Regulation of collagenase gene expression by IL-1 β requires transcriptional and post-transcriptional mechanisms

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ABSTRACT

Interleukin-1 β is believed to contribute to the pathophysiology of rheumatoid arthritis by activating collagenase gene expression. We have used a cell culture model of rabbit synovial fibroblasts to examine the molecular mechanisms of IL-1 β -mediated collagenase gene expression. Stimulation of rabbit synovial fibroblasts with 10 ng/ml recombinant human IL-1 β resulted in a 20-fold increase in collagenase mRNA by 12 h. Transient transfection studies using collagenase promoter – CAT constructs demonstrated that proximal sequences responded poorly to IL-1 β , possibly due to insufficient activation of AP-1 by this cytokine. More distal sequences were required for IL-1 β responsiveness, with a 4700 bp construct showing ~5-fold induction above control. To examine post-transcriptional mechanisms, transcript from a human collagenase cDNA was constitutively produced by the simian virus 40 early promoter. IL-1 β stabilized the constitutively expressed human transcript. Furthermore, mutation of the ATTTA motifs in the 3' untranslated region of the human gene also stabilized the transcript. Finally, the rabbit collagenase 3' untranslated region destabilized a constitutively transcribed chloramphenicol acetyltransferase transcript. These data indicate that in addition to activating transcription, IL-1 β increases collagenase transcript stability by reversing the destabilizing effects of sequences in the 3' untranslated region.

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of enzymes which degrade components of the extracellular matrix, thereby regulating connective tissue turnover (1). One member of this family, interstitial collagenase (MMP-1, E.C. 3.4.24.7), is responsible for degradation of collagens type I, II, and III within the extracellular matrix and is essential for efficient restructuring of this tissue (2,3). This enzyme is produced by a variety of cells in the body such as fibroblasts (4,5), macrophages (6), endothelial cells (7), keratinocytes (8) and chondrocytes (9) and plays a major

role in normal processes such as wound healing (10) and uterine resorption (11). Collagenase is inappropriately expressed in disease states such as tumor invasion (12) and rheumatoid arthritis (13). Indeed, synovial fibroblasts from the joints of patients with rheumatoid disease secrete high levels of collagenase (14) which contributes to cartilage degradation, bone resorption and ultimately joint destruction (15,16).

In cultured fibroblasts, collagenase can be induced by the protein kinase C agonist phorbol myristate acetate (PMA) (4,17), or more physiologically relevant stimuli such as urate crystals (18), IL-1 (19,20), TNF- α (21) and EGF (22). Of these agents, IL-1 is known to mediate cartilage degradation (23,24) and overexpression of this cytokine is observed in patients with chronic inflammation and connective tissue disease (22,23,25). Although IL-1 induction of collagenase is a major pathway in extracellular matrix degradation, relatively little is known of the molecular mechanisms by which this cytokine activates the collagenase gene.

In vitro, recombinant IL-1 β induces collagenase steady-state mRNA levels in cultured human and rabbit synovial fibroblasts (25–27), and this is due in part to increased transcription of the collagenase gene (26). The transcriptional response may involve the activator protein-1 (AP-1) binding site (5,28,29), which is necessary but not sufficient for mediating the PMA response in primary fibroblasts (30). However, regulation of the collagenase promoter by IL-1 may be more complex. A 73 bp human promoter construct containing the AP-1 site has been reported to respond to IL-1 stimulation, but when additional upstream sequence is added, IL-1 inducibility is dramatically reduced (31). Furthermore, a mutant form of IL-1 β synthesized *in vitro* induced the genes for c-Fos and c-Jun, without increasing expression of the collagenase gene, suggesting that AP-1 may not play a major role in IL-1 induction (32). These studies suggest that IL-1 does not activate collagenase gene expression through the same transcriptional mechanisms as PMA.

Recent data suggest that post-transcriptional processes also contribute to collagenase gene expression. The half-life of collagenase mRNA is modulated by PMA (33) and EGF (22). In epithelial cells, collagenase mRNA is undetectable in unstimulated cells, despite a transcriptionally active gene (34).

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One key to understanding the mechanisms of post-transcriptional regulation of collagenase may lie in sequence homology to other genes. The 3' untranslated region (UTR) of the collagenase gene contains three repeats of the sequence ATTTA (28), which has been shown by several laboratories to regulate the stability of cytokine and proto-oncogene mRNAs (35,36). These ATTTA elements are conserved in the human and rabbit collagenase genes (5,28), suggesting that they may play a role in gene regulation.

In the present study, we used a model of rabbit synovial fibroblasts (RF) to study the regulation of collagenase gene expression by IL-1 β . These cells resemble primary cultures of rheumatoid synovial cells in their response to experimental stimuli (4). Importantly, in addition to conservation of the ATTTA motif in their 3' UTRs, the human and rabbit collagenase genes share extensive homology in their promoter regions (28,29). We demonstrate that IL-1 induction of collagenase gene expression involves a modest transcriptional increase, which is accompanied by an increase in mRNA stability.

MATERIALS AND METHODS

Cell culture

Rabbit synovial fibroblasts were prepared as described previously (18). Cells were grown to confluency, then washed three times with HBSS to remove traces of serum, and placed in DMEM containing 0.2% lactalbumin hydrolysate (DMEM-LH). Cells were left unstimulated, or treated with 10⁻⁸ M PMA or 10 ng/ml recombinant IL-1 β (graciously provided by Immunex Research and Development Corp., Seattle, WA). Cells were used between the third and seventh passage.

Plasmids constructs

Plasmids containing between 1176 and 127 bp of the rabbit collagenase promoter linked to the chloramphenicol acetyl transferase gene (CAT) have been described previously (30). Larger promoter constructs were generated from a 4700 bp *HindIII* fragment of the collagenase promoter cloned into pUC18. A 2400 bp fragment was produced by *BglIII* digestion within the 4700 bp promoter, and a second 3900 bp construct was made by *PstI* digestion. The sticky ends produced from these digestion were filled in, *HindIII* linkers were added and the ends were ligated. Subsequently, partial digestion with *HindIII* produced the 2400 bp, 3900 bp and 4700 bp fragments of the rabbit collagenase gene promoter that were suitable for subcloning into the unique *HindIII* site within the multiple cloning site of the vector pSV0-CAT. pGemB-R contains a fragment consisting of 600 bp of the rabbit collagenase promoter and 251 bp of the CAT coding sequence cloned into the multicloning site of pGem-3 (Promega, Madison, WI). H9 is a cDNA corresponding to the terminal 530 bp of the rabbit collagenase gene cloned into pBR322. This fragment codes for the terminal 23 amino acids and contains the entire 3' UTR (18). The human glyceraldehyde phosphate dehydrogenase (GAPDH, ATCC) cDNA was used as a loading control for Northern analysis. The human collagenase expression plasmid pSG5HuCol (see below) was constructed by cloning the 1700 bp of the human collagenase cDNA into the *EcoRI* site of pSG5 (37). The 1700 bp human collagenase fragment was also cloned into pSG5 in the opposite orientation for riboprobe synthesis.

The ATTTA elements within pSG5HuCol were mutated to GCCCG by oligonucleotide-directed mutagenesis using the

Altered Sites Mutagenesis Kit (Promega, Madison, WI). To construct the plasmid SV₂CATH9, SV₂CAT was digested with *HpaI/BamHI* which removed the SV40 polyadenylation site. The vector was blunted with T4 polymerase and re-ligated with *BglIII* linkers (pSV₂CAT Δ PA). An *EcoRI* fragment of H9 was cloned into pBsSK⁺ (Stratagene, La Jolla, CA) and then removed as a *BglIII-BamHI* fragment. This fragment was then cloned into the *BglIII* site of pSV₂CAT Δ PA to give rise to SV₂CATH9. The rabbit sequence of SV₂CATH9 contains a polyadenylation site to replace that of SV₂CAT.

Assay of collagenase steady-state mRNA

Total cellular RNA was isolated using the guanidinium thiocyanate-CsCl method (38). Total RNA was quantitated by optical density, and 3 μ g of RNA was subjected to Northern analysis as previously described (39). Northern blots were hybridized with an [α -³²P]dCTP-labeled rabbit collagenase cDNA (H9) (18) for 20 h at 56°C. Blots were washed twice for 10 min at room temperature, followed by two 30 min washes at 56°C (0.2 $\times$ SSC, 0.5% SDS). Collagenase-specific mRNA was detected by autoradiography, and then the blots were stripped and reprobed with the housekeeping gene GAPDH. Autoradiographs were scanned using an E-C Apparatus Corp. scanning densitometer with a Hewlett-Packard 3390A integrator within the linear range of detection.

Transfection and CAT assay

Rabbit synovial fibroblasts were transfected using the calcium phosphate co-precipitation method as we have previously described (30). Briefly, 5 μ g of chimeric collagenase promoter-CAT constructs were transfected with calcium phosphate for 6 h, followed by a 3 min shock with 10% glycerol. Cultures were allowed to recover overnight, then washed and placed in DMEM containing 0.2% lactalbumin hydrolysate. Since the transfected cells were treated with IL-1 and PMA under serum-free conditions, the proliferative effects of these agents were not seen. Some cultures were stimulated with PMA (10⁻⁸ M) or IL-1 β (10 ng/ml) for 24 h. Cultures were then harvested, lysed by freeze-thaw, and total cellular protein was determined by the Bradford method (Bio-Rad). Three micrograms of protein were incubated in a CAT assay mixture for 1 h at 37°C, and acetylated [¹⁴C]chloramphenicol was separated from non-acetylated by TLC. Acetylated and non-acetylated ¹⁴C was quantitated by scintillation counting, and relative CAT activity was expressed as percent incorporation. All assays were within the linear range of the assay. Relative transfection efficiencies were determined by the Hirt's assay (40).

RNAse protection assay

The induction of mRNA from transfected collagenase promoter-CAT constructs was measured by RNAse protection assay (41). Briefly, 20 μ g of total RNA from transfected cells was hybridized to a radiolabeled CAT-specific antisense riboprobe overnight at 60°C with shaking. This antisense probe was transcribed from the pGemB-R plasmid linearized with *HindIII* using T7 polymerase and the Riboprobe *in vitro* transcription kit (Promega, Madison, WI). The hybridization products were subjected to digestion with RNAse A (40 μ g/ml) and RNAse T1 (2 μ g/ml) and protected fragments were separated on a sequencing gel. Samples containing no RNA or RNA from untransfected cells were included as negative controls. Expression

from the transfected human collagenase expression vector was also detected in rabbit fibroblasts by RNase protection. The probe used in these studies was derived from a plasmid in which the human cDNA was cloned into pSG5 in the opposite direction. This plasmid was linearized at the *Xba*I site within the cDNA. When this linearized plasmid was driven by the T7 RNA polymerase promoter, a 402 nucleotide antisense riboprobe was synthesized corresponding to the human 3' UTR. For detection of the mutated human collagenase transcript (pSG5HuCol μ), the mutated cDNA was cloned into pSG5 in the opposite direction and an antisense riboprobe was prepared as described above. For detection of SV₂CAT and SV₂CATH9 transcripts, the CAT-specific riboprobe described above was used.

Western blot analysis of collagenase protein

One milliliter of serum-free conditioned media from stimulated cultures was precipitated with 0.5 ml of cold 10% TCA for 30 min on ice. Protein was pelleted for 10 min at 15,000 $\times g$ at 4°C, washed with 0.1 M potassium acetate/95% ethanol, followed by an ethanol wash. Proteins were resuspended in sample buffer and separated by reducing SDS-PAGE (39). Gels were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot Cell (Bio-Rad). Collagenase protein was detected as previously described (27). Briefly, the membranes were blocked for 1 h with 10% FCS, then probed with rabbit collagenase-specific antiserum (27) at a dilution of 1:10,000 for 3 h. The membranes were then washed and specific antibody binding was detected with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Assay of DNA binding proteins

Nuclear protein extracts were prepared as previously described (42). Two 150 mm confluent plates of RF were stimulated as described, harvested and washed twice in Tris-buffered saline, pH 7.9. Cells were then resuspended in 400 ml of cold buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 1 mM PMSF) using a P200 pipetteman tip. After allowing the cells to swell on ice for 15 min, the cells were lysed by the addition of NP40 (final concentration 0.6%). Lysis was completed by vortexing vigorously for 10 s. The homogenate was centrifuged for 30 s in a microfuge and the nuclear pellet was resuspended in 50 μ l of cold buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM PMSF; 1 mM DTT). This suspension was agitated at 4°C for 15 min, and then pelleted in a microfuge for 5 min at 4°C. The resulting supernatant was removed and stored in small aliquots at -70°C. Protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA). For mobility shift assays, 5 μ g of nuclear extract was incubated for 15 min on ice in the presence of 2 μ g poly(dI:dC), 1 μ g BSA, 1 μ g salmon sperm DNA, 10% glycerol, 25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.1 M NaCl and 10,000 c.p.m. of ³²P-labeled AP-1 or mutant AP-1 oligonucleotide.

AP-1 oligonucleotide: 5'-GAAAGCATGAGTCACACAGCCCTCAGCT-3'
3'-CTTTCGTACTCAGTGTGTCGGGAGTCGA-5'
Mutant AP-1 oligonucleotide: 5'-GAAAGCAGGAGTCACACAGCCCTCAGCT-3'
3'-CTTTCGTCTCAGTGTGTCGGGAGTCGA-5'

Following a 15 min incubation at 37°C, samples were loaded onto a 4% polyacrylamide gel (1 $\times$ TBE) and electrophoresed at 150 V at 4°C. The gel was then dried and visualized by autoradiography.

RESULTS

IL-1 β increases secreted collagenase protein and steady-state levels of collagenase mRNA in rabbit synovial fibroblasts

We first confirmed that human IL-1 β could induce collagenase in rabbit fibroblasts. Confluent cultures of RF were placed in serum-free media and either left untreated, or treated with 10 nM PMA or various concentrations of IL-1 β . After 48 h, conditioned media were harvested and subjected to Western blot analysis for rabbit collagenase (Figure 1, panel A). IL-1 β increased production of collagenase protein in a dose-dependent manner, and the level of induction observed between 10 and 100 ng/ml IL-1 β was similar to that seen with PMA. To determine if this correlated with an increase in collagenase steady-state

Figure 1. Interstitial collagenase gene expression in rabbit synovial fibroblasts. (A) RF were cultured to confluency in DMEM plus 10% FCS, washed with HBSS, then cultured for 48 h in DMEM plus 0.2% lactalbumin hydrolysate (LH), LH plus PMA (10 nM) or LH plus IL-1 β at the concentrations indicated. One milliliter of conditioned media was precipitated with TCA, subjected to reducing SDS-PAGE, and transferred to PVDF filters. Collagenase protein was visualized using a monospecific antibody previously described (27) (see Materials and Methods). (B) Confluent cultures of RF were washed with HBSS and placed in DMEM-LH. PMA (10 nM) or IL-1 β (10 ng/ml) was added to selected cultures. At the time points indicated, total RNA was isolated and subjected to Northern analysis. Collagenase mRNA was detected using the rabbit collagenase cDNA H9 and visualized with autoradiography. Blots were then stripped and probed with the housekeeping gene GAPDH as a loading control.

mRNA, Northern blot analysis was performed on total RNA isolated from RF at various time points after stimulation with IL-1 β (Figure 1, panel B). Steady-state levels of collagenase mRNA in untreated cultures remained undetectable for 12 h, with a slight increase seen after 24 h under serum-free conditions. In contrast, cultures stimulated with 10 ng/ml IL-1 β demonstrated a rise in collagenase mRNA after 6 h, with peak induction at 12 h. Since unstimulated cultures showed no detectable message after 12 h, the maximal fold induction by IL-1 could not be determined. In other experiments, basal levels of collagenase mRNA were detectable at 12 h, and densitometric analysis showed 20-fold induction above control levels in IL-1 β -treated cells (data not shown). At 24 h, IL-1 β -stimulated collagenase mRNA began to decline and by 48 h, the signal was only 38% of the maximal level.

For comparison, in cells treated with PMA, collagenase mRNA levels rose more slowly, but continued to increase over 48 h. At 48 h, densitometric analysis showed ~20-fold induction above control levels in PMA-treated cells. These data confirm that increases in collagenase mRNA are paralleled by an increase in collagenase protein (18) and demonstrate that recombinant human IL-1 β can induce collagenase gene expression to a level comparable to that seen in PMA-stimulated cells. However, the differential time course for these two stimuli suggests different mechanisms of action.

The rabbit collagenase promoter is less responsive to IL-1 β than PMA

The increase in steady-state levels of collagenase mRNA has been attributed previously to transcriptional activation of the collagenase promoter (26,29,30). To determine if sequences in the collagenase promoter are responsive to IL-1 β , RF were transiently transfected with 5' deletion fragments of the collagenase promoter linked to the CAT reporter gene (30). As reported (30), we found that constructs containing 1176, 321, 182 or 127 bp of the collagenase 5' flanking region respond well to PMA (Figure 2, panel A). In contrast, IL-1 β activated only the 1176 bp constructs, and this was ~3-fold above control,

Figure 2. Expression of transiently transfected collagenase promoter constructs in RF. (A) RF were transfected in triplicate with pcCAT clones containing 1176, 321, 182, and 127 bp of the collagenase promoter using the calcium phosphate co-precipitation method. The cells were allowed to recover for 18 h in serum-containing media. Cultures were washed with HBSS, placed in LH and then left untreated, or treated with PMA (10 nM) or IL-1 β (10 ng/ml). Twenty four hours later, cytoplasmic protein was isolated and CAT activity was assayed. The percent incorporation for unstimulated cultures ranged from 4.2% (pcCAT1176) to 0.5% (pcCAT127), and remained within the linear range of the assay for induced cultures. CAT activity for PMA- and IL-1 β -stimulated cells is presented as fold induction above control. (B) Constructs containing 4700, 3900, 2400 and 1800 bp of the collagenase promoter were assayed for their ability to drive CAT gene expression in response to 10 nM PMA or 10 ng/ml IL-1 β . Induction is presented as fold above unstimulated cultures. (C) Rabbit synovial fibroblasts were transfected with constructs containing 4700, 1800 and 1200 bp of the collagenase promoter, and then allowed to recover for 18 h in serum-containing media. The cultures were then washed with HBSS and placed in LH, (C) or LH containing either 10 nM PMA (P) or 10 ng/ml IL-1 β (IL). After 24 h, total RNA was isolated, and CAT-specific mRNA was assayed by RNase protection assay as described in the Materials and Methods section. The molecular weight markers (MW) used consisted of a kinase labeled 1 kb DNA ladder (BRL, Bethesda, MD). The 'No Plasmid' lanes refer to RNA samples isolated from untransfected cultures. Samples were assayed by Northern analysis to ensure equal loading (Data not shown).

despite the fact that the endogenous gene was activated to a level comparable to that seen with PMA (data not shown). Thus proximal promoter sequences which can drive collagenase gene transcription in response to PMA do not respond to IL-1 β .

We next tested more distal regions which contained as much as 4700 bp of the collagenase promoter (Figure 2, panel B). All distal promoter constructs responded well to induction with PMA. In contrast to studies with the 1176 bp promoter, transcriptional induction by IL-1 β improved as larger segments of the promoter were included. A clone containing 4700 bp of collagenase 5' flanking sequence demonstrated ~5-fold induction above unstimulated levels in response to IL-1 β . A similar level of transcriptional activation was also observed in nuclear run-on analysis of IL-1 β -treated cells (data not shown).

We used RNase protection assays to confirm that the observed levels of CAT protein represented increases in CAT mRNA.

Figure 3. Activation of AP-1 binding activity and *c-jun* gene expression in RF in response to PMA and IL-1 β . (A) Nuclear extracts were isolated from untreated, PMA-treated or IL-1 β -treated RF at 6 and 24 h. Five micrograms of extract was incubated with a 32 P-labeled oligonucleotide corresponding to -83 to -57 of the collagenase promoter, which contains the AP-1 site. The mutant AP-1 oligo has a T $\rightarrow$ G transversion which has been shown to ablate DNA binding activity (29). DNA-protein complexes were run on a 4% polyacrylamide gel and exposed to X-ray film for 48 h. (B) RF were untreated or stimulated with 10 nM PMA or 10 ng/ml IL-1 β and total RNA was isolated at the time points indicated. Total RNA was subjected to Northern analysis and probed with a radiolabeled *c-jun* cDNA (ATCC). Equal loading of total RNA is demonstrated by ethidium bromide stained 18S and 28S ribosomal RNA.

(Figure 2, panel C). As was seen in the CAT assays, PMA and IL-1 induced CAT gene expression from the 4700 bp construct, and this induction was considerably more pronounced in PMA-treated cells. In contrast, cells transfected with the smaller promoter constructs (1800 and 1176 bp) showed CAT transcript only in PMA-treated cells. Interestingly, CAT activity, but not CAT transcript, was detectable in cells transfected with the 1176 bp construct and stimulated with IL-1 β (Figure 2, panels A and C). This is probably due to the fact that CAT protein is very stable and serves as a record of total transcriptional activity from transiently transfected plasmids. In contrast, expression of the CAT transcript is a more transient event. It is therefore likely that the small amount of transcript produced by the 1176 bp construct declined below the level of detection of the RNase protection assay. This is supported by our finding that the induction of collagenase mRNA by IL-1 β is transient, and declines after 12 h (Figure 1, panel B). Taken together, the CAT assay and RNase protection data demonstrate that compared to stimulation with PMA, IL-1 β treatment results in only a moderate level of transcriptional activation which depends on the presence of additional upstream promoter sequences.

Expression of AP-1 binding activity and *c-jun* mRNA in IL-1 β -treated cells

The transient transfection experiments with the collagenase promoter constructs indicated that PMA and IL-1 β utilize different promoter elements for transcriptional activation. While the transactivation of collagenase by phorbol esters is mediated through the activating protein (AP-1) binding site located in the proximal promoter (29,30), the proximal constructs containing an intact AP-1 site do not respond well to IL-1 β (Figure 2, panels A and B). To examine if this is due to repression, or a lack of AP-1 binding activity, nuclear extracts from PMA- or IL-1 β -treated cells were assayed by mobility shift assay (Figure 3, panel A). There is no detectable AP-1 activity after 6 h of culture in serum-free media, or in cells treated for 6 h with IL-1 β . Cells treated with PMA show AP-1 binding activity after 6 h, and by 12 h this represents a 12-fold increase above control levels. However, in contrast to PMA, cells cultured for 12 h in IL-1 β show only minimal induction above control levels.

Figure 4. Expression of SV₂CAT in rabbit synovial fibroblasts. Rabbit synovial fibroblasts were transfected with SV₂CAT and cultured in DMEM plus 10% FCS for 18 h. Cultures were then washed with HBSS and placed in LH. Triplicate cultures were then treated with nothing (control), PMA (10 nM) or IL-1 β (10 ng/ml). Twenty four hours later, cytoplasmic protein was isolated and CAT activity was assayed.

A mutant AP-1 probe, containing a T→G transversion at nucleotide -77 of the collagenase promoter, was also tested. This mutation has been shown to ablate AP-1 binding at its recognition site (29). PMA-treated extracts did not bind to the mutant AP-1 probe, indicating that the binding activity observed with the wild-type probe was specific (43). Thus we conclude that IL-1 β is not a strong inducer of AP-1 binding activity in RF.

The AP-1 protein is composed of homodimers of members of the Jun family of transcription factors, or heterodimers of Jun and Fos proteins (44). Activation of AP-1 binding by phorbol esters is mediated through post-translation (45,46) modification of Fos and Jun, as well as transcriptional activation of the genes for these transcription factors (47). To determine if the low level of AP-1 activity observed in IL-1 β -treated cells was at the pre- or post-translational level, we assayed *c-jun* mRNA in RF (Figure 3, panel B). In unstimulated cells, there is a detectable increase in *c-jun* mRNA after 6 h of culture in serum-free media. Cells treated with PMA demonstrate a maximal increase in *c-jun* message at 2 h. The induction of *c-jun* by PMA is transient, and decreases by 40% at 6 h. In contrast to PMA-treated cells, IL-1 β -treated cells show no such increase at 2 h. After 6 h of IL-1 β treatment, *c-jun* mRNA is only 1.2-fold above that seen in control cultures. This is below the level of induction (2.5-fold) observed for the declining signal of PMA-treated cells. These data suggest that the minimal proximal promoter response and AP-1 activity observed in IL-1 β -treated cells is due, at least in part, to poor activation of the *c-jun* gene.

Post-transcriptional regulation of the collagenase gene

The discrepancy between the large increase in steady-state levels of collagenase mRNA and the modest transcriptional induction following IL-1 β treatment suggest that post-transcriptional mechanisms may be important in IL-1 β -dependent collagenase gene expression. Previous studies have shown that transcription inhibitors such as actinomycin-D and DRB either are toxic to

Figure 5. Expression of the transfected human collagenase gene pSG5HuCol in rabbit synovial fibroblasts. (A) To construct the clone pSG5HuCol, 1700 kb of the human collagenase cDNA was cloned into the *EcoRI* site of pSG5 (37) in the orientation shown. The 1700 bp fragment was also cloned in the opposite orientation, with the 3' UTR proximal to the T7 RNA polymerase promoter of pSG5. When linearized at the *XbaI* site the reverse orientation plasmid yields a template for a human collagenase 3' UTR-specific riboprobe. This riboprobe was used to detect human collagenase transcripts in pSG5HuCol-transfected rabbit synovial fibroblasts. The riboprobe did not protect endogenous transcripts from untransfected rabbit synovial fibroblasts (data not shown). (B) Rabbit synovial fibroblasts were transfected with pSG5HuCol and then cultured in DMEM plus 10% FCS for 18 h. Serum was then washed out and the cells were placed in LH or LH plus IL-1 β (10 ng/ml). At the time points indicated, total RNA was isolated by the guanidinium-CsCl method. Twenty micrograms of total RNA were analyzed by RNase protection assay and transcripts from the transfected human gene were visualized using the human 3' UTR-specific anti-sense riboprobe. The molecular weight markers (MW) used consisted of a kinase labeled 1 kb DNA ladder (BRL, Bethesda, MD), and the 'No RNA' lane corresponds to the riboprobe hybridized in the absence of cellular RNA. (C) Expression of the endogenous rabbit collagenase gene in pSG5HuCol-transfected rabbit synovial fibroblasts. Three micrograms of total RNA from the RNA samples analyzed in panel B were also subjected to Northern blot analysis. Expression of the endogenous rabbit collagenase gene was visualized by probing with the rabbit cDNA H9. Equal amounts of total RNA were used in the experiments depicted in panels B and C as demonstrated by ethidium bromide stained 18S and 28S ribosomal RNAs.

rabbit fibroblasts or stabilize the collagenase message (22, Vincenti, unpublished observations), thereby making these reagents unsuitable for studies of mRNA decay in this system. Instead, we placed the collagenase gene under the constitutive transcriptional control of the SV40 early promoter, and transiently transfected the SV40–collagenase chimeric constructs into rabbit fibroblasts. The SV40 promoter drives transcription at high levels in mammalian cells (48,49) and is not modulatable in primary fibroblasts by external stimuli (48). This approach eliminates transcriptional activation of collagenase by IL-1 β , so that the effects of IL-1 β on mRNA stability can be determined.

We confirmed our previous finding (48) that the SV40 promoter drives transcription of the CAT gene at high levels in primary rabbit fibroblasts, and that this level of transcription is not modulatable by PMA (Figure 4). Furthermore, the SV40 early promoter is not modulatable in RF by IL-1 β (Figure 4). Thus transient transfection of collagenase constructs under the transcriptional control of this promoter is an effective system for examining post-transcriptional regulation of the collagenase gene.

First we created the pSG5HuCol construct by placing 1.7 kb of the human collagenase gene under the constitutive transcrip-

tional expression of the SV40 promoter (Figure 5, panel A). The human collagenase cDNA was used for these experiments because sequence divergence in the 3' UTR of the human (5) and rabbit genes (28,50) allows specific detection of a human transcript in rabbit cells, but does not detect endogenous rabbit sequence. pSG5HuCol (Figure 5, panel A) was transfected into RF which were then allowed to recover in media containing 10% serum for 18 h (51). At this point, serum was removed and half of the cultures were treated with IL-1 β . Total RNA was harvested at various time points after serum wash-out and the stability of transfected gene transcripts was assayed by RNase protection assay (Figure 5, panel B). The figure shows that cells cultured for 18 h in serum contain high levels of human collagenase mRNA. After 4 h in serum-free conditions, the human transcript has not decayed in untreated or IL-1 β -treated cells. In untreated cells, this transcript decays to about one half of its original level after 12 h. In contrast, cells placed in serum-free media containing IL-1 β contained pSG5HuCol transcript levels at 12 h comparable to those at the 0 h time point. This IL-1 β -dependent enhancement of pSG5HuCol transcripts was concomitant with an IL-1 β -dependent rise in steady-state collagenase mRNA from the

Figure 6. Expression of the transfected wild-type and ATTTA mutant-containing human collagenase cDNA in RF. (A) Homology analysis of part of the coding sequence and the 3' UTR sequence of the human and rabbit collagenase genes using the University of Wisconsin GCG 'GAP' program. Each line represents a point of nucleotide identity. The stop codons are in bold and the polyadenylation signals are underlined. The consensus ATTTA sequences described by Shaw and Kamen (35) are in bold and underlined. (B) The wild-type and mutant pSG5HuCol cDNAs were transfected into RF and assayed as in Figure 5 using anti-sense riboprobes of the wild-type and mutant 3' UTRs.

endogenous rabbit gene (Figure 5, panel C). These results suggest that IL-1 β increases steady-state levels of collagenase mRNA by increasing the stability of the transcript.

Next we tested the role of the ATTTA motif in collagenase mRNA stability (35,36). The 3' UTRs of the human and rabbit collagenase genes each contain three copies of the mRNA instability motif ATTTA (Figure 6, panel A) and the conservation of this motif suggests that it may play a role in gene regulation. Thus the ATTTA elements of the human gene were mutated in the context of pSG5HuCol (see Figures 5A and 6A). The resulting clone, pSG5HuCol μ , was transiently transfected into RF, and some cultures were treated with IL-1 β . The stability of transcripts from the mutated human gene was then assayed by RNase protection (Figure 6 panel B). In untreated cells, the wild-type transcript decays between 5 and 10 h of culture in serum-free media, while it is stable during this time period in IL-1 β -treated cells. In contrast, transcripts from the construct with mutated ATTTA elements are stable between 5 and 10 h in untreated cells. In IL-1 β -treated cells, the mutant transcript appears to be even further stabilized. A lower molecular weight band is present in pSG5HuCol- and pSG5HuCol μ -transfected cells which appears to be a product of RNA decay. These data suggest that the ATTTA elements, which have been shown to destabilize cytokine and proto-oncogene transcripts (35,36), also destabilize human collagenase mRNA (see Discussion).

We have shown that the ATTTA motifs in the human collagenase 3' UTR contribute to instability of the mRNA.

However, it is not clear if the destabilizing function of the ATTTA motif is conserved in the rabbit gene. Furthermore, it is not known if the collagenase 3' UTR is a destabilizing element in and of itself, as has been described for the GM-CSF 3' UTR (35), or requires additional upstream sequences. To answer these questions, we have tested the ability of the rabbit collagenase 3' UTR to act as a destabilizing element for the constitutively expressed construct SV₂CAT. For these studies, we cloned the rabbit 3' UTR downstream of CAT coding sequences in the SV₂CAT construct. The resulting construct, SV₂CATH9 (Figure 7, panel A) was then transfected into RF, and CAT gene expression was assayed (Figure 7, panels B and C). The presence of the rabbit collagenase 3' UTR resulted in decreased CAT gene expression, as determined both by CAT activity assay and RNase protection of CAT transcript. The decreased CAT gene expression was not due to reduced transfection efficiency of SV₂CATH9, as determined by Hirt's assay (Figure 7, panel D). Thus, the 3' UTR of the rabbit collagenase gene is capable of acting as a destabilizing element, and this ability does not require upstream coding sequences.

DISCUSSION

In the present study, we have examined the induction of collagenase gene expression in synovial fibroblasts treated with IL-1 β . IL-1 β treatment induced collagenase mRNA levels comparable to PMA, but the time course for this cytokine was

Figure 7. Expression of SV₂CAT and SV₂CATH9 in RF. (A) The construct SV₂CATH9 was cloned by replacing the SV40 polyadenylation sequence of SV₂CAT with the rabbit 3' UTR. The rabbit 3' UTR contains 108 nt of coding sequence, three ATTTA elements, and a polyadenylation site (see Figure 6A). (B) CAT protein expression in cells transfected with SV₂CAT and SV₂CATH9 was assayed as previously described. Transfection efficiency was determined by Hirt's assay (40). (C) CAT transcript levels were assayed by RNase protection assay in SV₂CAT- and SV₂CATH9-transfected cells.

more rapid than PMA. In addition, induction by IL-1 did not involve a large transcriptional component, and depended on the presence of distal promoter sequences. Finally, transcripts from a transfected human collagenase gene decayed more slowly in IL-1 β -treated cells. The decay rate observed in untreated cells depends, at least in part, on the presence of ATTTA sequences in the 3' UTR, since mutation of these sequences stabilized mRNA. These results suggest that IL-1 β increases collagenase gene expression by mechanisms that are distinct from PMA and that involve increased mRNA stability.

We found that regions of the collagenase promoter which are known to be responsive to PMA (29,30) are not sufficient for IL-1 β -mediated transcriptional activation. The 1176 bp collagenase promoter construct transfected into fibroblasts demonstrated a transcriptional response to IL-1 β of only 2.9-fold above control values. The minor IL-1 β response of the 1176 bp construct contrasted with the 18-fold induction observed for the same construct in PMA-treated cells. The positive IL-1 β -responsive elements appear to reside farther 5' in the collagenase promoter, with increasing responsiveness observed with increasing promoter size. Given the gradient of responsiveness observed with increasing promoter size, multiple elements probably cooperate for IL-1 β -mediated transcriptional induction, as has already been demonstrated for phorbol ester activation of collagenase (30,43).

Transcriptional activation of the collagenase gene by PMA is mediated at least in part by the AP-1 site located 77 bp 5' of the transcriptional start site (29,30). This does not seem to be the case for IL-1 β , since this cytokine is not a strong inducer of *c-jun* gene expression or AP-1 binding activity. It has been reported (31) that IL-1 stimulates collagenase gene expression through the AP-1 site in human rheumatoid synovial cells. However, unlike our cells, these rheumatoid synovial cells displayed high basal levels of *c-jun* gene expression. Such high expression of this proto-oncogene might circumvent the normal transcriptional activation pathways stimulated by IL-1.

The moderate transcriptional response observed with IL-1 β was inconsistent with the dramatic increase seen in the level at steady-state. This implied that post-transcriptional mechanisms may be important for IL-1 β -mediated collagenase gene expression. Indeed, post-transcriptional regulation of collagenase gene expression has been demonstrated by others (22,34). However, assessing the role of collagenase mRNA stability can be problematic. This is due in part to the fact that mRNA levels in unstimulated cells are too low to obtain accurate measurements of half-life through pulse-chase methodology. Furthermore, treatment of cells with transcription inhibitors can have a profound effect on cellular physiology, and thus confound interpretation of the results (52). MacCachren *et al.* (26) induced collagenase mRNA with IL-1, then assayed the half-life in transcriptionally arrested cells, both in the presence and absence of IL-1. In contrast to our findings, these authors did not observe increased mRNA stability in the presence of IL-1. However, since transcriptional inhibitors themselves can stabilize the collagenase mRNA (22, Vincenti, unpublished observations), experiments with these drugs may be difficult to interpret.

To circumvent these difficulties, we used a constitutive promoter system that does not require induction with cytokine, and that does not need transcription inhibitors (52). It is important to note that the plasmid SV₂CAT, which is driven by the same promoter as pSG5HuCol, is not modulatable by IL-1 β , indicating that transcriptional activation of the SV40 promoter is not the

mechanism of IL-1 β -mediated enhanced expression. With this system, we found increased stability of the transfected human transcript in IL-1 β -treated cells, and this increase is concomitant with an increase in steady-state mRNA from the endogenous rabbit gene, thus correlating transcript stabilization with increased steady-state levels.

We also found that the 3' UTR of collagenase transcripts play an important role in mediating mRNA stability. The 3' UTR of the rabbit collagenase gene acts as a destabilizing sequence when inserted 3' of the CAT reporter gene. Interestingly, this region of the gene was not sufficient to mediate CAT mRNA stabilization in response to IL-1 β (data not shown), implying that additional upstream sequences also play a role. Indeed, regulation of *c-fos* mRNA stability is dependent upon sequences within the 3' UTR as well as elements within the coding region (53). Destabilizing elements, such as those which exist in the coding region of the *c-fos* transcripts, may be required for IL-1 β -mediated stabilization of collagenase mRNA.

The 3' UTRs of the rabbit and human genes contain three copies of the ATTTA motif which has been shown to contribute to the strict post-transcriptional control of proto-oncogene and cytokine transcripts (35,36). Our finding that mutation of these elements stabilizes the collagenase transcript in untreated and IL-1 β -treated cells implies a role for these elements in the collagenase gene as well.

A labile destabilizer has been described which mediates rapid turnover of the *c-myc* transcript (54). This cytosolic factor binds to A+U-rich elements in the 3' UTR of the *c-myc* transcript and mediates selective degradation of this mRNA (36). An mRNA destabilizing factor, such as that described for *c-myc*, may be present in resting cells, and may interact with the ATTTA destabilizing motif found in the 3' UTR of the collagenase gene. Mutation of this motif would ablate binding of a destabilizing factor, resulting in increased transcript stability relative to the wild-type. Similarly, upon stimulation with IL-1 β , this factor may be inhibited or degraded. This could explain the increased stability observed for the wild-type human transcript in IL-1 β -treated cells.

Previous studies of the molecular events involved in collagenase gene induction by cytokines such as IL-1 and TNF have concentrated on transcriptional activation (31,55). However, optimal activation of the native collagenase promoter with these cytokines has not been clearly demonstrated. A recent study indicates that EGF induction of collagenase and stromelysin involves post-transcriptional events (22). This cytokine induced collagenase steady-state mRNA levels 10-fold above control, with only a 2-fold increase in transcription rate. Studies such as this emphasize the importance of examining post-transcriptional mechanisms when studying cytokine induction of collagenase gene expression.

IL-1 β is a potent inducer of interstitial collagenase gene expression in fibroblasts. In the present study, we have demonstrated that the molecular mechanism of IL-1 β -mediated collagenase gene expression has both a transcriptional and a post-transcriptional component.

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