM-1 contained about 11,000 bp of the 5′-flanking region. Sequencing of exons revealed that the murine gene contains 13 exons, which correspond in size to those of the human gene (Fig. 1, Table I). The only significant differences are exons 9 and 13 which, respectively, contain 54 and 15 base pairs more than in the human gene. Furthermore, introns 1, 2, 3, and 12 in the mouse gene are about half the size of corresponding human introns (21).

The initiation site for transcription as determined by primer extension revealed a double start site located 19 and 20 bp upstream of the translated sequence (Fig. 2). Sequencing of about 2800 base pairs of the 5′-flanking region revealed several common promoter elements (Fig. 1). There is a TATA box-like motif TTAAA at positions −30 to −25 but no CCAAT box. There are three GC boxes that may serve as binding sites for the transcription factor Sp1 (46) (located at positions −62 to −57, −451 to −446, and −598 to −589). Four AP-1-like binding sites were also identified (−50 to −44, −88 to −80, −472 to −465, and −1080 to −1072). Two of those correspond to similar sequences in the human gene, but sites corresponding to the first one (−50 to −44) and the most upstream one have not been reported in the human gene. Several conserved sequence elements with similarity to the polyoma virus enhancer A-binding protein-3 sites (47) were found in the 5′-flanking sequence (−365 to −360, −479 to −474, −658 to −653, and −901 to −896), as well as in the first intron (Fig. 1). One consensus sequence (5′-CCCCAGGC-3′) for AP-2 (−590 to −483), several microsatellite segments of alternating CA residues, as well as one NF-κB motif (−527 to −519) were also present. A putative tumor growth factor-β1-inhibitory element found in the human gene was absent in the murine promoter. During the course of this work, characterizations of the MMP-9 gene from mouse (48) and rabbit (49) were published.

Generation of Transgenic Mice—In order to explore the regulatory mechanisms of the MMP-9 gene, we generated trans-

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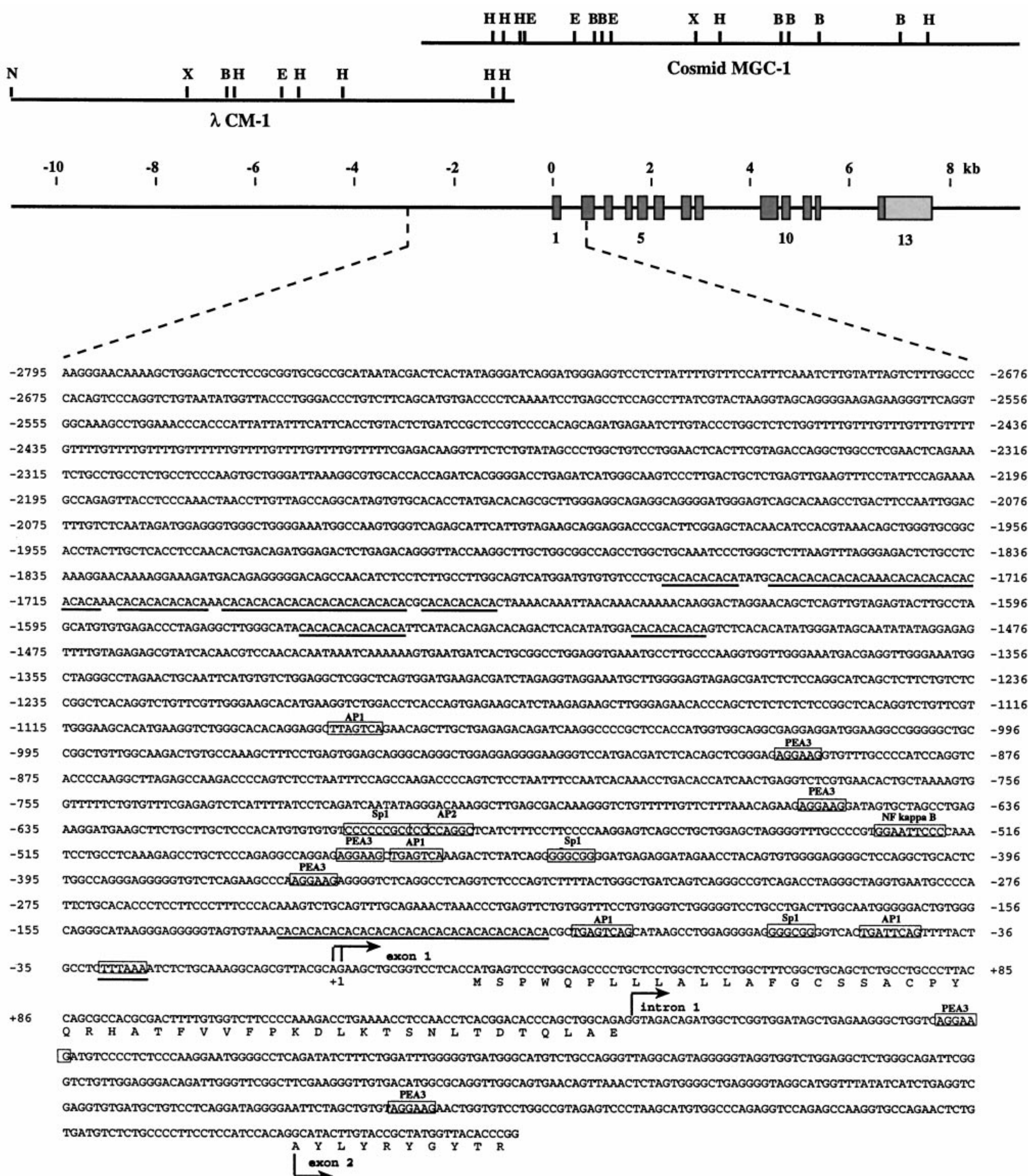

FIG. 1. **Structure of the mouse MMP-9 gene and sequence of the 5′-end.** *Top*, the entire gene and flanking regions were contained in the MGC-1 cosmid clone, which reached 23 kilobases downstream from the last exon. The CM-1 λ clone further provided about 11 kilobases of the 5′-flanking region. The restriction map was obtained using *Not*I (*N*), *Xho*I (*X*), *Bam*HI (*B*), *Eco*RI (*E*), and *Hin*dIII (*H*). *Middle*, the exons of the gene are depicted by *boxes*, numbered from the 5′-end, and the introns and flanking sequences are shown by a *solid line*. Scale in kilobases is shown. *Bottom*, nucleotide sequence of the 5′-end flanking region. The *bent arrow* indicates the transcription initiation site as determined by primer extension. The numbering of nucleotides starts at the transcription initiation site. The TATA motif, GC boxes, AP-1-like, AP2, polyoma virus enhancer A-binding protein-3, and NF-κB binding consensus sequences are *boxed*. Alternating CA-rich sequences are *underlined*.

genic mice by microinjection of different promoter-reporter gene constructs into fertilized oocytes. A total of six constructs containing different portions of the 5′-end of the gene and the β-galactosidase gene as a reporter were made (Fig. 3). Three constructs, 645-*LacZ*, 2700-*LacZ*, and 7700-*LacZ*, contained 0.65, 2.7, and 7.7 kb of the 5′-flanking region, and three constructs, 645ExIn-*LacZ*, 2700ExIn-*LacZ*, and 7700ExIn-*LacZ*, contained additionally the first exon and intron of the MMP-9 gene in front of the *LacZ* gene. The constructs containing exon 1 had a mutation in the ATG initiator codon for translation (ATG → ATC) to allow translation of the transcript to start from the ATG methionine initiator codon in the *LacZ* gene.

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TABLE I
Exon-intron junctions of the mouse gene for MMP-9

Nucleotide sequences at the intron (lowercase letters) and exons (uppercase letters) junctions are shown. The derived amino acid sequence and corresponding position in the polypeptide are displayed below. Amino acids encoded by split codons are listed twice and partially in parentheses. Asterisks indicate translation stop codon.

Exon/intron number	Exon-intron junctions	Exon size	Intron size
		bp	*bp*
1	.... ATG AGT CCC... ....CTG GCA GAG gtagacagat Met Ser Pro Leu Ala Glu 1 47	141 + 19	435
2	ccatccacag GCA TAC TTG... ....ATC ACA TAC TG gtgagatgtc Ala Tyr Leu Ile Thr Tyr Tr(p) 48 124	230	223
3	tcacctccag G ATC CAA AAC... ....GGT GTG GCG G gtgagaattc Tr(p) Ile Gln Asn Gly Val Ala Gl(u) 125 174	149	270[a]
4	cctcttgcag AG CAC GGA GAC... ....AAA GGC GTC G gtgagatcct Gl(u) His Gly Asp Lys Gly Val Va(l) 175 217	129	106
5	ctttctacag TG ATC CCC ACT... ....CCT AGT GAG A gtgagtatgc (Va)l Ile Pro Thr Pro Ser Glu Ar(g) 218 275	174	176
6	gtcaggtcag GA CTC TAC ACA... ....CCT ACC CGA G gtacctctgc (Ar)g Lys Tyr Thr Pro Thr Arg Va(l) 276 333	174	350
7	gtctctccag TG GAC GCG ACC... ....CCA GAC CAA G gtgagcgggg (Va)l Asp Ala Thr Pro Asp Gln Gl(y) 334 392	177	105
8	taccttccag CC TAC AGC CTG... ....TAT CTG TAT G gtgaggctgg (Gl)y Tyr Ser Leu Tyr Leu Tyr Gl(y) 393 444	156	1150[a]
9	ttgtttttag GT CGT GGC TCT... ....TTC AAG GAC GG gtaagcaggg (Gl)y Arg Gly Ser Phe Lys Asp Gl(y) 445 555	334	102
10	ttcttctcag T TGG TAC TGG... ....TTC TTC TCT G gttagtttgt (Gl)y Trp Tyr Trp Phe Phe Ser (Gl)y 556 602	140	270
11	tcttccgcag GA CGT CAA ATG... ....CGT GTC TGG AG gtaagagcga (Gl)y Arg Gln Met Arg Val Trp Ar(g) 603 652	151	103
12	ccttctgcag A TTC GAC TTG... ....CAG TAC CAA G gtgagggctg (Ar)g Phe Asp Leu Gln Tyr Gln As(p) 653 687	104	1125[a]
13	tttcccgtag AC AAA GCC TAT... ....CAG TGC CCT TGA (As)p Lys Ala Tyr Glu Cys Pro *** 688 730	131 + 943	3′-UTR[b]

[a] Size determined by polymerase chain reaction with specific oligonucleotides.
[b] Untranslated region.

Intron 1 was included in some of the construct, since it has been shown to contain enhancer elements in other genes such as that for the α1 chain of type I collagen (50). Three to eight lines of mice were generated with each construct to ensure that the expression pattern obtained with each construct was repeatable. Polymerase chain reaction and Southern analyses were carried out to establish integration of the inserts into the genome, and histochemical analyses with X-gal provided evidence about cell-specific expression patterns of the transgene.

Expression of MMP-9 Promoter/LacZ Reporter Constructs in Transgenic Mice—Transgenic mice were first generated with constructs containing the minimum promoter, 645-*LacZ*, and a longer one, 2700-*LacZ*, containing 2.7 kb of the upstream region. However, mice and mouse embryos made with these constructs did not yield any expression of the *LacZ* gene in cells that normally express MMP-9 in transgenic embryos or adult tissues. Ectopic expression could be observed in some lines with both constructs, but its pattern was neither uniform nor repeatable in four founder lines analyzed (data not shown). The addition of the 5′-end of the MMP-9 gene, including intron 1, to constructs 645-*LacZ* and 2700-*LacZ* (*i.e.* the 5′-untranslated region and the first exon and intron) did not alter the expression pattern. Consequently, it could be concluded that the first intron does not contain cis-acting elements conferring tissue-specific expression of the endogenous MMP-9 gene.

Due to the lack of expression with the constructs described above, we made another one, 7700-*LacZ*, containing 7.7 kb of the upstream sequence and generated transgenic mice. In contrast, to the shorter constructs, mouse embryos harboring 7700-*LacZ* revealed expression of the *LacZ* gene in bones of 14.5–16.5-day-old embryos. For example, at embryonic day 15.5, distinct expression could be observed in the scapula, long bones of fore and hind limbs, ribs, and the lower jaw (Fig. 4). Additionally, expression was observed in hair follicles in different mouse lines made with this construct. Construct 7700ExIn-*LacZ*, containing additionally the first exon and intron, yielded similar expression pattern in bones as 7700-*LacZ* when analyzed in whole X-gal-stained embryos, with the exception that no expression was present in hair follicles (Fig. 4).

The −2722 to −7745 Upstream Region of the MMP-9 Gene Confers Expression to Osteoclasts—Only mice made transgenic with constructs containing 7.7 kb of the 5′-flanking region of the MMP-9 gene yielded expression of the *LacZ* gene in bones as shown in whole embryos in Fig. 4. In order to assess the expression pattern at the cellular level, we carried out microscopic histochemical analysis, partially combined with *in situ* hybridization, to establish if the *LacZ* expression corresponds to that of the endogenous gene. In Fig. 5, *A* and *B*, staining with

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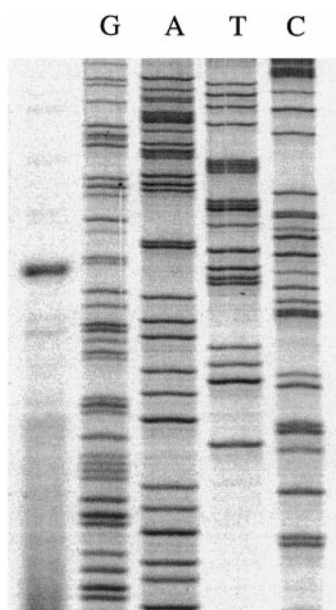

FIG. 2. **Determination of the transcription initiation site.** The mRNA start site was localized by primer extension analysis using poly(A) RNA from mouse skull as described under "Experimental Procedures." *Lanes 1–4*, results from a co-run of the sequencing reactions of cloned genomic DNA with guanidine (*G*), adenosine (*A*), thymidine (*T*), and cytosine (*C*) indicated at the *top*. The transcription start site is shown in *lane 5*.

X-gal shows expression of the transgene in single cells located at the site of endochondral ossification in the diaphysis of long bones, beneath hypertrophic chondrocytes of the epiphysis. This result is practically identical to what we have previously shown for the endogenous gene by *in situ* hybridization (30). In that report, we also assigned the endogenous gene expression specifically to cells that were shown to be osteoclasts by histochemical staining with tartrate-resistant acid phosphatase. In order to demonstrate that the cells expressing 7700-*LacZ* were indeed osteoclasts, we carried out *in situ* hybridization of X-gal-stained tissues, and these experiments showed the signals to be present in cells positive for the blue color produced by β-galactosidase (Fig. 5*C*). These experiments demonstrated that expression of the 7700-*LacZ* construct was confined to osteoclasts in developing bone. Transgenic mice harboring insert 7700ExIn-*LacZ* showed exactly the same expression pattern as construct 7700-*LacZ*, demonstrating that the upstream segment −2722 to −7745 includes the cis-regulatory element(s) required for osteoclast expression.

Localization of 7700-LacZ Expression to Migrating Keratinocytes—MMP-9 has been shown to be expressed in cultured keratinocytes (31), but our previous *in situ* hybridization studies in developing embryos and adult mice did not reveal expression of the gene in normally developing epithelia (30). However, we have shown by *in situ* hybridization that the MMP-9 gene is expressed in migrating keratinocytes of healing skin wounds, indicating a role for the enzyme in the repair process (28). To examine if any of the MMP-9 promoter-*LacZ* gene constructs

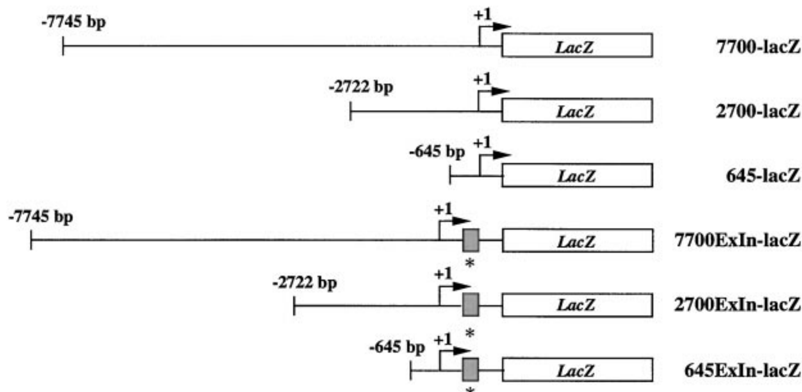

FIG. 3. **Schematic illustration of the MMP-9 promoter-*LacZ* reporter gene constructs.** The *numbers* on the constructs are the distance (in base pairs) from the transcription initiation site (+1). The three lowest constructs (7700ExIn-*LacZ*, 2700 ExIn-*LacZ*, and 645ExIn-*LacZ*) contain the first exon (141 bp) and intron (435 bp) of the MMP-9 gene. The *asterisk* in these constructs depicts the presence of a point mutation introduced to the ATG codon (ATG mutated to ATC), so that translation starts from the ATG codon in the *LacZ* gene.

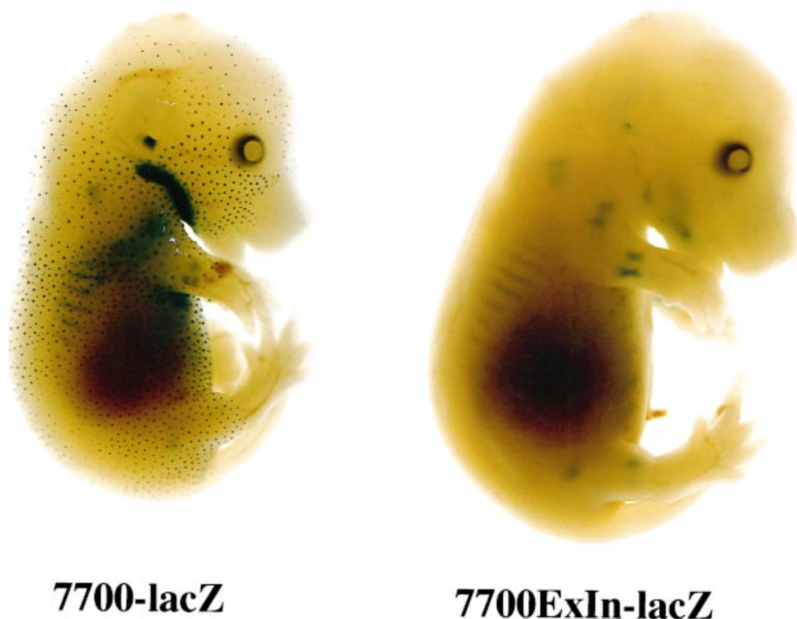

FIG. 4. **Expression of MMP-9 promoter/*LacZ* constructs in 15.5-day-old transgenic mouse embryos.** Embryos containing 7700-*LacZ* yield expression in the scapula, long bones, fore and hind limbs, ribs, and the lower jaw. Furthermore, strong expression is present in hair follicles. Embryos transgenic for construct 7700ExIn-*LacZ* reveal essentially the same expression pattern as 7700-*LacZ*, except that the expression in hair follicles is absent.

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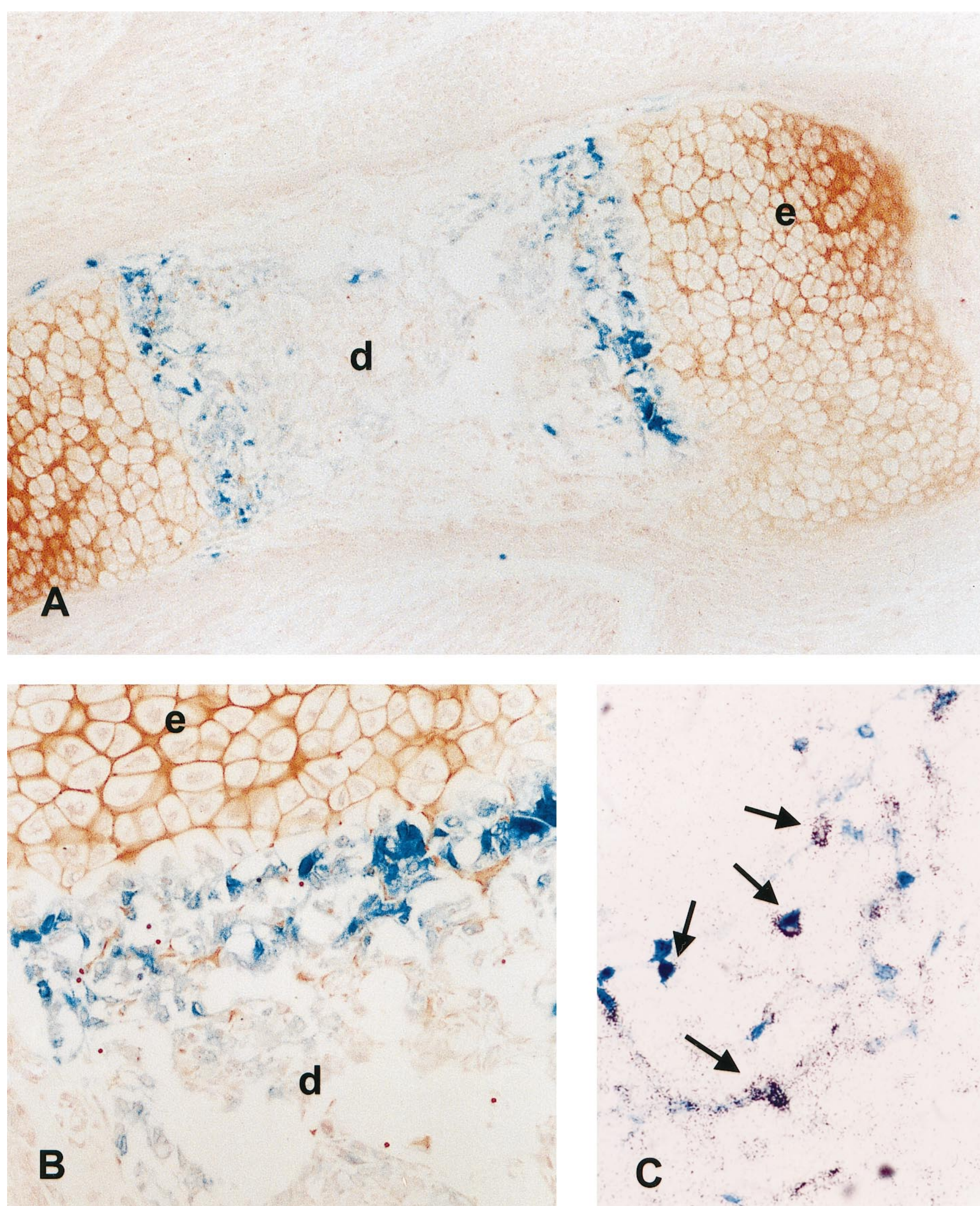

FIG. 5. **Expression of 7700ExIn-*LacZ* in developing bone.** In *A* (4× objective) and *B* (10× objective) expression of *LacZ* is present in single cells located at the site of endochondral ossification in the diaphysis (*d*) of tibia beneath hypertrophic chondrocytes of the epiphysis (*e*) shown with safranin and X-gal stain. *C*, *in situ* hybridization and X-gal staining demonstrate that the 7700ExIn-*LacZ* transgene and endogenous MMP-9 are coexpressed in most osteoclasts (*arrows*).

are expressed in epithelial cells, we analyzed tissues from mice made transgenic with the different constructs for expression of β-galactosidase. In general, we did not observe expression of the transgene in epithelia of organs such as skin, lung, or gastrointestinal tract with any of the six constructs made in this study.

We then analyzed if keratinocytes of healing skin wounds expressed the constructs. Incision wounds of about 1 cm were introduced into the dorsal skin and sutured with a couple of stitches to bring the wound edges together. When pieces of whole recovering wounds were stained with X-gal 1–7 days later, expression of *LacZ* could be followed in mice harboring constructs containing 7.7 kb of the 5′-flanking region of the MMP-9 gene (Fig. 6, *A*–*G*). When the surface of a wound was stained with X-gal, one could macroscopically observe cells expressing β-galactosidase at the wound edges (Fig. 6*A*). Staining of tissue sections from the wounds demonstrated strong positive reaction in keratinocytes migrating in under the fibrin clot covering 2-day-old wound (Fig. 6, *B* and *C*). Fig. 6*D*, showing double staining of sections with X-gal and anti-MMP-9 antibodies, demonstrated that most migrating keratinocytes expressing X-gal also costained with the MMP-9 antibody. Furthermore, scattered cells, presumably macrophages, beneath the wound contained the protein, and some of them also expressed the *LacZ* reporter gene. The fact that all cells and their immediate surroundings stained with the MMP-9 antibody but not X-gal may be due to secretion of the MMP-9 enzyme. Identification of β-galactosidase-expressing cells as keratinocytes was carried out by counterstaining with cytokeratin antibodies (Fig. 6, *E* and *F*). Keratinocytes resting on the normal basement membrane adjacent to the wound did not show any staining reaction. At day 7, the reepithelialization process was

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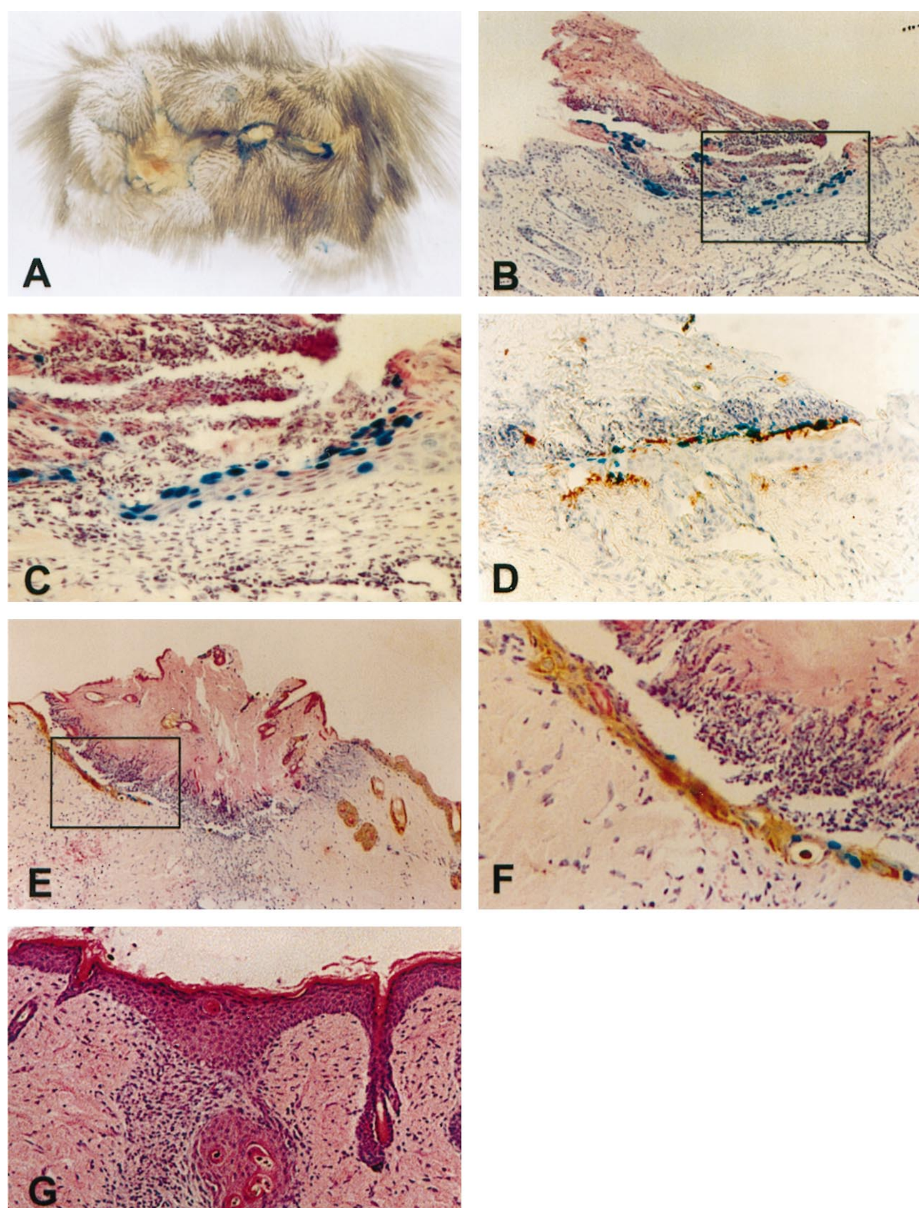

FIG. 6. **Expression of 7700ExIn-*LacZ* in incision skin wounds.** *A*, X-gal staining of the surface of a 3-day-old wound reveals positive staining of cells located at the wound edge. *B*, in a cross-section of a 2-day old wound, single cells migrating over the wound beneath the fibrin clot show expression of β-galactosidase. In contrast, keratinocytes resting on a mature basement membrane on both sides of the wound do not express the transgene, shown with hematoxylin-eosin and X-gal staining. *C*, amplification of the boxed area in *A*. *D*, double staining of a 2-day-old wound with X-gal and anti-MMP-9 antibodies. Most of the migrating keratinocytes exhibit codistribution of X-gal and MMP-9, while keratinocytes of the normal epithelium at the edges of the wound are negative for both. Some cells in the dermis and their immediate surrounding, presumably macrophages, show the presence of the MMP-9 protein, and some of those cells also show reaction with X-gal. *E*, immunostaining of a 2-day-old wound with anti-cytokeratin antibodies of a hematoxylin-eosin and X-gal-stained tissue demonstrates that cells expressing 7700ExIn-*LacZ* are keratinocytes. *F*, amplification of the region boxed in *E*. *G*, 7-day-old wound. Reepithelialization is complete. The new epithelium over the fibrotic scar tissue is thicker than the adjacent normal epithelium. Expression of 7700ExIn-*LacZ* has ceased. Hematoxylin-eosin and X-gal stains were used.

complete. The new epidermis was thicker, and the presence of fibrotic tissue was apparent, but expression of β-galactosidase by keratinocytes had ceased (Fig. 6*G*). In all founder lines, cells at wound edges expressed β-galactosidase.

DISCUSSION

It is well established that some of the members of the large family of MMPs exhibit highly restricted temporal and spatial expression patterns *in vitro*, indicating tissue-specific roles for these enzymes in extracellular matrix turnover. However, there are currently no reports on the regulatory mechanisms driving these cell lineage-specific expression patterns. The present study provides the gene structure and sequence of the promotor region of mouse MMP-9 as well as evidence that cis-regulatory element(s) necessary for the most of the cell-specific expression patterns of MMP-9 reside in a region between 2722 and 7745 base pairs upstream of the transcription initiation site.

The mouse gene was shown to be 7.7 kilobases and to contain 13 exons as previously shown for the human, mouse, and rabbit genes (21, 48, 49). The present study revealed the presence of two potential transcription initiation sites at positions 19 and 20 upstream of the ATG translation initiator codon. One of those sites corresponds to the site mapped for the human gene (21), but they differ slightly from that reported by Masure *et al.* (48). Sequencing of the upstream region revealed high sequence conservation between −1 and −600 bases in the mouse and human genes. Both genes have a TATA box-like motif, TTAAA, at about position −30 and lack a CCAAT box. The mouse gene has three GC boxes and three AP-1-like binding sites, as opposed to one and two in the human gene, respectively (21). Additionally, the mouse gene has a fourth AP-1-like motif further upstream. Several CA repeat microsatellite segments were observed in the 2722-base pair upstream sequence in the mouse gene, including one located close to the transcription initiation site as in the human counterpart. Computational analysis of the 5′-flanking region of the gene revealed several additional putative binding sites whose functionality and necessity for MMP-9 gene regulation still remain to be assigned. However, one or more of those binding sites have been implicated in mediating the effects of a diverse set of agents, which include tumor necrosis factor α, 12-*O*-tetradecanoyl-phorbol-13-acetate (51), v-Src (52), and Ha-Ras (53). While those studies focused on determining the transcriptional requirements for MMP-9 induction, they do not provide any information on regulatory requirements driving cell lineage-specific expres-

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sion of the MMP-9 gene.

The present results from the studies with transgenic mice demonstrated that the 2722-base pair 5′ upstream region does not confer cell-specific expression *in vivo*. However, some of the numerous transcription factor consensus binding motifs contained within this sequence are probably essential for the basic activity of the MMP-9 promoter, as reported in several studies. Thus, Yokoo and Kitamura (54) showed in transiently transfected glomerular mesangial cells that AP-1 activation is essential for the induction of MMP-9 by interleukin-1, which is also mediated through NF-κB stimulation. Himelstein *et al.* (55) also showed in cell transfection studies that motifs of the MMP-9 promoter, such as NF-κB, Sp1, Ets, AP-1, and a retinoblastoma element participate in transcriptional regulation of the MMP-9 expression. Furthermore, Gum *et al.* (56) have reported that mutation of the most downstream AP-1 motif practically abolishes the activity of a MMP-9 promoter-driven CAT reporter.

The experiments with promoter/*LacZ* reporter gene constructs in transgenic mice showed that expression of the MMP-9 gene in osteoclasts and migrating keratinocytes requires the region between −2722 and −7745. Constructs containing this segment yielded strong expression in osteoclasts and in migrating keratinocytes of a healing wound. The present results also demonstrated that the first intron does not contain an enhancer, as this intron does in several extracellular matrix genes (50, 57). However, this intron may be important for restricting ectopic expression, since the 7700-*LacZ* construct yielded ectopic expression in epithelial hair follicle cells, while mice expressing construct 7700ExIn-*LacZ* only exhibited expression in cells normally expressing MMP-9.

At the cellular level, both the 7700-*LacZ* and 7700ExIn-*LacZ* constructs were shown to yield highly specific expression in osteoclasts of developing bone, cells that normally strongly express MMP-9 (30). Coexpression of β-galactoctisidase with that of the endogenous gene was verified *in situ* hybridization analysis of the same tissue sections with an MMP-9 probe (see Fig. 5*C*). In addition to the major expression in osteoclasts, expression of MMP-9 has been shown to occur in migrating keratinocytes of a healing wound (27). In this study, both constructs 7700-*LacZ* and 7700ExIn-*LacZ* were expressed in corresponding keratinocytes of transgenic mice, and we also showed extensive codistribution of the MMP-9 protein and *LacZ* expression by double staining. Therefore, cis-regulatory element(s) for this expression pattern must also be present in the sequence between −2722 and −7745. Furthermore, as in the *in vivo* situation for the endogenous MMP-9 gene, keratinocytes resting on a normal mature basement membrane did not express the reporter gene in transgenic mice or contain the MMP-9 protein, as determined by immunohistochemical staining. Following complete healing and reepithelialization of the skin wound at day 7, expression of the reporter gene ceased, essentially as has been shown for the endogenous gene (27). It has previously been shown that MMP-9 is expressed in invading trophoblasts of the implanting embryo (33, 34) as well as by macrophages infiltrating invasive breast and colon cancers, while the actual cancer cells do not express the enzyme (29, 58). We have recently shown that trophoblasts of implanting embryos of mice transgenic for constructs 7700-*LacZ* and 7700ExIn-*LacZ* express the reporter gene, and furthermore, in such transgenic mice macrophages located around invading exogenous carcinoma cells also express the reporter gene. This suggests that the −2722 to −7745 segment also contains element(s) necessary for induction of expression of the gene in both trophoblasts and macrophages.[2]

The results of the present study represent an initial step in identifying the exact mechanisms of cell lineage-specific expression of an MMP, which has a highly cell-specific expression pattern and presumably specific function. Further work will be aimed at narrowing down the region necessary for this expression and finally the actual nucleotide sequences responsible for the activities. It will also be particularly interesting to find out whether a single or separate elements direct expression to osteoclasts and macrophages that are both derived from monocytes as well as epithelial keratinocytes that appear to express the MMP-9 gene only during migration.

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