Cloning of the Genes for Human Stromelysin and Stromelysin 2: Differential Expression in Rheumatoid Synovial Fibroblasts†

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ABSTRACT: Stromelysin is a member of a gene family of metalloproteinases involved in extracellular matrix remodeling in normal and diseased processes. Primary cultures of rheumatoid synovial cells produce large amounts of metalloproteinase mRNA and proteins. We cloned a cDNA for human stromelysin from a rheumatoid synovial cell cDNA library, and we used the cDNA to isolate the gene for human stromelysin and a related gene, stromelysin 2. We sequenced parts of the genes and found that both are contained on ~14 kilobase pairs of DNA. Using an exon-containing fragment of the stromelysin 2 genomic clone as a specific probe in Northern blot analysis, we demonstrate the differential expression of stromelysin and stromelysin 2 in rheumatoid synovial cells, human foreskin fibroblasts, and rabbit synovial fibroblasts. In addition, using chimeric constructs of the stromelysin promoter linked to the bacterial gene chloramphenicol acetyltransferase (CAT), we show that the elements required for the tumor promoter phorbol myristate acetate (PMA), epidermal growth factor (EGF), and interleukin 1β (IL-1β) induction are contained on a 307 base pair fragment which includes ~270 base pairs (bp) of 5′-flanking DNA. The cloning of the human stromelysin and stromelysin 2 genes, the documentation of their differential expression, and the identification of transcriptional regulatory regions in the stromelysin gene will facilitate the study of metalloproteinase gene expression in normal processes and in diseases such as rheumatoid arthritis.

Stromelysin is a neutral metalloproteinase that has the ability to degrade non-collagen components of connective tissue including proteoglycans, fibronectin, and laminin (Okada et al., 1986). Collagenase is a related metalloproteinase with the singular ability to initiate breakdown of the interstitial collagens (Harris, 1985). The ~50% homology between the human collagenase (Brinckerhoff et al., 1987; Whitham et al., 1986; Goldberg et al., 1986) and stromelysin cDNA and amino acid sequences (Whitham et al., 1986, Wilhelm et al., 1987; Saus et al., 1988; this paper) suggests that these enzymes are members of a multigene family of metalloproteinases. Collagenase and stromelysin are often, but not always (Edwards et al., 1987), coordinately expressed, implying that some mechanisms in their regulatory pathways may be shared.

Human stromelysin has 75% homology to rat stromelysin (transin) (Matrisian et al., 1986). Rat stromelysin mRNA is expressed in rat fibroblasts treated with epidermal growth factor (EGF) or transformed with certain oncogenes, therefore linking metalloproteinase expression with cellular transformation (Matrisian et al., 1986). We and others (Saus et al., 1988) have isolated the stromelysin cDNA from primary cultures of rheumatoid synovium, a tissue isolated from the joints of patients with rheumatoid arthritis.

Rheumatoid arthritis is a proliferative and invasive but nonmalignant disease of the synovial fibroblast cells that line the joints. In this disease, the synovium proliferates into a mass that produces large amounts of metalloproteinases capable of degrading articular cartilage, tendon, and bone, resulting in extensive joint destruction (Harris, 1985). The invasive and proliferative capabilities of rheumatoid synovium liken this disease to a localized malignancy (Harris, 1985). One focus of our research has been to identify the factors that induce these enzymes and the mechanisms controlling this induction.

Cytokines and growth factors, such as interleukin 1 (IL-1) (Dayer et al., 1986; Dinarello, 1988) and epidermal growth factor (EGF) (Breathnach et al., 1987; Kerr et al., 1988; Machida et al., 1988), as well as the tumor promoter phorbol myristate acetate (PMA) (Fini et al., 1987; Frisch et al., 1987) stimulate production of the metalloproteinases collagenase and stromelysin. IL-1 is a major mediator of the pathological events in rheumatoid arthritis (Harris, 1985), and the ubiquitous nature of EGF (Carpenter, 1981) suggests that it may also be involved in rheumatoid pathology. How these cytokines increase metalloproteinase gene expression is not known. In contrast, the mechanism controlling the induction of the human collagenase gene by PMA is at least partially understood: a PMA-responsive element, TGAGTCAG, to which the transcription factor AP-1 binds, is located 60 bp upstream of the transcription start site and is required for collagenase mRNA induction upon treatment with PMA (Angel et al., 1987). To date, it has been shown that sequences with a high percentage of sequence identity to the collagenase AP-1 binding site are present in the rabbit (Frisch & Ruley, 1987) and rat (Matrisian et al., 1986) stromelysin genes, the rat stromelysin 2 gene (Breathnach et al., 1987), and the rabbit collagenase gene (Fini et al., 1987).

In the rat, cDNA and genomic clones for stromelysin and stromelysin 2 (transin and transin 2) have been isolated, and the expression of the two genes was shown to be differentially regulated (Breathnach et al., 1987). In addition, the cDNAs for human stromelysin and stromelysin 2 have been cloned and sequenced, and they were also shown to be differentially expressed in some human tumors (Muller et al., 1988). Here we report the isolation and characterization of the genes for human stromelysin and stromelysin 2.

Using an exon-containing fragment of the gene for stromelysin 2 that serves as a specific probe, we measure stromelysin 2 mRNA expression. We show the differential regulation of the stromelysin and stromelysin 2 genes in human foreskin fibroblasts, rheumatoid synovial fibroblasts, and rabbit

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synovial fibroblasts. We also show that a 307 bp fragment of stromelysin 5′-DNA, beginning 18 bp upstream of the ATG start codon and representing ~270 bp of untranscribed 5′-flanking DNA, is responsive to PMA, EGF, and IL-1. Since EGF and IL-1 do not detectably affect stromelysin 2 expression by Northern blot analysis, our data suggest that these genes may be under distinct regulatory mechanisms.

Materials and Methods

Screening of cDNA and Genomic Libraries. The methods for library screening and Northern and Southern blots are given in detail because they contain modifications of standard methods that may be critical for probe specificity. The λgt11 rheumatoid synovial cell cDNA library was previously described (Brinckerhoff et al., 1987). A total of 10^5 cDNA plaques were screened with a rabbit stromelysin cDNA (Fini et al., 1987). The λ Charon 4A human genomic library was obtained from American Type Culture Collection (Rockville, MD). Plating and growth conditions were as described (Maniatis et al., 1982). The λ EMBL 3 human genomic library was obtained from Stratagene (LaJolla, CA) and was plated and grown as described by the information supplied with the library; 2×10^6 Charon 4A plaques and 1×10^6 EMBL 3 plaques were screened with the full-length human stromelysin cDNA described in this paper. Two positive clones from the Charon 4A library and four positive clones from the EMBL 3 library were obtained. A total of 1×10^6 λ EMBL 3 plaques were screened with the 160 bp *Eco*RI/*Xba*I 5′ fragment of the human stromelysin cDNA (Figure 1), and four positive clones were obtained.

The libraries were plated at a density of 1.5×10^4 plaques per 100-mm plate. The plaques were lifted onto Colony/Plaque Screen filters (New England Nuclear, Boston, MA) according to the manufacturer's instructions with the modifications described below. Duplicate lifts were made from each plate: the first for 2 min and the second for 5 min. The filter-bound DNA was denatured 2 times for 5 min each in 0.5 M NaOH/1.5 M NaCl followed by one neutralization for 5 min in 0.5 M Tris-HCl, pH 8.0/1.5 M NaCl. The filters were then rinsed in 2× SSC (1× SSC contains 150 mM NaCl/15 mM sodium citrate; Maniatis et al., 1982). Drying the filters prior to prehybridization was not required. Prehybridization was carried out for as little as 15 min to as long as 16 h at 65 °C in a solution containing 10% dextran sulfate (Pharmacia), 1% SDS (sodium dodecyl sulfate), 1× Denhardt's (Maniatis et al., 1982), and 6.6× SSC. To hybridize, 1–10 ng of denatured oligo-labeled (Feinberg & Vogelstein, 1983) stromelysin cDNA insert isolated by standard methods (Maniatis et al., 1982) per milliliter of prehybridization solution was added along with 0.1–1.0× nonspecific polynucleotide solution. A 10× stock of nonspecific polynucleotide solution contains 33.3 mM Tris, pH 8.1, 80 μg/mL poly(A) (Sigma), 80 μg/mL poly(C) (Sigma), 2 mg/mL yeast sRNA (Sigma), and 0.5 mg/mL salmon sperm DNA (Worthington). The filters were hybridized at 65 °C for ~16 h. The filters were rinsed in 65 °C double-distilled H_2O and washed for 1 h at 65 °C in 2× SSC/1% SDS and for 1 h at 65 °C in 0.2× SSC/0.1% SDS. A liter of solution was used to wash 20–30 filters. The filters were exposed to Kodak XAR film for ~16 h at −70 °C with a Dupont Cronex intensifying screen.

DNA Sequencing. The longest cDNA isolated from the λgt11 rheumatoid synovial cell library was excised by restriction endonuclease *Eco*RI digestion and ligated into the *Eco*RI site of M13 mp18 and -19 sequencing plasmids (Messing, 1983) by standard methods (Maniatis et al., 1982). The resulting plasmids were sequenced by the dideoxy method (Sanger et al., 1977) using [γ-^{35}S]dATP. M13 clones containing restriction fragments of the full-length clone were also isolated and sequenced. The remaining four positive cDNA isolates were mapped by restriction enzyme analysis (Maniatis et al., 1982) and shown to be truncated isolates of the same stromelysin cDNA. Restriction fragments containing exons and 5′-flanking DNA from the genomic stromelysin and stromelysin 2 clones were sequenced as described above.

Southern Blots. Human genomic DNA was isolated as previously described (Aldridge et al., 1984), and λ DNA was isolated by the DEAE-cellulose method (Silhavy et al., 1984). Restriction endonuclease digestion and agarose gel electrophoresis were performed by standard methods (Maniatis et al., 1982). The DNA was transferred to a Gene Screen Plus membrane (New England Nuclear, Boston, MA) as described by the manufacturer with modifications as indicated below. The Southern gel was depurinated for 15 min at room temperature with agitation in 0.25 N HCl and then denatured for 15–30 min in 0.4 N NaOH/0.6 M NaCl. The DNA was transferred from the gel using 10 sheets of Whatman 3MM paper cut to the size of the gel and soaked in the denaturing solution. No additional denaturing solution, except that contained in the saturated Whatman paper, was used in the ~16-h transfer. After transfer, the Gene Screen filter was neutralized by soaking for 15 min in 1.5 M NaCl/0.5 M Tris, pH 7.5–8.0. The subsequent steps are the same as described for plaque lifts above.

Northern Blots. RNA was isolated from human foreskin fibroblasts, rabbit synovial fibroblasts, or rheumatoid synovial cells as described (Brinckerhoff et al., 1987; Maniatis et al., 1982). Each gel lane contained 10 μg of whole cell RNA in 47% formamide (Bethesda Research Labs), 6.1% formaldehyde, and 4.7 mM NaH_2PO_4. The sample was heated at 55 °C for 15 min, glycerol was added to 10%, and the sample was electrophoresed on a 1% agarose gel containing 10 mM NaH_2PO_4, pH 7.4, and 6.6% formaldehyde. Electrophoresis was for ~4 h at 75 V in the presence of 10 mM NaH_2PO_4, pH 7.4 at 4 °C, with buffer recirculation. The RNA was transferred to Gene Screen Plus membranes essentially as described for Southern blots except the depurination and denaturation steps were omitted and 10× SSC was used as the transfer solution. After a ~16-h transfer, the filter was baked at 80 °C for 2 h. The subsequent steps are the same as described for plaque lifts above except the probe concentration was 0.1–1.0 ng/mL of prehybridization solution. The stromelysin and stromelysin 2 probes are described in this paper. The human collagenase cDNA probe has been described previously (Brinckerhoff et al., 1987). The human glyceraldehyde-3-phosphate dehydrogenase (GAPD) 1.2 kilobase pair (kbp) cDNA was obtained from the American Type Culture Collection (Rockville, MD). We have found that GAPD gene expression is not affected by the treatments used on the human and rabbit fibroblasts cells, and we use this gene to control for differences in RNA quantitation and transfer. Quantitation of the autoradiographs was performed by scanning with a densitometer (E-C Apparatus Corp., St. Petersburg, FL), using a Hewlett-Packard 3390-A peak area integrator (Hewlett-Packard, Palo Alto, CA).

Cell Cultures. Specimens of rheumatoid synovial tissue, obtained from surgery at the Mary Hitchcock Hospital (Hanover, NH), were dissociated into a single cell suspension by treatment with bacterial collagenase and trypsin (Dayer et al., 1976). Cells were plated as primary cultures at 30–50% confluence in 100-mm-diameter cultures dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY)

with 20% fetal calf serum (FCS; Gibco). After 7–10 days in culture, the cells were confluent. At this time, they were washed 3 times in Hanks balanced salt solution to remove traces of serum and cultured in serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate (LH). The amount of collagenase synthesized and secreted was determined by collagen fibril assay (Dayer et al., 1976; Brinckerhoff et al., 1979). RNA was harvested at appropriate intervals for individual experiments. As a control for some experiments, primary cultures were passaged with trypsin (0.25%; Gibco) 1:2 or 1:3 and grown to confluence in DMEM/10% FCS. At confluence, these passaged cells were placed in DMEM/LH, and RNA was harvested.

Fibroblasts derived from newborn human foreskins, obtained from the Newborn Nursery at Mary Hitchcock Memorial Hospital, were also used in some experiments; the collagenase produced by these cells is identical with that synthesized by synovial cells (Whitham et al., 1986; Goldberg et al., 1986; Brinckerhoff et al., 1987). Fibroblasts isolated by digestion of the minced tissue with bacterial collagenase, as described above, were plated in DMEM/20% FCS, grown to confluence, and passaged 1:3 in DMEM/10% FCS. For the experiments described here, cells were used between passages 3 and 7. The cells were treated for 24 h with 10 ng/mL EGF (Collaborative Research Inc., Bedford, MA) or 100 units/mL IL-1β (Immunex, Seattle, WA) in DMEM/5% FCS. Some cells were treated with 10^{-8} M PMA (Sigma Chemical Co., St. Louis, MO) in DMEM/LH. Control untreated cultures were incubated in DMEM/5% FCS or DMEM/LH.

Rabbit synovial fibroblasts were obtained from the knees of 4-week-old New Zealand white rabbits (Brinckerhoff et al., 1979). The synovium was removed, minced, and processed as described for human foreskin fibroblasts.

Stromelysin Promoter–CAT Reporter Chimeric Plasmids. *Eco*RI and *Dde*I were used to excise a promoter fragment spanning −325 to −18 relative to the start site of translation. The *Eco*RI/*Dde*I ends of the stromelysin fragment and the *Hin*dIII ends of the pSVOCAT-linearized plasmid were made blunt with the Klenow fragment of DNA polymerase I. The 307 bp fragment of stromelysin 5′-flanking DNA was inserted into the unique *Hin*dIII site of the promoterless plasmid pSVOCAT (Gorman et al., 1982) in either orientation relative to the chloramphenicol acetyltransferase (CAT) gene. $CaCl_2$ transformation into the DH5 α strain of *Escherichia coli*, and plasmid preparations were as described (Maniatis et al., 1982).

Transfection. Rabbit synovial fibroblasts, seeded at 3×10^5 cells per 60-mm dish, were transfected the following day with 10 μg of the CAT plasmids by the calcium phosphate method with glycerol shock (Davis et al., 1986; Graham & van der Eb, 1973). Some cells were transfected with the pSV2CAT plasmid as a positive control (Gorman et al., 1982). Sixteen hours after transfection, the cells were transferred to serum-free medium (see above) and incubated for an additional 24 h with 10^{-8} M PMA, 400 units/mL IL-1β, or 10 ng/mL EGF. The cells were then harvested, and CAT activity was measured as described (Gorman et al., 1982) using 3 μg of protein extract. The CAT assay measures the acetylation of [^{14}C]chloramphenicol. Reaction products were separated by thin-layer chromatography (TLC) where the acetylated product and the unacetylated substrate migrate differently. TLC spots were cut out, and the ^{14}C in each was counted in a liquid scintillation counter. Percent incorporation of chloramphenicol substrate into acetylated product was then calculated. Since the data obtained were within the linear range of the assay, the percent incorporation is a reflection of the amount of CAT enzyme present in the extract (Gorman et al., 1982). The relative percent incorporation, normalized to the level obtained with untreated cells transfected with the stromelysin promoter fragment in the correct orientation relative to the CAT gene, is given under Results.

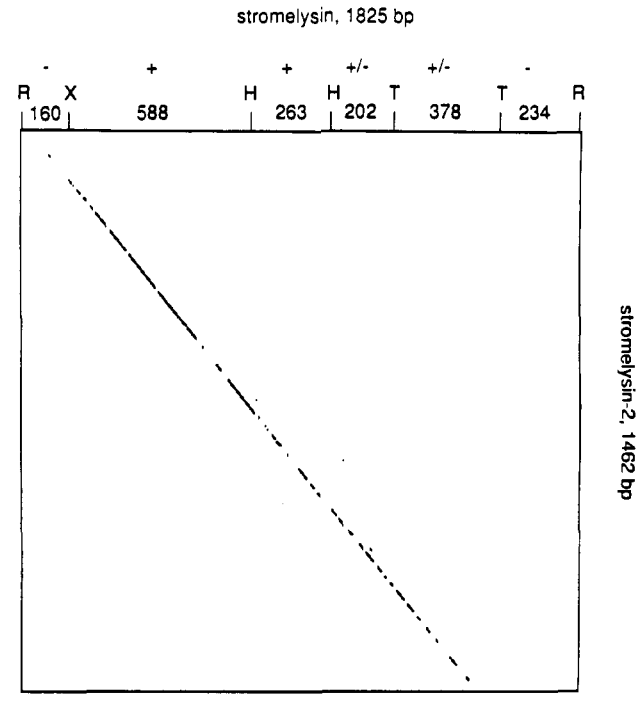

FIGURE 1: Sequence homologies in the human stromelysin and stromelysin 2 cDNAs. The human rheumatoid synovial cell stromelysin sequence of 1825 nucleotides and the human stromelysin 2 cDNA sequence of 1462 nucleotides (Muller et al., 1988) were analyzed with the DNA Inspector Ile program (Gross, 1986) using a search element length of 12 with 1 mismatch allowed. Any stretch of 12 nucleotides with 11 or more identical residues is represented as a dot. The restriction map of the stromelysin cDNA is shown: R = *Eco*RI, X = *Xba*I, H = *Hin*dIII, T = *Taq*I. Sizes for each restriction fragment are given in base pairs. The relative degree of homology between the two cDNAs in each of the regions defined by the restriction fragments is shown above each fragment with the following symbols: (-) no homology; (+/-) a moderate degree of homology; (+) a high degree of homology.

Results

Isolation of Human Stromelysin cDNA from Rheumatoid Synovial Cells. Five overlapping clones for human synovial cell stromelysin, including a full-length 1.8 kbp cDNA, were isolated by screening a cDNA library generated from the mRNA in primary cultures of rheumatoid synovial cells with the rabbit stromelysin cDNA (Fini et al., 1987). The restriction enzyme map of the full-length clone (Figure 1) and the DNA sequence is essentially identical with that isolated by others (Whitham et al., 1986; Wilhelm et al., 1987; Saus et al., 1988), except that our synovial cell clone has a five-base sequence, GTTTT, immediately upstream of the poly(A) tail that is not present in gingival stromelysin (Whitham et al., 1986). To facilitate sequencing, 5′ to 3′ ordered restriction fragments of the stromelysin cDNA were generated and subcloned. These fragments were used subsequently as ordered 5′ to 3′ probes in Southern blot analysis of genomic stromelysin and stromelysin 2 clones (Figure 2).

Our human synovial cell stromelysin cDNA has 78% overall homology to human stromelysin 2, a cDNA isolated from certain human tumors (Muller et al., 1988). In these tumors, there is generally a greater amount of stromelysin 2 mRNA than stromelysin mRNA. Figure 1 also shows a homology

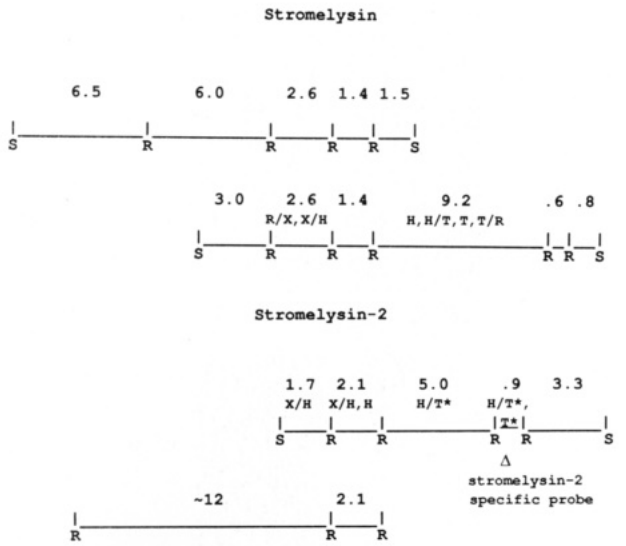

FIGURE 2: Human genomic stromelysin and stromelysin 2 clones: restriction maps and stromelysin cDNA restriction fragment hybridization pattern. The *Eco*RI restriction maps for two overlapping isolates of the stromelysin gene and two overlapping isolates of the stromelysin 2 gene are shown where R = *Eco*RI sites and S = *Sal*I sites introduced into the genes during the construction of the λ EMBL 3 genomic library. The fragment sizes are given in approximate kilobase pairs. Restriction fragments of the human stromelysin cDNA (Figure 1) are designated above the genomic clone fragment to which they hybridize in Southern blot analysis. From the 5′ to the 3′ direction, the ordered restriction fragments of the cDNA are identified as follows: R/X = the 160 bp *Eco*RI/*Xba*I fragment; X/H = the 588 bp *Xba*I/*Hin*dIII fragment; H = the 263 bp *Hin*dIII fragment; H/T = the 202 bp *Hin*dIII/*Taq*I fragment; T = the 378 bp *Taq*I fragment; and T/R = the 234 bp *Taq*I/*Eco*RI fragment. An asterisk indicates that the designated stromelysin cDNA fragment will only hybridize under low stringency conditions (hybridization and washes at 50 °C: first wash in 2× SSC/1.0% SDS and second wash in 1× SSC/0.1% SDS). The 0.9 kbp *Eco*RI fragment of the stromelysin gene used in subsequent Southern and Northern blot analysis is indicated.

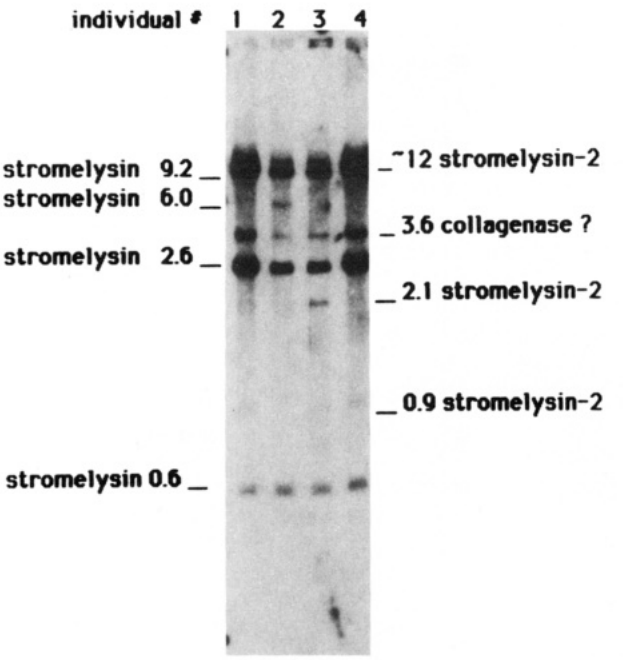

FIGURE 3: Southern blot analysis of human genomic DNA probed with the stromelysin cDNA. *Eco*RI-digested human genomic DNA, isolated from four samples of normal human lymphocytes, was probed with the oligo-labeled stromelysin cDNA under high stringency conditions (see Materials and Methods). The sizes of each fragment hybridizing to the probe are given in approximate kilobase pairs. The gene to which each fragment corresponds, as defined by the restriction maps for the human stromelysin and stromelysin 2 genes in Figure 2, is indicated. The questionable identity of the 3.6 kbp band is described under Discussion.

matrix analysis, comparing the cDNA sequence of stromelysin to stromelysin 2 (Muller et al., 1988). A perfect line would indicate 100% identity between the two sequences. The figure shows that restriction fragments at the 5′ and 3′ ends of the stromelysin cDNA have little homology to the stromelysin 2 cDNA. The 588 bp *Xba*I/*Hin*dIII fragment and the 263 bp *Hin*dIII fragment define regions that are highly conserved between the two sequences.

Isolation of Human Genomic Clones for Stromelysin and Stromelysin 2. The human stromelysin cDNA was used to screen two human genomic libraries under high stringency conditions (see Materials and Methods). Eight positive clones were isolated and characterized. Restriction mapping and Southern blot hybridization with ordered fragments of the stromelysin cDNA (Figure 1) indicate that five of the clones are overlapping isolates of the stromelysin gene. Three of the clones are overlapping isolates of a second gene, stromelysin 2. Figure 2 shows the *Eco*RI restriction maps for two overlapping isolates of each gene. Each gene is encoded on ~14 kbp of DNA. In addition, there are isolates for each gene that have ~12 kbp of 5′-flanking DNA. By sequencing exon-containing portions and comparing the sequences to those of the stromelysin and stromelysin 2 cDNAs, we established that these two sets of clones represent the genes for either human stromelysin or human stromelysin 2. The exon sequences obtained from the sequencing of restriction fragments of the genomic stromelysin and stromelysin 2 clones were identical with their respective cDNA sequences.

The stromelysin cDNA was used to probe a human genomic Southern blot shown in Figure 3. Genomic DNA from four normal human lymphocyte samples was digested with the *Eco*RI restriction endonuclease, and the fragments were separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with the stromelysin cDNA under conditions of high stringency. All of the hybridizing restriction fragments, except the 3.6 kbp fragment, can be accounted for by the presence of two stromelysin genes, stromelysin and stromelysin 2, as defined by the restriction maps in Figure 2. The 3.6 kbp fragment could represent another stromelysin-related gene, or it could be the *Eco*RI fragment of the collagenase gene containing exons 1 and 2 (Collier et al., 1988). See Discussion for details. This Southern blot demonstrates that the genes isolated from the λ libraries are an accurate representation of those found in the human genome.

Characterization of a Stromelysin 2 Specific Probe. As illustrated in Figure 2, a 900 bp 3′ *Eco*RI fragment of the stromelysin 2 genomic clone does not hybridize to the stromelysin cDNA 202 bp *Hin*dIII/*Taq*I or 378 bp *Taq*I restriction fragments under high stringency conditions, but the genomic fragment does hybridize when the stringency is lowered. These stromelysin cDNA restriction fragments define regions between the stromelysin and stromelysin 2 cDNAs with only a moderate degree of sequence homology (Figure 1). The 900 bp *Eco*RI fragment of the stromelysin 2 genomic clone serves as a stromelysin 2 specific probe under high stringency conditions and was used to measure stromelysin 2 mRNA expression in Northern blot analysis. To demonstrate the specificity of the stromelysin 2 probe, it was hybridized under high stringency to a Southern blot of the genomic clones for stromelysin and stromelysin 2. As shown in Figure 4, the 900 bp stromelysin 2 *Eco*RI fragment hybridizes to a 900 bp *Eco*RI fragment and a ~12 kbp *Hin*dIII fragment of the stromelysin 2 clone while it does not cross-hybridize to the

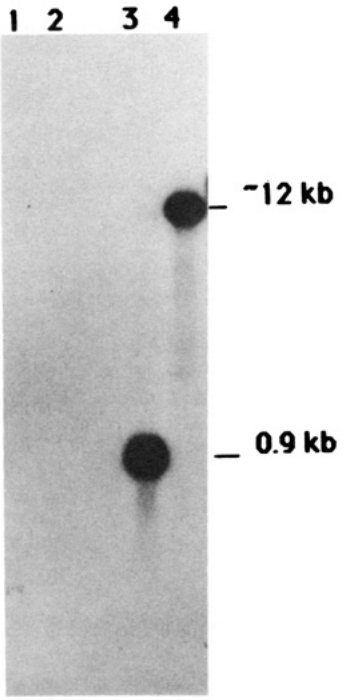

FIGURE 4: Southern blot analysis of stromelysin 2 probe specificity. λ DNA from the stromelysin (lanes 1 and 2) and stromelysin 2 (lanes 3 and 4) genomic clones was digested with restriction enzymes, either *Eco*RI (lanes 1 and 3) or *Hin*dIII (lanes 2 and 4), Southern transferred, and probed with the oligo-labeled 0.9 kbp *Eco*RI fragment of the stromelysin 2 clone under high stringency conditions (see Materials and Methods).

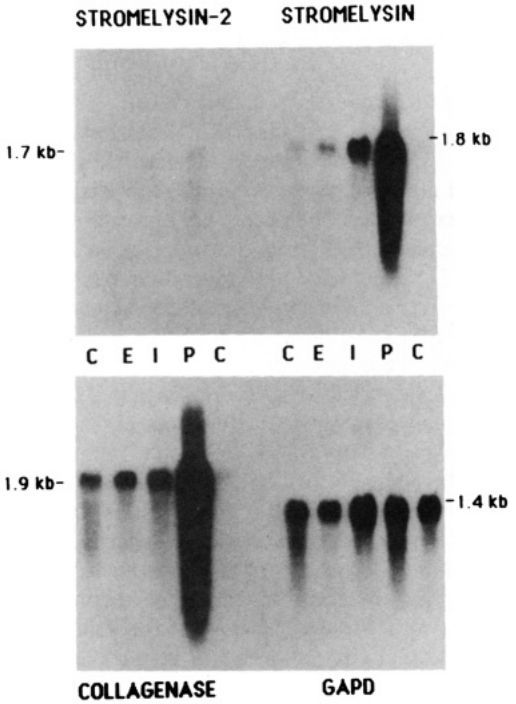

FIGURE 5: Northern blot analysis of human foreskin fibroblast RNA. RNA was isolated from monolayer cultures of human foreskin fibroblasts treated for 24 h with 10 ng/mL EGF (E) or 100 units/mL IL-1β (I), in DMEM/5% FCS, or with 10^{-8} M PMA (P) in DMEM/LH. Control cultures (C) were incubated in either DMEM/5% FCS (first lane) or DMEM/LH (last lane). Cells were harvested, and 10 μg of RNA per lane was Northern transferred and probed with one of the ollowing oligo-labeled inserts as indicated in the figure: the 0.9 kbp *Eco*RI fragment of the human stromelysin 2 genomic clone; the human stromelysin cDNA; a 1.7 kbp *Eco*RI fragment of the human collagenase cDNA; or a 1.2 kbp cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPD). Sizes of the hybridizing mRNAs are given in kilobases.

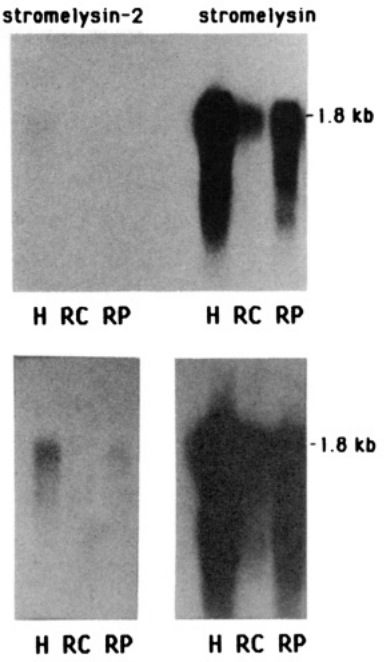

FIGURE 6: Northern blot analysis of human rheumatoid synovial cell RNA and rabbit synovial fibroblast RNA. RNA was isolated from primary cultures of human rheumatoid synovial cells (H) in DMEM/10% FCS, rabbit synovial fibroblast control cells (RC) in DMEM/LH, or rabbit synovial fibroblasts treated with PMA for 24 h (RP) in DMEM/LH. Cells were harvested, and 10 μg of RNA per lane was Northern transferred and probed with the oligo-labeled 0.9 kbp *Eco*RI fragment of the stromelysin 2 genomic clone or the stromelysin cDNA as indicated. The sizes of the hybridizing mRNAs are indicated in kilobases. The top two panels represent a 5-h exposure of the blot to the film; the bottom panels represents a 16-h exposure to the same blots.

stromelysin clone, even after a 3–4-fold longer (~16 h) exposure than that required to detect the stromelysin 2 signal. These data demonstrate that under the conditions detailed under Materials and Methods, the stromelysin 2 probe is specific for stromelysin 2 sequences.

Relative Expression of Stromelysin and Stromelysin 2 mRNAs. The stromelysin 2 specific probe and the stromelysin cDNA were used under high stringency conditions to measure the relative expression of the two stromelysin mRNAs in human foreskin fibroblasts, rheumatoid synovial cells, and rabbit synovial fibroblasts. Figure 5 shows companion Northern blots of RNA isolated from untreated human foreskin fibroblasts or from human foreskin fibroblasts treated with EGF or IL-1β in the presence of serum or with PMA in the absence of serum. The blots were probed with the specific 900 bp *Eco*RI fragment of the stromelysin 2 genomic clone, the full-length stromelysin cDNA, a 1.7 kbp fragment of the human collagenase cDNA (Brinckerhoff et al., 1987), or a 1.2 kbp human cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPD), a gene whose expression is not changed with these treatments and was used to normalize for differences in the amount of RNA loaded per lane. A 5-h exposure of the blot to the film is shown. Densitometric scanning of the autoradiographs and normalization to GAPD housekeeping gene expression indicate that PMA induces collagenase and stromelysin mRNA levels 46-fold over control levels in untreated cells while stromelysin 2 is only minimally induced, by 50%, upon treatment with PMA. EGF induces collagenase and stromelysin expression by 2-fold while IL-1β induces collagenase mRNA by 2-fold and stromelysin mRNA levels by 4-fold. Neither EGF nor IL-1 had a detectable affect on stromelysin 2 mRNA levels.

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                                EcoR 1
                                GAATTCCCAGAAGAAAAAAAATG
                                  *    *   *   *
AAGCTTGTCAGCTCTCTTTAAGTAAATACAGAGACGTGAATTATCTAGCC
                                                  -304

CTCCAGTTTTCTCCTCTACCAAGACAGGAAGCACTTCCTGGAGATTAATC
      *****  ** *** ** **** ** ** ******* ********
AATTCTTTTTCGTCTTTACAAACACAGAAATCATTTCCTGGGGATTAATC
                                                  -254

ACTGTGTTGCCTTGCAAAATTGGGAAGGTTGAGAGAAATTAGTAAAGTAG
* *      * ** ** * **  *** ***** **** ** * * ****
A-T-----TC-TTTCA-AGTTTAGAAAGTTGAAGGAAAATACTGATGTAG
                                                  -204

GTTGTATCATCCTACTTTGAATTTGGAATGTTTGGAAATGGTCCTGCTGC
* ******* * *    * *      ********  **   **   **
GCTGTATCACCATTACATTA------AATGTTTGTGAACATTCAGACTTA
                                                  -154

CATTTGGATGAAAGCAAGGATGAGTCAAGCTGCGGGTGATCCAAACAAAC
       *  **  ** * **** ***  ***  *******  *     *
-------AAAAACACATGCATGAATCATACTGTTGGTGATCTCA----GC
                                                  -104

ACTGTCACTCTTTAAAAGCTGCGCTCCCGAGGTTGGACCTTACAAGGAGG
* **** ** * ******    **  *   **        ** ***
ATTGTCTCTGTATAAAAGAAAGGCCACTAGGGGGTAGAGCTAGAAGCCCA
                                                  -54
                                    Dde 1
CAGGCAAGACAGCAAGGCATAGAGACAACATAGAGCTAAGTAAAGCCAGT
   *                                *   * **** * ****
GTAGAC--------------------------AAAGAAGGTAAGGGCAGT
                                                  -4

GGAAATG
*  ****
GAGATG
   0
```

FIGURE 7: DNA sequence analysis and comparison of the human stromelysin and human stromelysin 2 5′-flanking regions. Sequences were determined as described under Materials and Methods. The human stromelysin (upper lines) and stromelysin 2 (lower lines) sequences were aligned for maximum homology, and the gaps introduced for this purpose are indicated by dashes. Matches are indicated by asterisks. Regions similar to the AP-1 binding site are underlined, the putative TATA boxes are overlined, and the ATG start codons for translation are in boldface type. The *Eco*RI and *Dde*I restriction sites that were used to create the stromelysin promoter–CAT reporter chimeric plasmids are indicated.

As shown in Figure 6, primary rheumatoid synovial cells cultured in the presence of 10% serum express high levels of stromelysin mRNA while stromelysin 2 mRNA is expressed to a very low but still detectable level. Similar results are obtained when the cells are cultured in the absence of serum (data not shown). The relationship of these data to the effects of serum on rat stromelysin and stromelysin 2 expression is described under Discussion. Primary cultures of rheumatoid synovial cells also express high levels of collagenase mRNA while passaged cultures express very low levels of collagenase and stromelysin mRNA and no detectable stromelysin 2 (data not shown). Even when primary rheumatoid synovial cells were treated with EGF or IL-1β, stromelysin 2 mRNA levels were not detectably affected (not shown). When passaged rheumatoid synovial cells were treated with PMA in the presence of serum, the expression of stromelysin mRNA was induced to high levels, similar to those seen in primary cultures, while stromelysin 2 mRNA was increased to the same low levels seen in primary cultures. Together, these results demonstrate that the expression of stromelysin and stromelysin 2 is differentially regulated in rheumatoid synovium.

Figure 6 also shows that like human foreskin fibroblasts, rabbit synovial fibroblasts treated with PMA express high levels of stromelysin mRNA compared to untreated cultures, while stromelysin 2 mRNA expression induced by PMA is weak. We demonstrated that the human stromelysin 2 probe is specific for unique rabbit sequences by probing a rabbit genomic Southern blot with the human stromelysin 2 specific probe (data not shown).

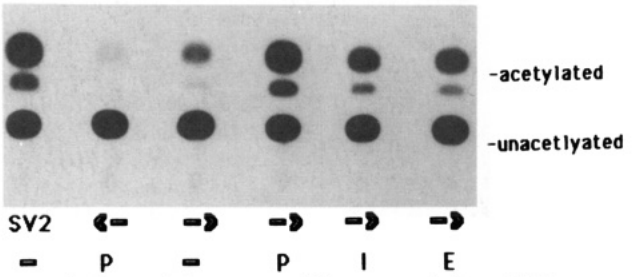

FIGURE 8: Stromelysin promoter driven transcription. CAT enzyme activity was measured in rabbit synovial fibroblasts transiently transfected with either pSV2CAT containing the SV40 promoter as a positive control (SV2), pSVOCAT containing the *Eco*RI/*Dde*I stromelysin promoter fragment in the reverse orientation relative to the CAT gene (←), or the *Eco*RI/*Dde*I fragment of the stromelysin promoter in the correct orientation relative to the CAT gene (→). Sixteen hours after transfection, the cells received no treatment (–) or were treated for 24 h with either 10^{-8} M PMA (P), 10 ng/mL EGF (E), or 400 units/mL IL-1β (I) in DMEM/LH. Thin-layer chromatography was used to resolve the acetylated product from the unacetylated substrate in the CAT assay.

Stromelysin and Stromelysin 2 Promoter Sequence Comparison. The sequence of ~300 bp of human stromelysin and stromelysin 2 5′-flanking DNA immediately upstream of the ATG start codon is shown in Figure 7. Aligning the sequences as shown demonstrates that they have ~50% overall homology, with stretches that are up to 80% homologous. Located ~35–40 nucleotides upstream from their TATA sequences, both genes contain a sequence with 77–88% sequence identity to the AP-1 binding site, ATGAGTCAG, known to be required for PMA induction of the human collagenase gene (Angel et al., 1987).

Stromelysin Promoter Function Assays. To better understand stromelysin gene expression at the level of transcription, a 307 bp *Eco*RI/*Dde*I fragment of stromelysin 5′-flanking DNA, 18 bp upstream of the ATG start codon, was inserted in either orientation upstream of the chloramphenicol acetyltransferase (CAT) reporter gene in the promoterless plasmid pSVOCAT. Plasmids were transiently transfected into rabbit synovial fibroblasts, and transfectants were treated with PMA, EGF, or IL-1β. Twenty-four hours after treatment, the cells were harvested, and lysates were assayed for CAT activity. Figure 8 shows the results for a representative experiment. All experiments were qualitatively similar, and the range is given below.

The 307 bp stromelysin promoter fragment in the correct orientation relative to the CAT gene gives a low level of constitutive expression that can be induced 5–8-fold upon treatment with PMA. In the control construct, transcription driven by the 307 bp promoter fragment in the opposite orientation does not result in CAT activity, even under conditions where CAT activity driven by the promoter construct in the correct orientation is maximally induced by PMA. IL-1β induced CAT gene expression driven by the 307 bp stromelysin promoter fragment 3–4-fold, and EGF had a 2.4–3-fold inducing effect. The relative inductions with IL-1 and EGF treatment correlate with those seen at the level of mRNA in Northern blot analysis, suggesting that the elements required for EGF and IL-1 regulation of stromelysin expression are contained on this 307 bp stromelysin promoter fragment. While it is clear that the 307 bp promoter fragment is PMA-responsive, additional upstream elements may be required for modulation of the magnitude of the PMA response. This has also been observed for the human (Angel et al., 1987) and

rabbit (Brinckerhoff & Auble, 1989) collagenase genes.

DISCUSSION

Using our rheumatoid synovial cell cDNA for stromelysin as a probe, we have cloned the genes for human stromelysin and stromelysin 2 and determined that they are encoded by ~14 kbp of DNA. We have identified and used a fragment of the stromelysin 2 gene as a stromelysin 2 specific probe, and we show that stromelysin and stromelysin 2 are differentially expressed in rheumatoid synovium, human foreskin fibroblasts, and rabbit synovial fibroblasts. In addition, we provide evidence for PMA, IL-1, and EGF transcriptionally functional elements contained on 307 bp of human stromelysin 5'-flanking DNA.

The cloned human stromelysin and stromelysin 2 genes can account for all except the 3.6 kbp *Eco*RI band detected when the human rheumatoid synovial cell stromelysin cDNA is used as a probe under high stringency conditions in human genomic Southern blot analysis. The strong hybridization of this 3.6 kbp band suggests the presence of yet another stromelysin-related gene. The pump 1 cDNA (Muller et al., 1988) has only 49% homology stromelysin, and, therefore, its putative gene would probably not be detected under the high stringency conditions.

Alternatively, the 3.6 kbp band could represent the *Eco*RI restriction fragment of the collagenase gene containing exons 1 and 2 (Collier et al., 1988). There are two 50 bp exon-containing stretches in this fragment that have 80% similarity to the stromelysin cDNA. Indeed, the first 0.3 kbp of the human collagenase cDNA (Brinckerhoff et al., 1987) will hybridize under high stringency conditions to the stromelysin cDNA (unpublished data). It is important to point out that the remaining 1.7 kbp of the collagenase cDNA (Brinckerhoff et al., 1987) does not hybridize to the stromelysin cDNA under these conditions and was used as a collagenase-specific probe in the Northern blot analysis shown.

Because the genes for stromelysin and stromelysin 2 have a high degree of homology in some regions, this raises the possibility that when the stromelysin cDNA is used as probe, even under high stringency conditions, it may also be detecting stromelysin 2. For this reason, we believe that a stromelysin 2 specific probe is needed. We demonstrated that a fragment of the stromelysin 2 genomic clone, that has exon sequences with a low degree of homology to stromelysin, can be used as a stromelysin 2 specific probe. We used the probe to show that rheumatoid synovial cells express very low levels of stromelysin 2 mRNA, that stromelysin 2 expression is only weakly induced by PMA in rabbit and human fibroblasts, and that stromelysin 2 expression is not detectably affected by treatment with either EGF or IL-1 in human fibroblasts.

All the cloned metalloproteinase gene promoters, i.e., rabbit (Brinckerhoff & Auble, 1989) and human collagenase (Angel et al., 1987); rabbit (Frisch & Ruley, 1987), rat (Matrisian et al., 1986), and human stromelysin (Quinones et al., 1989; this paper); and rat (Breathnach et al., 1987) and human stromelysin 2 (this paper), are PMA-inducible to a large extent, except for human stromelysin 2. However, the data for the rat genes are less clear. Both rat stromelysin and stromelysin 2 can be induced by PMA in only some batches of serum (Breathnach et al., 1987). We show that human stromelysin can be induced to high levels by PMA in the presence or absence of serum while human stromelysin 2 is only weakly induced under the same conditions. The AP-1 sequence of the human stromelysin 2 gene is different from that of the human stromelysin gene, the rat stromelysin gene (Matrisian et al., 1986), and the rat stromelysin 2 gene (Breathnach et al., 1987). Like the consensus, ATGAGTCAG, all of the metalloproteinase genes have a G residue at the fifth position except human stromelysin 2 which has an A. The G residue in position 5 of the consensus was shown by dimethyl sulfate footprinting (Harshman et al., 1988) to be in close proximity to the protein when AP-1 is bound to DNA. In addition, position 9 of the consensus has a greater degree of variability in the metalloproteinase genes. It is interesting to note that both rat and human stromelysin 2 have a T in this position while none of the other metalloproteinase genes do. It was shown that mutating the A and G in positions 8 and 9 to a C and T caused a decrease in PMA responsiveness to ~25% of the levels seen with the unmutated consensus (Angel et al., 1987). We hypothesize that the human stromelysin 2 promoter G to A transition in position 5 and the presence of a T in position 9 may account for the low level of PMA responsiveness seen for the human stromelysin 2 gene. These conversions may not allow the factor to bind as well.

The human stromelysin promoter has a high percentage of sequence similarity to the rat (Matrisian et al., 1986) and rabbit (Frisch & Ruley, 1987) stromelysin promoters, and the human stromelysin 2 promoter has a high percentage of sequence similarity to the rat stromelysin 2 promoter (Breathnach et al., 1987). Approximately 700 bp of rabbit stromelysin promoter confers IL-1 induction and dexamethasone, a gluccocorticoid, repression of CAT reporter gene expression (Frisch & Ruley, 1987). Recently, it has been shown that a 468 bp fragment of the human stromelysin promoter is also responsive to IL-1 and dexamethasone (Quinones et al., 1989). We extend these data by showing that 307 bp of the human stromelysin promoter is sufficient for IL-1, EGF, and PMA induction. This fragment is the smallest human stromelysin promoter fragment shown to date that retains PMA and IL-1 responsiveness. Rat stromelysin mRNA expression is induced by treatment with EGF while rat stromelysin 2 expression is not detectably affected (Breathnach et al., 1987). This is in agreement with our data for the human stromelysin and stromelysin 2 genes. In addition, the 307 bp human stromelysin promoter fragment is sufficient to confer EGF responsiveness on a CAT reporter gene.

In summary, this 307 bp *Eco*RI/*Dde*I fragment, representing ~270 bp of untranscribed 5'-flanking DNA of the human stromelysin gene, contains the elements required for PMA, IL-1, and EGF transcriptional regulation. Further analysis of the transcriptional regulation of the stromelysin and stromelysin 2 genes will provide insight into the molecular mechanisms governing this differential metalloproteinase gene expression in normal and diseased states. Particularly, it will be interesting to determine why stromelysin 2 expression is greater than that of stromelysin in certain human tumors, while the reverse is true in rheumatoid synovium and in PMA-treated fibroblasts.

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Registry No. PMA, 16561-29-8; EGF, 62229-50-9; stromelysin, 79955-99-0.

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