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# Coordinate regulation of stromelysin and collagenase genes determined with cDNA probes

(cycloheximide inhibition/macrophage/capillary endothelial cells/phorbol esters/metalloproteinases)

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ABSTRACT **Secreted proteinases are required for tumor metastasis, angiogenesis, and tissue remodeling during wound healing and embryonic growth. Thus, the regulation of the genes of secreted proteinases may serve as an interesting model for growth-controlled genes in general. We studied the genes of the secreted proteinases stromelysin and collagenase by using molecularly cloned cDNAs from each proteinase. Stromelysin cDNA was cloned by differential screening of a total cDNA library from rabbit synovial cells treated with phorbol 12-myristate 13-acetate, which yielded a clone of 1.2 kilobase pairs; collagenase cDNA was obtained by cloning reverse transcripts of anti-collagenase-immunoadsorbed polysomal mRNA, which yielded a clone of 0.8 kilobase pairs. Stromelysin and collagenase mRNA species of 2.2 and 2.4 kilobases, respectively, were detected on hybridization blots of RNA from phorbol 12-myristate 13-acetate-treated but not untreated rabbit synovial cells. Expression of stromelysin mRNA was also induced in rabbit alveolar macrophages and rabbit brain capillary endothelial cells treated with phorbol 12-myristate 13-acetate. Stromelysin and collagenase mRNA were both induced by phorbol 12-myristate 13-acetate and cytochalasin B at a constant ratio of the two gene products; this suggests coordinate regulation. The fact that induction was blocked after inhibition of protein synthesis by cycloheximide implicates an indirect signal transduction pathway that requires new protein synthesis.**

In addition to its structural role, extracellular matrix (ECM) interacts with and stabilizes, in many cell systems, the cytoskeleton (1–3), which in turn stabilizes the phenotype of the cell by influencing gene expression (3–9). Thus, the regulation of the synthesis of secreted proteinases that are capable of degrading ECM proteins is a potential control point for intercellular signaling. An understanding of the regulation of the genes of secreted proteinases may also contribute to the characterization of normal and pathologic processes for which these proteinases are apparently required, such as tumor cell metastasis (10), wound healing, angiogenesis (11), and connective tissue destruction accompanying rheumatoid arthritis (12).

The complex protein composition of ECM motivated a search for proteinases that could act in concert with collagenase to achieve complete remodeling of ECM. Collagenase is secreted as proenzymes of 53 and 57 kDa (13–15); we recently identified and characterized a 51-kDa protein that is the proenzyme form of a metalloproteinase, stromelysin, which is secreted by rabbit synovial fibroblasts (RSF) treated with phorbol 12-myristate 13-acetate (PMA). Stromelysin is capable of degrading fibronectin, laminin, elastin, IgG, and proteoglycans (13).

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In the study reported here, we molecularly cloned stromelysin and collagenase cDNAs from RSF and used them to study the regulation of the stromelysin and collagenase genes at the mRNA level by hybridization analysis. Our results suggest that PMA and other inducers impinge on an indirect signal transduction pathway containing newly induced, or very labile, protein components and that protein synthesis is required in the induction process. Two other cell types, rabbit alveolar macrophages and rabbit brain capillary endothelial cells, which were previously reported to synthesize collagenase (11, 14, 15), are shown here to synthesize a 2.3-kilobase (kb) stromelysin mRNA when treated with PMA; this indicates that several cell types have the potential for ECM turnover.

## MATERIALS AND METHODS

**Cell Culture, Treatment with Inducing Agents, Labeling of Metabolic Proteins, Immunoprecipitation, Isolation of RNA, *In Vitro* Translation, and Gel Electrophoresis.** These procedures were carried out as described (14, 16–19).

**Synthesis and Cloning of Double-Stranded cDNA.** The total cDNA library of 20,000 recombinants that was used to isolate stromelysin clones was synthesized essentially by the hairpin loop-primer method of Wickens *et al.* (20) using 16.5 μg of mRNA from PMA-treated RSF. The double-stranded cDNA was tailed with oligo(dC) residues and inserted into the *Pst* I site of oligo(dG)-tailed, *Pst* I-cut pBR322 (21). For differential screening, colonies were transferred into 0.2-ml aliquots of culture medium in 96-well microtiter plates, grown, and then used to inoculate nitrocellulose filters on agar (21). Two filters were screened with $^{32}$P-labeled cDNA prepared from mRNA from untreated RSF, and two filters were screened with $^{32}$P-labeled cDNA prepared from mRNA from PMA-treated RSF. Twelve colonies that hybridized preferentially in both filters that contained the cDNA probe from the treated cells were used to prepare plasmid DNA. The DNA was linearized with *Sal* I, bound to nitrocellulose filters, and used for hybrid-selected translation with total RNA from PMA-treated RSF. For collagenase cDNA cloning, polysomes were isolated by the sucrose gradient method (22) and were incubated with 75 μg of S99 anti-collagenase IgG (23) per $A_{260}$ unit of polysomes for 4 hr at 4°C. Immunoadsorbed polysomal mRNA was prepared by successive chromatography on protein A-Sepharose and oligo(dT)-cellulose (22). Approximately 100 ng of purified mRNA was used to synthesize double-stranded cDNA by the RNA replacement synthesis method (24); the cDNA was tailed with oligo(dC) residues and inserted into *Pst* I-cut, oligo(dG)-tailed pBR322.

Abbreviations: ECM, extracellular matrix; RSF, rabbit synovial fibroblasts; PMA, phorbol 12-myristate 13-acetate.

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**Hybrid-Selected Translation.** Hybrid-selected translation was performed as described by Miller *et al.* (25). Briefly, 50 μg of total RNA was hybridized with 10 μg of plasmid (immobilized on a nitrocellulose filter) in 60% formamide/10 mM Pipes, pH 6.4/0.4 M NaCl at 47°C. Filters were washed at 50°C in 2× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% $NaDodSO_4$ and then in 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$. An actin cDNA probe (26) used as a control was the gift of M. Kirschner (University of California, San Francisco).

**Colony Screening, Southern Blots, and RNA Hybridization Blots of Formaldehyde-Agarose Gels.** These procedures were performed according to the methods of Maniatis *et al.* (21). Hybridization was performed in 50% formamide/5× NaCl/Cit/50 mM phosphate buffer, pH 7/2× Denhardt's solution (21)/100 μg of sonicated salmon sperm DNA per ml at 42°C.

**Cytoplasmic Dot Hybridization.** Cytoplasmic dot hybridization (cytodot analysis) was performed on cells grown in 12-well culture dishes according to the method of White and Bancroft (27) except that the detergent composition of the lysis buffer was 0.5% Brij 35 and 0.5% deoxycholate instead of Nonidet P-40. In undiluted samples, cytoplasmic RNA from a maximum of $10^5$ cells was applied to dots on the nitrocellulose filters.

## RESULTS

**Molecular Cloning of Stromelysin and Collagenase cDNAs.** Nine of the 12 hybrid-selected RNAs encoded a protein of about 51 kDa. The translation product of the clone with the largest cDNA insert [800 base pairs (bp)] immunoprecipitated with anti-stromelysin antibody (13) but failed to immunoprecipitate with nonimmune IgG or anti-collagenase IgG (Fig. 1), which demonstrates that the insert encoded stromelysin. The hybrid-selected protein also had a partial peptide map and two-dimensional gel mobility similar to that of biosynthetically labeled prostromelysin (data not shown). The 800-bp *Pst* I insert was electroeluted from an agarose gel, nick-translated, and used to screen a second cDNA library. A stromelysin clone (pSL2) with a 1.2-kbp insert, whose restriction map is shown in Fig. 2, was obtained.

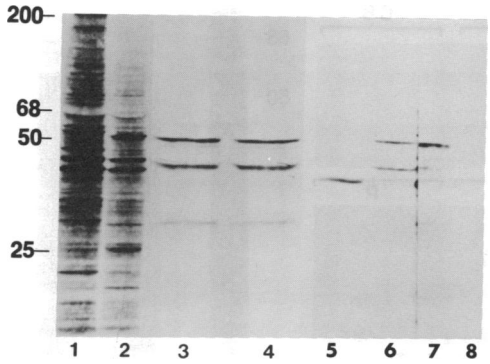


FIG. 1. Hybrid-selected translation analysis of stromelysin clone pSL2. $NaDodSO_4$/polyacrylamide gel analysis of rabbit reticulocyte lysate translation products from: RNA from untreated RSF (lane 1); RNA from PMA-treated RSF (lane 2); hybrid-selected RNA using the initial 800-bp stromelysin clone (lane 3); hybrid-selected RNA using pSL2 DNA (lane 4); as a control, hybrid-selected RNA using pA1, a chicken β-actin clone (lane 5); anti-stromelysin immunoprecipitate of 800-bp stromelysin clone-selected protein (lane 6); anti-stromelysin immunoprecipitate of pSL2-selected protein (lane 7); anti-collagenase immunoprecipitate of pSL2-selected protein (lane 8). Molecular weight markers (in kDa) are shown on the left. The lower band in the hybrid-selected translations is an endogenous rabbit reticulocyte lysate product that is observed in "minus RNA" translations (e.g., Fig. 3, lane 7).

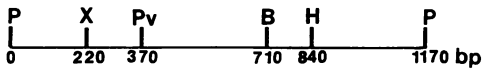


FIG. 2. Restriction map of pSL2 insert. The approximately 1.2-kbp insert from pSL2 was obtained by electroelution from an agarose gel of *Pst* I-digested pSL2 and was subjected to single- or double-enzyme restriction digestions, which were analyzed by both agarose and polyacrylamide gel electrophoresis to measure fragment sizes accurately. Molecular weight markers were *Hin*dIII-digested λ DNA, *Hae* III-digested PhiX DNA, and *Hin*fI-digested pBR322 DNA (Bethesda Research Laboratories). P, *Pst* I; X, *Xho* I; Pv, *Pvu* II; B, *Bst*EII; H, *Hin*fI.

*In vitro* translation of immunoprecipitated collagenase mRNA revealed a translation product of nearly homogeneous collagenase (Fig. 3). Plasmid DNA was purified from random recombinants in the cDNA library generated from purified collagenase mRNA and was subjected to hybrid-selected translation analysis (data not shown). During the course of this study, a rabbit collagenase cDNA clone with a 650-bp insert (28) was provided to us by C. Brinckerhoff (Dartmouth College). A positive cDNA clone from our library with an 800-bp insert (pCL1) that hybridized strongly to that collagenase cDNA and had a similar restriction map was used for further experiments. The collagenase and stromelysin cDNA clones (pCL1 and pSL2, respectively) failed to cross-hybridize.

**Coordinate Regulation of Stromelysin and Collagenase Expression.** Nick-translated stromelysin and collagenase cDNAs were hybridized to blots of RNA from untreated or PMA-treated RSF (Fig. 4); both RNA species (migrating at 2.2 and 2.4 kb, respectively) were highly inducible by PMA. The size of collagenase mRNA was similar to that reported previously (28, 29).

The kinetics of the induction of the stromelysin and collagenase genes by PMA were compared by hybridizing dot blots of total cytoplasmic RNA (cytodots), which were normalized to the DNA content per nuclear pellet. Stromelysin and collagenase RNAs appeared by 8 hr after the addition of PMA (Fig. 5*a*) and accumulated with strikingly similar kinetics. After treatment with cytochalasin B, the induction kinetics were slower; stromelysin mRNA and collagenase mRNAs were not detected until 16 hr after cytochalasin B addition. Similar results were obtained when

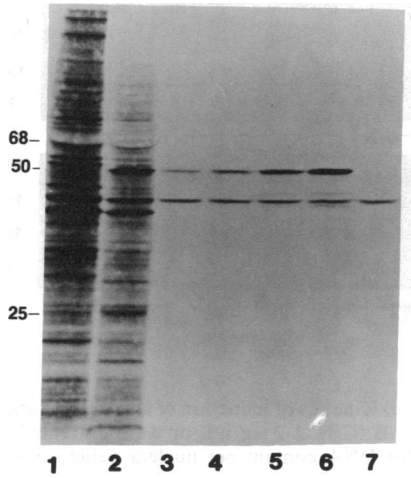


FIG. 3. *In vitro* translation of immunoprecipitated collagenase mRNA. $NaDodSO_4$/polyacrylamide gel analysis of 5-μl aliquots of 15-μl translation reactions: 2 μg of total RNA from untreated RSF (lane 1); 2 μg of total RNA from PMA-treated RSF (lane 2); 1 or 3 μl of collagenase mRNA from preparation 1 (lanes 3 and 4); 1 or 2 μl of collagenase mRNA from preparation 2 (lanes 5 and 6); minus RNA translation (lane 7).

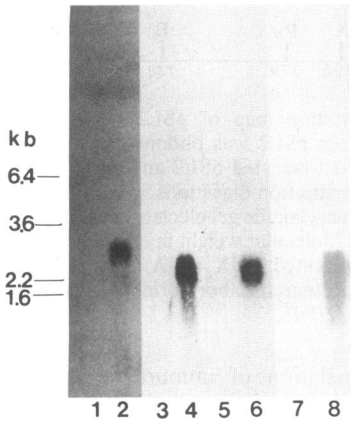


FIG. 4. Blot-hybridization analysis of stromelysin and collagenase mRNAs. Ten micrograms of total RNA from untreated RSF (lanes 1 and 3) or RSF treated with PMA (100 ng/ml) for 24 hr (lanes 2 and 4) were fractionated on a formaldehyde/1.2% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pCL1 insert DNA (lanes 1 and 2) or pSL2 insert DNA (lanes 3 and 4). Molecular weight markers (at left) are yeast, *Escherichia coli*, and rabbit ribosomal RNAs (P-L Biochemicals). Lanes 5–8 show RNAs from rabbit alveolar macrophages (lanes 5 and 6) or rabbit brain capillary endothelial cells (lanes 7 and 8) hybridized with pSL2 DNA. Cells were untreated (lanes 5 and 7) or treated with PMA (20 ng/ml) for 24 hr (lanes 6 and 8). Autoradiographic exposures were 5 hr, except for lanes 5 and 6, which were 30 hr.

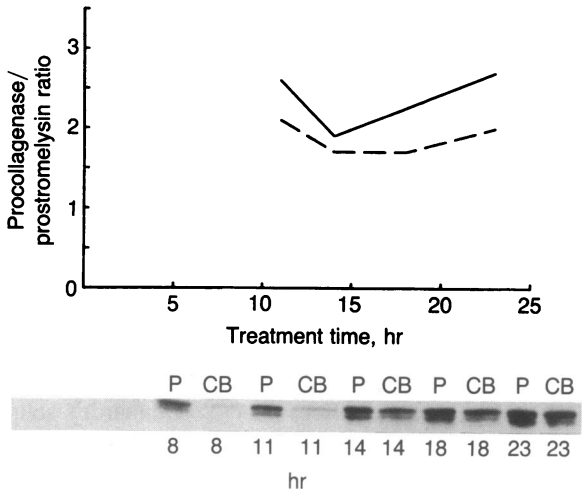


FIG. 6. Kinetics of induction of stromelysin and collagenase protein. RSF were treated with PMA (P) at 100 ng/ml or cytochalasin B (CB) at 1 μg/ml, and secreted proteins were labeled for 2 hr with [$^{35}$S]methionine in methionine-free medium containing one of the inducers. (*Lower*) The secreted proteins were analyzed on a 10% $NaDodSO_4$/polyacrylamide gel, and the bands corresponding to procollagenase (upper bands) and prostromelysin (lower band) were identified (13, 16). (*Upper*) The calculated ratio of procollagenase/prostromelysin protein obtained from a Hoefer densitometer scan is plotted. ——, Cytochalasin B; ---, PMA.

the synthesis of stromelysin and collagenase proteins were studied directly; the ratio of collagenase/stromelysin protein was about 2:1 throughout the induction period (Fig. 6).

The long lag period preceding the appearance of stromelysin and collagenase mRNAs led us to ask whether the induction of stromelysin and collagenase was a direct con-

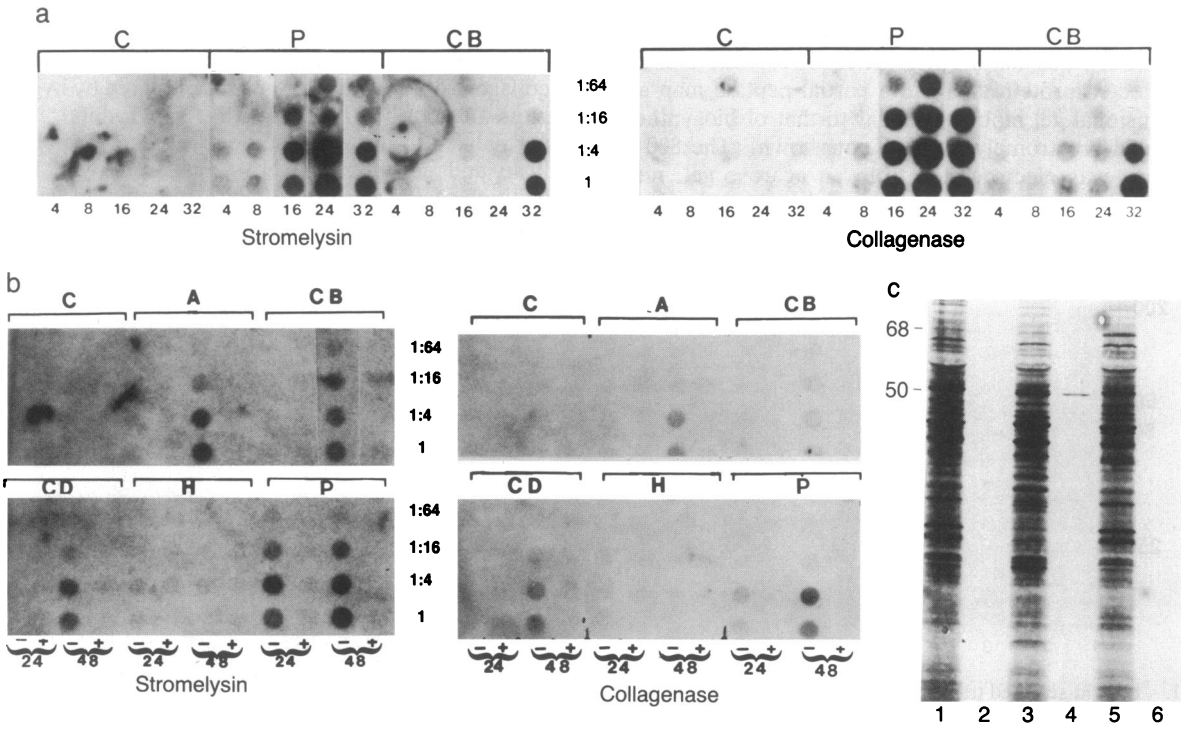


FIG. 5. (*a*) Kinetics of induction of stromelysin and collagenase mRNA expression. RSF were treated for 4–32 hr with PMA (P) at 100 ng/ml, cytochalasin B (CB) at 2 μg/ml, or were untreated (C), and cytoplasmic RNAs were prepared (21). The sample volumes were adjusted to normalize for DNA content per nuclear pellet, and 1:4 serial dilutions were applied to the nitrocellulose filter, which was hybridized with nick-translated pSL2 or pCL1 insert DNA. Time of treatment (in hours) is given below each lane. (*b*) Effect of cycloheximide on induction of stromelysin and collagenase mRNAs in response to various inducers. RSF were treated with PMA (P) at 100 ng/ml, A23187 (A) at 20 ng/ml, cytochalasin B (CB) at 2 μg/ml, cytochalasin D (CD) at 2 μg/ml, 1X HB101 protein supplement (H), or were untreated (C) for 24 or 48 hr in the presence (+) or absence (−) of cycloheximide at 10 μg/ml. Cytoplasmic RNAs were prepared, normalized to DNA content per nuclear pellet, and hybridized with nick-translated pSL2 or pCL1 insert DNA. Time of treatment (in hours) is given below each pair of lanes. (*c*) *In vitro* translation of RNA from cycloheximide-treated RSF. RNAs (5 μg) isolated from RSF treated with cycloheximide alone (as in *b*) (lane 1), PMA alone (lane 3), or PMA and cycloheximide (lane 5) were translated in reticulocyte lysates. Lanes 2, 4, and 6 are S99 anticollagenase immunoprecipitates of lanes 1, 3, and 5, respectively.

sequence of interaction of the inducer with RSF or whether the induction of these genes might be a secondary effect resulting from the induction of "inducer" genes. Inhibition of protein synthesis with cycloheximide blocked induction of stromelysin and collagenase by PMA, cytochalasins B and D, and an additional inducer, the calcium ionophore A23187 (30). The serum-free growth medium HB101 (New England Nuclear), which increases overall protein synthesis and secretion (14), was used as a control for PMA (Fig. 5*b*). RNA was obtained in normal yield from the cycloheximide-treated RSF, and it translated *in vitro* with normal efficiency (Fig. 5*c*); in fact, inspection of the translations shows that certain mRNAs that were absent in PMA-treated RSF appeared in RSF treated with cycloheximide or cycloheximide and PMA. Thus, the cycloheximide blockage of PMA-inducible stromelysin and collagenase mRNAs was unlikely to be due to a generalized RNA degradation process. These results are consistent with an indirect signal transduction pathway, using newly synthesized proteins, for all the inducers tested.

**Expression of Stromelysin mRNA in Rabbit Alveolar Macrophages and Rabbit Brain Capillary Endothelial Cells.** Two other cell types known to express collagenase and stromelysin when appropriately stimulated, rabbit alveolar macrophages (15) and rabbit brain capillary endothelial cells (11, 14), were tested for stromelysin expression by RNA blot-hybridization analysis. Lanes 5–8 of Fig. 4 show that both cell types expressed 2.2-kb stromelysin mRNAs when they were treated with PMA, but untreated cells did not. The macrophages expressed 10–20 times less stromelysin mRNA on an RNA basis than did the RSF or endothelial cells; this is in keeping with the much lower amounts of enzyme protein and activity seen for both collagenase and stromelysin (data not shown). The expression of different stromelysin mRNAs having various degrees of homology with RSF stromelysin mRNA cannot be ruled out by these data.

## DISCUSSION

We report here the molecular cloning by differential hybridization of cDNA encoding part of stromelysin, a broad-specificity secreted metalloproteinase (13). We have also isolated a collagenase cDNA clone, which cross-hybridizes with a previously reported clone (28), by using the strategy of collagenase mRNA purification by immunoadsorption of polysomes. Cloning and sequencing of a full-length cDNA for human collagenase has been reported recently (29), and it has been shown to be related, at the amino acid level, to transin, a gene induced in Fisher rat 3T3 cells by transformation, growth factors, and cytochalasin B (31). Our preliminary data indicating that transin cDNA cross-hybridizes with our stromelysin cDNA suggest that these two proteins may be very similar (unpublished observations).

Stromelysin and collagenase mRNAs were induced coordinately with several inducers, including PMA and cytochalasin B; however, the kinetics of induction for both mRNA species were slower with cytochalasin B than with PMA. Combined with the observation that every agent that induced collagenase protein also induced stromelysin (13, 17, 32), this finding suggests tight coordinate regulation of the collagenase and stromelysin genes. This might possibly arise from a common signal transduction pathway operating on both genes. It is unlikely that stromelysin and collagenase are related to each other by a protein processing mechanism because (*i*) stromelysin and collagenase proteins are distinct immunoprecipitable products of *in vitro* translations using mRNA from PMA-treated RSFs, (*ii*) stromelysin cDNA failed to cross-hybridize with collagenase cDNA, (*iii*) anti-stromelysin and anti-collagenase antibodies fail to cross-react (13), and (*iv*) peptide maps of stromelysin and collagenase are distinct (13).

The lag time of appearance of stromelysin and collagenase mRNAs after treatment of cells (at least 4 hr for PMA and at least 8 hr for cytochalasin B) was unusually long. In contrast, the rate of mouse mammary tumor virus mRNA synthesis is stimulated within minutes after the addition of dexamethasone, and maximal accumulated mRNA levels are reached within about 2 hr (33). The mouse metallothionein-I (MT-I) gene is stimulated to produce maximal MT-I mRNA at about 3 hr after the addition of cadmium (34). The long lag period in our system seems diagnostic of an indirect induction mechanism and suggests that the synthesis of new signal transduction proteins is involved in the induction of proteinase genes. The experiments with cycloheximide support this view. A general mRNA degradation elicited by cycloheximide seems unlikely because (*i*) RNA was reproducibly obtained in normal yield from cycloheximide-treated RSF, and it translated *in vitro* with normal efficiency and (*ii*) reports in the literature using similar cycloheximide treatments show superinduction or no inhibition for other genes examined (35). Our results are consistent with those of Rabin *et al.* (36) who found that the PMA induction of a 38-kDa protein of unknown function called MEP was also indirect. The induction of the transin gene by various agents is also inhibited by cycloheximide (37).

Because we observed the induction of stromelysin mRNA in three distinct cell types—RSF, macrophages, and endothelial cells—it will be of interest to compare the regulatory pathways used in various cell types. Determining the nature of the putative inducer protein(s) is of considerable interest. It has previously been reported (3, 17, 32) that all the agents capable of inducing the collagenase gene also altered RSF morphology including a reorganization of actin microfilaments. A signal transduction mechanism involving obligatory actin cytoskeletal reorganization was postulated (3, 17), but it is unlikely that the putative inducer protein causes the cytoskeletal reorganization because cycloheximide does not prevent cells from responding morphologically to PMA or cytochalasin B (unpublished observations).

**Note.** Recently we have observed significant cross-hybridization and sequence homology between our stromelysin clone and the rat cDNA clone pTR-1 of Matrisian *et al.* (31). The mRNA that hybridizes with pTR-1 was reported to be induced by epidermal growth factor, phorbol esters, cytochalasin B, and transformation by *ras* or *src* oncogenes (37), and its mRNA induction was inhibited by cycloheximide. Thus, the "transin" protein encoded by pTR-1 mRNA is likely to be the rat homologue of rabbit stromelysin, implying that the stromelysin gene is transformation-induced.

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