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Interleukin-6 Increases Rat Metalloproteinase-13 Gene Expression through Stimulation of Activator Protein 1 Transcription Factor in Cultured Fibroblasts*

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The role of IL-6 in collagen production and tissue remodeling is controversial. In Rat-1 fibroblasts, we measured the effect of IL-6 on matrix metalloproteinase-13 (MMP-13), c-*jun*, *junB*, and c-*fos* gene expression, binding of activator protein 1 (AP1) to DNA, amount of AP1 proteins, immunoreactive MMP-13 and TIMP-1 proteins, and Jun N-terminal kinase activity. We show that IL-6 increased MMP-13-mRNA and MMP-13 protein. These effects were exerted by acting on the AP1-binding site of the MMP-13 promoter, as shown by transfecting cells with reporter plasmids containing mutations in this element. Mobility shift assays demonstrated that IL-6 induced the DNA binding activity of AP1. This effect was accompanied by a marked increase in c-Jun, JunB, and c-Fos mRNA, as well as in c-Jun protein and its phosphorylated form. The latter is not due to increased Jun N-terminal kinase activity but to a decreased serine/threonine phosphatase activity. We conclude that IL-6 increases interstitial MMP-13 gene expression at the promoter level. This effect seems to be mediated by the induction of c-*jun*, *junB*, and c-*fos* gene expression, by the binding of AP1 to DNA, by increasing phosphorylated c-Jun, and by the inhibition of serine/threonine phosphatase activity. These effects of IL-6 might contribute to remodeling connective tissue.

Interleukin-6 (IL-6)[1] is a multifunctional glycoprotein produced by activated monocytes, macrophages, endothelial cells, and hepatic stellate cells that induces a wide variety of biological activities on many kinds of target cells, including fibroblasts, hepatocytes, and hepatic stellate cells (1). IL-6 promotes cell growth and differentiation and regulates specific gene expression of a variety of cells (2). IL-6 induces the expression of the acute phase proteins in the liver by inducing the binding of NF-IL6 and STAT3 to the promoter region of acute phase genes and promotes a rapid and transient tyrosine phosphorylation of the cytoplasmic domains of the IL-6 receptor (gp130) (1, 3). Acute and chronic liver diseases, particularly alcoholic liver diseases, are similar to the acute phase response in some respects. Thus, patients with acute alcoholic hepatitis show fever, muscle wasting, neutrophilia, and increased production of C-reactive protein, α_1-antitrypsin, and amyloid A (4, 5). High levels of IL-6 have been detected in the sera of patients with alcoholic liver cirrhosis (6–8), hepatitis B virus infection (9), and acute hepatitis (10, 11). Some authors have shown a correlation between circulating concentrations of IL-6 and serum concentrations of C-reactive protein (12, 13). Thus, IL-6 seems to be one of the most important factors regulating inflammatory responses in the liver.

Matrix metalloproteinases (MMPs) constitute a family of structurally related zymogens (collagenase-1 (MMP-1), collagenase-3 (MMP-13), gelatinases A and B (MMP-2 and MMP-9), and stromelysin (MMP-3), among others) capable of degrading a wide variety of extracellular matrix components (14). In rats and mice, there is only one interstitial collagenase (MMP-13); it shares 86% homology with human MMP-13 but not with the human or rabbit MMP-1 (15, 16). A variety of biologically active agents, such as tumor necrosis factor-α and interleukin-1, modulates the synthesis of these enzymes and their natural inhibitors, tissue inhibitors of MMP (TIMPs) (17–19). Although IL-6 shares many biological activities with IL-1, the role of IL-6 on the regulation of synthesis of MMPs and TIMPs remains controversial (17, 20–22). Whereas some authors found evidence for increased collagenase production (20, 23–25), others could not demonstrate any effect of IL-6 on the expression of MMP (21) or showed that IL-6 induces the synthesis of TIMP (17, 22, 26–28). We have undertaken the present study to elucidate the effect of IL-6 on rat MMP-13 (collagenase-3) gene expression in Rat-1 fibroblasts. We demonstrate that this cytokine stimulates MMP-13 expression by acting on an AP1-binding site in the MMP-13 promoter, after inducing the synthesis and phosphorylation of AP1 proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat-1 fibroblasts obtained from American Type Culture Collection were grown at 37 °C in an atmosphere of 5% CO_2, 95% air in cell culture flasks using 10 ml of Dulbecco's minimum essential medium with Earle's salts (Life Technologies, Inc.) containing 5% fetal bovine serum (Flow, Irvine, Ayrshire, Scotland), 0.5 mg/ml L-glutamine, 100 units/ml penicillin G and 0.1 mg/ml streptomycin. In some experiments we used protein extract from J-Jahn cells (29). This is a human lymphoblastoid cell line derived from Jurkat cells that expresses large amount of c-Jun after being stimulated with phorbol 12-myristrate

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[1] The abbreviations used are: IL-6, interleukin-6; AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinases; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element; RSV, Rous sarcoma virus; PEA-3, polyomavirus enhancer activator-3.

13-acetate (PMA). This cell line was a gift from Dr. J. Alcami (Madrid, Spain) (29).

Recombinant Plasmids—The luciferase reporter gene p2TRE-Luc contains two copies of the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) upstream of the herpes simplex virus-tk promoter (30) (gift of K. Chien, University of California, San Diego, CA) inserted into p19-Luc. pMMP-13-CAT plasmids (p(−2200) MMP-13-CAT, p(−1000)MMP-13-CAT, p(−500)MMP-13-CAT, p(−284)MMP-13-CAT, and p(−76)MMP-13-CAT) contain 76 base pairs to 2.2 kilobase pairs 5′-flanking DNA of the MMP-13 gene promoter inserted into a vector that encodes the enzyme chloramphenicol acetyltransferase (CAT) (pSVO-CAT) (31). p(−76)MMP-13-CATm is a construct similar to p(−76)MMP-13-CAT, but containing a TRE, in which the wild-type sequence GTGACTCA have been scrambled into GTTCCAAG. p*Fos*-Luc contains the *Hin*dIII/*Xba*I fragment (760 base pairs) of the human c-*fos* promoter inserted into p19-Luc (32). Construction of pRSV-β-galactosidase containing the Rous sarcoma virus (RSV) promoter and the β-galactosidase reporter gene has been described elsewhere (32). pJun-Luc contains the *Salt*I fragment (2.2 kilobase pairs) of the human c-Jun promoter inserted into p19-Luc vector (33). Human pBR 18 S contains the *Eco*RI fragment (5.8 kilobase pairs) subcloned into the pBR 322 vector.

Transfection and Luciferase Assays—Rat 1 fibroblasts were transiently transfected by the LipofectAMINE technique. Briefly, after overnight incubation, cells were washed twice with Opti-MEM medium (Life Technologies, Inc.) and incubated with a cotransfection mix containing 1.5 μg of CAT plasmids and 11 μg of LipofectAMINE reagent (Life Technologies, Inc.) in Opti-MEM medium at 37 °C for 8 h. After incubation, the transfection mix was aspirated and replaced with growth medium containing 10% fetal calf serum. After 24 h, the cells were washed with phosphate-buffered saline, and a new medium without fetal calf serum was added. After 2 h, cells were treated with IL-6 for various periods of time. Afterward, cells were washed with cold phosphate-buffered saline. Cell lysates were prepared, and CAT activity was determined as described elsewhere (34). Because CAT activity in cells transfected with constructs containing the smaller portions of the MMP-13 promoter was very low, the amount of cell lysate used for the CAT assays was 2 (p(−500)MMP-13-CAT), 8 (p(−284)MMP-13-CAT), and 18 times higher (p(−76)MMP-13-CAT and p(−76)MMP-13-CATm) than the amount of cell lysate used when cells were transfected with p(−1000)MMP-13-CAT or p(−2000)MMP-13-CAT constructs. In some experiments, cells were cotransfected with 0.5 μg of luciferase reporter plasmids and 0.5 μg of β-galactosidase pRSV-β-galactosidase as an internal control of transfection efficiency. In these cases, luciferase activity was determined using the enhanced luciferase assay kit according to the manufacturer's protocol (Analytical Luminescence, San Diego, CA). Cell lysates were prepared in 125 μl of of cell lysis buffer. Luciferase activity was determined using 50-μl aliquots, and protein concentrations determined with 5-μl aliquots using the Bradford protein assay (Bio-Rad). Published procedures were used to measure β-galactosidase activity (35). Transfections were performed in duplicate or triplicate.

RNA Preparation and Northern Analysis—Total RNA was prepared from cultured Rat-1 fibroblasts as described by Chomczynski and Sacchi (36). Five-microgram RNA samples were separated by electrophoresis on 2.2 M formaldehyde, 1% agarose gels and transferred to nylon membranes (MSI, Westboro, MA). cDNA probes for rat interstitial collagenase, c-Jun (37), JunB (38), and c-Fos (39) and 18 S RNA (*Eco*RI fragment of the pBR322 plasmid) were labeled using a random priming DNA labeling kit (Roche Molecular Biochemicals). Membranes were hybridized and washed with a final stringency of 0.1× SSC, 0.1% SDS and then analyzed by autoradiography. The autoradiograms were quantitated by scanning laser densitometry (Desk Top™ Scanner Plus, Amersham Pharmacia Biotech).

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—Nuclear proteins from Rat-1 fibroblasts untreated and treated with IL-6 were extracted by the method of Dignam *et al.* (40). The pellet was resuspended in 50 μl of Dignam C buffer and protein concentration was determined by the Bio-Rad assay according to the manufacturer's instructions. An oligonucleotide representing the consensus AP1 binding site from the human collagenase gene (41) was synthesized with a Cyclone Plus oligonucleotide synthesizer (Milligen, Novato, CA) and purified by high-performance liquid chromatography. The DNA sequences of this oligonucleotide were as follows: sense, 5′-TAAAGCAT-GAGTCAGACACCTC-3′; antisense, 3′-ATTTCGTACTCAGTCTGTG-GAG-5′. Double-stranded oligonucleotides probes were end labeled using the Klenow fragment and [^{32}P]dCTP (Amersham Pharmacia Biotech). Prior to adding ^{32}P-labeled oligonucleotides, 10 μg of nuclear protein extracts were incubated for 15 min with a mixture of 8 μl of a mixture mixture containing 0.5 μl of 5 mg/ml poly(dI-dC) (Roche Molecular Biochemicals), 1 μl of 100 mM $MgCl_2$, and 6.5 μl of distilled H_2O in the presence of 2× NDB (20 mM Hepes, pH 7.6, 100 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol, 20% glycerol). End-labeled oligonucleotides (20,000 cpm) were incubated with this mixture for 20 min. For competition experiments, 200-fold unlabeled annealed oligonucleotide was added to binding reactions. The autoradiograms were quantitated by scanning laser densitometry.

Western Blot Analysis—Whole cell protein extracts were prepared from Rat 1 fibroblasts and PMA-treated Junkat cells cultured on plastic until confluency. Cells were washed with phosphate-buffered saline and lysed by adding an equal volume of Dignam C buffer (40). After mixing at 4 °C for 30 min, the samples were centrifuged at 16,000 × *g* for 5 min at 4 °C, and the supernatant was collected and stored at −80 °C. For the analysis of cell free MMP-13 protein level, culture medium from cells unstimulated or stimulated with 20 and 40 ng/ml IL-6 for 24 h in Dulbecco's modified Eagle's medium without fetal calf serum was collected and centrifuged at 1500 × *g* to remove particles. For the analysis of TIMP-1 protein level, 500 μl of culture medium, after being centrifuged, were concentrated to 20 μl (Microcon-10 concentrator, Amicon, Beverly, MA). Protein assays were performed using the Bradford assay (Bio-Rad). Proteins (25 μg) were separated in an 8% SDS-polyacrylamide gel and transferred onto an Immobilon membrane (Millipore, Bedford, MA) overnight at 30 V at 4 °C. Equal loading was confirmed by Ponceau S staining. After electrotransfer the filter was incubated in Blotto (5% nonfat dry milk in TBS-Tween 20 (25 mmol/liter Tris-HCl, pH 8.0, 144 mmol/liter NaCl, 0.075% Tween-20)) overnight at 4 °C. The filter was then washed three times, 7 min each wash, in TBS-Tween 20 at 4 °C. The filter was then incubated with polyclonal MMP-13, (Chemicon International Inc. Temecula. CA), TIMP-1, C/EBP, c-Jun, or c-Fos antibodies or monoclonal phosphorylated c-Jun antibodies (Santa Cruz Biotechnology, Santa Cruz, CA.) at 1:400 (diluted in Blotto) for 2 h at 4 °C. The polyclonal MMP-13 antibody reacts with human and rat MMP-13 protein, whereas TIMP-1 antibody was specific for rat protein. c-Jun, phosphorylated c-Jun, C/EBP, and c-Fos antibodies react with rat as well as with human and mouse proteins. The filter was washed with TBS-Tween 20, as described previously, and then incubated with the secondary antibody, goat anti-rabbit-IgG-alkaline phosphatase (Amersham Pharmacia Biotech) diluted 1:2000 in Blotto for 1 h at 4 °C. The filter was washed, incubated at room temperature with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Madison. WI) for approximately 1 min, and finally rinsed with water.

Kinase Assay—c-Jun N-terminal kinase (JNK) assay were performed as described (41). Briefly, 20 μg of whole cell protein were incubated with recombinant GST-c-Jun substrate (amino acids 1–79) (42) conjugated to glutathione-agarose beads (Amersham Pharmacia Biotech), extensively washed, and then incubated in kinase reactions containing [γ-^{32}P]ATP as a phosphate donor. Substrate protein was resolved by gel electrophoresis, and phosphate incorporation was assessed by autoradiography and phosphorimager analysis.

Serine/Threonine Phosphatase Assay—Untreated or IL-6-treated Rat-1 fibroblasts were washed three times with ice-cold phosphate-buffered saline and lysed in Dignam C buffer lacking phosphatase inhibitors (sodium molibdate, sodium vanadate, β-glycerophosphate, and *p*-nitrophenylphosphate). Endogenous phosphates were removed by passing cell lysate through a Sephadex G-25 spin column. Protein concentration of the phosphate-reduced sample was measured using the Bradford protein assay reagent from Bio-Rad. Serine/threonine phosphatase activity was measured using a commercially available assay following the manufacturer's instructions (Promega).

RESULTS

IL-6 Induces MMP-13 Gene Transcription—To determine the effects of IL-6 on MMP-13 gene expression, we examined the steady-state levels of MMP-13 mRNA in Rat-1 fibroblasts treated with increasing concentrations of IL-6 for 24 h. The level of MMP-13 mRNA in each sample was normalized to the level of 18 S RNA. This treatment resulted in a marked increase in steady-state levels of MMP-13 mRNA (Fig. 1). Thus, 40 ng/ml IL-6 increased MMP-13 levels 3.4-fold over the control level (Fig. 1*A*). Likewise, these levels were increased to 1.2-, 2-, 2.9-, and 3.2-fold more than the control level after 3, 6, 12, and 24 h, respectively, of incubation with 20 ng/ml IL-6 (Fig. 1*B*). This increase was in the range of the increase induced with 0.6 nM tumor necrosis factor-α (2.4-fold) or 2 ng/ml IL-1 (3.8-fold) in

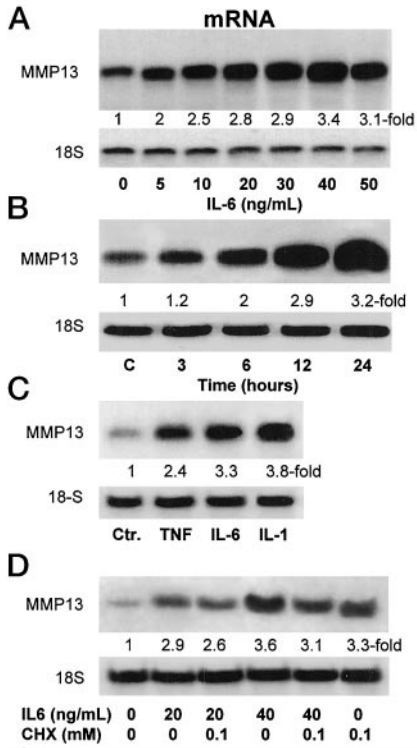

FIG. 1. **Northern blot analysis of Rat-1 fibroblasts MMP-13 mRNA.** *A,* dose-response effect of IL-6 on MMP-13 mRNA. Confluent Rat-1 fibroblasts were incubated for 24 h in Dulbecco's modified Eagle's medium without fetal calf serum in the absence or presence of increasing concentrations of IL-6. *B,* time-response effect of 20 ng/ml IL-6 on MMP-13 mRNA. *C,* effect of 0.6 nmol/liter tumor necrosis-α, 20 ng/ml IL-6, and 2 ng/ml IL-1 for 24 h on MMP-13 mRNA. *D,* MMP-13 mRNA in cells incubated for 24 h in control medium or in medium containing 20 or 40 ng/ml IL-6 in the presence or absence of 0.1 mM cycloheximide (*CHX*). Five micrograms of total RNA were electrophoresed on a formaldehyde, 1% agarose gel and transferred to a nylon filter by capillary blotting. Bound RNA was hybridized to ^{32}P-labeled cDNA probe as described under "Experimental Procedures." The filters were exposed to x-ray film at −70 °C with an enhancing screen. The autoradiograms were quantitated by scanning laser densitometry. The level of MMP-13 mRNA in each sample was normalized to the level of 18 S RNA (*18S*). These blots are representative of three separate experiments.

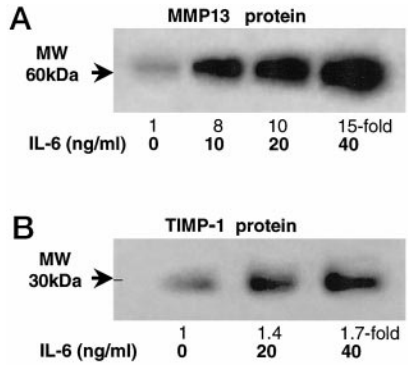

FIG. 2. **Effect of treatment of cells with IL-6 on immunoreactive MMP-13 and TIMP-1 proteins.** *A,* samples of medium without fetal calf serum (0.5 μg of protein) harvested from cells untreated and treated with 10, 20, or 40 ng/ml IL-6 for 24 h were analyzed by Western blot with polyclonal antibody against human MMP-13 as described under "Experimental Procedures." *B,* samples of medium harvested from cells untreated or treated with 10–40 ng/ml IL-6 were concentrated, and 30 μg protein from each sample were analyzed by Western blot with specific polyclonal antibody against rat TIMP-1. *MW,* molecular mass.

these cells (Fig. 1*C*). The IL-6-induced increase in MMP-13 mRNA was abrogated by pretreating cells with 0.1 mM cycloheximide (Fig. 1*D*), which decreases protein synthesis by 95% in these cells (data not shown). As it has been described for c-Fos and c-Jun (43), preincubation of cells with cycloheximide resulted in an 3-fold increase in MMP-13 mRNA levels. However, this level was not further increased by incubating cells with 20 or 40 ng/ml IL-6. These effects on MMP-13 mRNA were associated with a dose-dependent increase in the amount of immunoreactive MMP-13. This protein increased 5.9-, 8-, and 12-fold, respectively, after incubating the cells with 10, 20, or 40 ng/ml IL-6 for 24 h (Fig. 2*A*). The same experimental conditions enhanced immunoreactive TIMP-1 only slightly. Treatment of cells with 40 ng/ml IL-6 for 24 h increased TIMP-1 only 1.7-fold (Fig. 2*B*).

To identify the mechanism by which IL-6 increases collagenase mRNA, a CAT reporter gene driven by a portion of the MMP-13 gene promoter (p(−1000)MMP-13-CAT) was transfected into cultured Rat-1 fibroblasts and the levels of CAT activity determined. This activity was normalized to the β-galactosidase activity of the cotransfected pRSV-β-galactosidase plasmid. Four separate experiments showed that IL-6 increased CAT activity and that this increase was dose- and time-related (Fig. 3).

To determine the sequences on which IL-6 exerts its effects on MMP-13 transcription, we transiently transfected cells with a series of constructs obtained by progressively deleting more 5′-flanking sequences of the MMP-13 gene promoter and inserting them into a CAT reporter plasmid. Deletion of promoter sequences upstream of base pair −76 relative to the transcription start site did not abrogate the effect of IL-6 on MMP-13 gene expression (Fig. 4, *A–E*). On the contrary, this effect of IL-6 disappeared in cells transfected with a mutant construct in which the proximal AP1 binding site have been converted from GTGACTCA into GTTCCAAG (Fig. 4*F*).

To assess the role of the TRE sequence in the stimulation of the MMP-13 gene, we transfected Rat-1 fibroblasts with a plasmid containing two copies of the TRE upstream of the herpes simplex virus-tk promoter (p2xTRE-Luc) and measured the effect of increasing concentrations of IL-6 on the luciferase activity in cell lysates. We found that 20 ng/ml IL-6 increased luciferase activity 1.4-fold the control level at 6 h and reached 1.9- and 2.6-fold at 12 and 24 h, respectively (Fig. 5). The increased luciferase activity was more marked in cells incubated with 40 ng/ml IL-6, where there was a 1.9-, 2.7-, and 3.1-fold induction at 6, 12, and 24 h, respectively (Fig. 5).

IL-6 Increases AP1 Binding to DNA—To determine whether IL-6 induces the DNA binding activity of AP1, we examined the kinetics of AP1 binding activity in IL-6 stimulated cells. Nuclear extracts were prepared from Rat-1 fibroblasts treated with 20 ng/ml IL-6 for 1–6 h. A ^{32}P-labeled oligonucleotide containing the AP1 consensus sequence was used as a probe. AP1 binding activity increased 1.9-fold (Fig. 6*A*) and 2.4-fold (Fig. 6*B*) over the control level after 6 and 24 h, respectively. Likewise, AP1 binding to DNA was only slightly enhanced (1.2-fold) in cells treated with 5 ng/ml IL-6 for 24 h, but rose to 1.8-, 2.4-, and 2.6-fold with 10, 20, and 30 ng/ml IL-6, respectively, for the same period of time (Fig. 6*B*). This binding was efficiently competed with 200-fold molar excess of the same unlabeled oligonucleotide (Fig. 6), but not with 200-fold molar excess of an unlabeled oligonucleotide containing a C/EBP consensus binding site (Fig. 6*A*). On the other hand, incubation of the nuclear extract with a phosphorylated c-Jun-specific antibody prior to the gel retardation assay led to the formation of two supershifted complexes (Fig. 6*A*), demonstrating that phosphorylated c-Jun was a member of this complex.

IL-6 Enhances c-fos and c-jun Gene Expression—Because AP1 is a collection of transcriptional factors composed of mem-

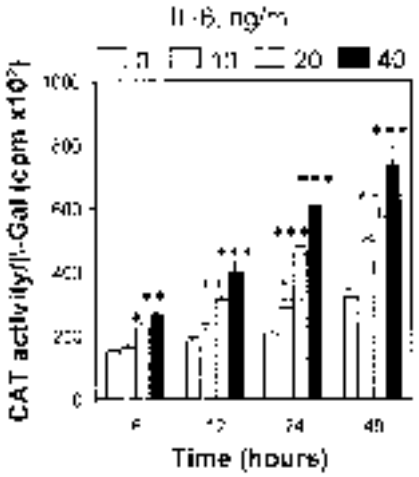

FIG. 3. **Effect of IL-6 on collagenase gene promoter in Rat-1 fibroblasts.** Cells were transfected by the LipofectAMINE method with 1.5 μg of p(−1000)MMP-13-CAT reporter plasmid and 0.6 μg of pRSV-β-galactosidase plasmid. One day after transfection, the cells were incubated with 0, 10, 20, or 40 ng/ml of IL-6 in the absence of fetal calf serum for 6, 12, 24, or 48 h. After the indicated time, cells were harvested, and CAT and β-galactosidase activities were measured in the homogenates as described under "Experimental Procedures." CAT activity was normalized to β-galactosidase activity as an internal standard for transfection efficiency. Values are given as cpm corrected by the β-galactosidase activity. The results represent the mean ± SD of one experiment performed in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, as compared with control cells.

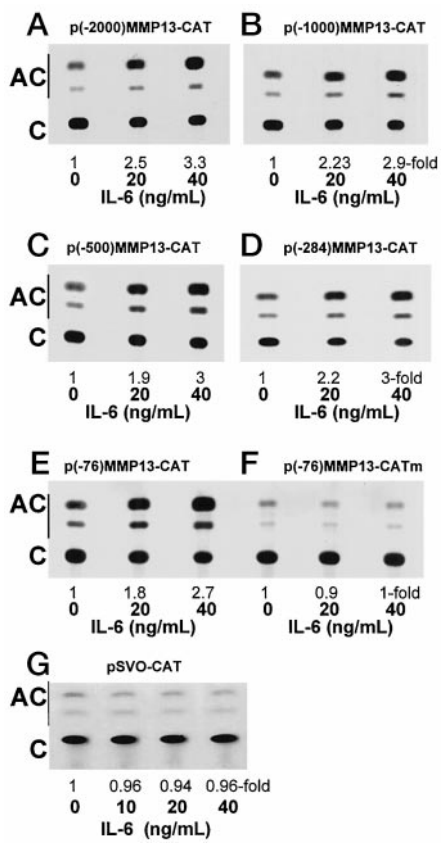

FIG. 4. **Deletion analysis of the MMP-13 gene promoter.** Rat 1 fibroblasts were transiently cotansfected with pRSV-β-galactosidase plasmid, and a series of MMP-13-CAT constructs was obtained by removing progressively more 5′-flanking sequences of the MMP-13 promoter. p(−2000)MMP-13-CAT contained nucleotides −2000 to +27 of the MMP-13 promoter inserted into the vector plasmid pSVO-CAT. Fragments of MMP-13 promoter contained in plasmids p(−1000)MMP-13-CAT, p(−500)MMP-13-CAT, p(−284)MMP-13-CAT, and p(−76)MMP-13-CAT were −1000, −500, −284 and −76 to +27, respectively. The mutated plasmid p(−76)MMP-13-CATm was similar to p(−76)MMP-13-CAT, but the proximal TRE has been scrambled. Cell lysates were assayed for CAT and β-galactosidase activities as described in Fig. 2 and under "Experimental Procedures." Because CAT activity in cell lysate decreased with the length of the portion of MMP-13 promoter contained in each of these constructs, we used increasing amounts of cell lysate to measured CAT activity. Thus, we used 2.5, 2.5, 5, 20, 45, and 45 μl of cell lysate from cells transfected with p(−2000)MMP-13-CAT (*A*), p(−1000)MMP-13-CAT (*B*), p(−500)MMP-13-CAT (*C*), p(−284)MMP-13-CAT (*D*), p(−76)MMP-13-CAT (*E*), and p(−76)MMP-13-CATm (*F*), respectively. *G*, the effect of IL-6 on the empty vector pSVO-CAT. The results presented are representative of three separate experiments. *AC,* acetylated chloramphenicol; *C,* radiolabeled chloramphenicol.

bers of the Jun and Fos families (44), we investigated whether IL-6 treatment of cells was associated with an activation of the *jun* or *fos* genes. Incubation of cells with 5–30 ng/ml IL-6 for 24 h resulted in enhanced induction of c-Jun, JunB, and c-Fos mRNA. Thus, in cells treated with 20 ng/ml IL-6, mRNA levels of these three proteins were increased 5.5-, 4.3-, and 3.9-fold (Fig. 7). To assess the role of IL-6 on c-*jun* and c-*fos* transcription, cells were transfected with the plasmids pJun-Luc or pFos-Luc. Cells transfected with pJun-Luc and treated with 20 or 30 ng/ml of IL-6 for 7 h resulted in an increase in luciferase activity by 2.6- and 3.6-fold, respectively. At 12 h, this activity was slightly lower than at 7 h, and at 24 h, these levels were decreased only to 1.6- and 1.7-fold (Fig. 8*A*). Treatment of cells transfected with pfos-Luc with 20 and 30 ng/ml of IL-6 for 7 h increased luciferase activity only by 43 and 61%, respectively, over the control level, and 24 and 17%, respectively, after 24 h (Fig. 8*B*).

To assess whether IL-6 induces AP1 proteins, whole cell protein extracts were prepared from Rat-1 fibroblasts treated with increasing concentrations of IL-6 for 12 h. IL-6 induced a dose-dependent increase in a 39-kDa protein, the nonphosphorylated c-Jun, (41, 42) (Fig. 9, *A* and *B*). Likewise, Western blots using specific polyclonal c-Fos antibody showed an increase in the expression of c-Fos p62 protein (Fig. 9, *C* and *D*). Because phosphorylation of c-Jun plays a critical role in the activation of gene transcription, we analyzed phosphorylated c-Jun with a specific monoclonal antiphosphorylated c-Jun antibody, directed at the phosphorylated activation domain. As a positive control for this protein, we included whole cell proteins extracted from Junkat cells treated with 25 ng/ml of PMA. Western blot analysis revealed one single band located at 41 kDa, the density of which increased after incubation with IL-6 (20 ng/ml), reached its maximal density after 12 h (8.5-fold) (Fig. 10*A*), and then declined slowly over the next 12 h (Fig. 10*B*). Treatment of cells with higher doses of IL-6 (80 ng/ml) enhanced phosphorylated c-Jun only 1.9-fold (Fig. 10*C*).

Because IL-6 increased phosphorylation of the c-Jun activation domain, we measured JNK activity in Rat-1 fibroblasts treated with IL-6 for 15 min. JNK activity was measured using a previously described solid-state assay using GST-c-Jun as substrate (41). Incubation of cells with 10 and 20 ng/ml IL-6 decreased JNK activity to 80 and 60%, respectively, of that of control cells (Fig. 10*D*).

IL-6 Inhibits Serine/Threonine Phosphatase—Because IL-6 enhanced phosphorylation of the c-Jun activation domain without enhancing JNK activity, we wanted to determine whether IL-6 inactivates a serine/threonine phosphatase. Measurement of the serine/threonine phosphatase activity in Rat-1 fibroblasts treated with increasing concentration of IL-6 for 6 h showed that IL-6 decreased phosphatase activity in a dose-dependent fashion. Treatment of cells with 20 or 40 ng/ml IL-6 decreased this activity to 39 or 22%, respectively, of control activity (Fig. 11*A*). Likewise, incubation of cells with 20 ng/ml IL-6 for 3–24 h resulted in a decrease of serine/threonine phos-

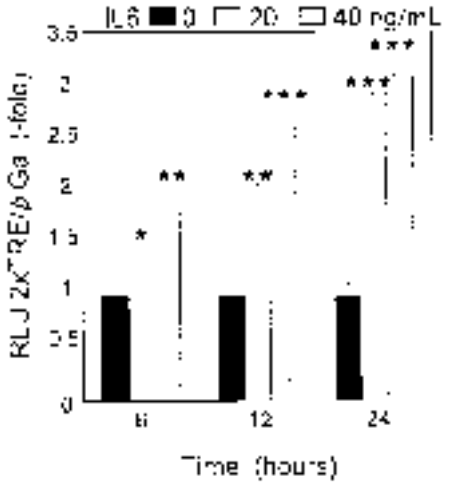

FIG. 5. **Effect of IL-6 on TRE in Rat-1 fibroblasts.** Cells were transfected as indicated under "Experimental Procedures" with 0.5 μg of p2xTRE-Luc, 0.5 μg of pRSV-β-galactosidase, and 1.1 μg of pUC19 and cultured without fetal calf serum in the absence or presence of 20 or 40 ng/ml IL-6 for 6, 12, or 24. After the indicated time, luciferase and β-galactosidase activities were measured as mentioned. Values are given as fold over the activity in cells incubated without IL-6. Results represent mean values ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, as compared with control cells.

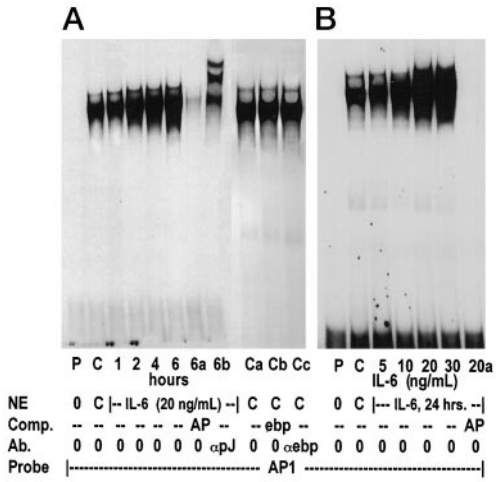

FIG. 6. **Mobility shift assays using the AP1 probe.** DNA binding was analyzed by gel retardation assay as described under "Experimental Procedures." *A,* time course of AP1 activation in response to IL-6 for 1–6 h. A double-stranded radiolabeled AP1 oligonucleotide was incubated with nuclear proteins extracted from control cells or cells treated with 20 ng/ml IL-6. *B,* nuclear extracts from cells treated for 24 h with increasing concentrations of IL-6 were incubated with radiolabeled AP1 probe. *Lanes P,* AP1 radiolabeled probe without addition of nuclear extract. *Lanes C,* probe incubated with nuclear proteins extracted from control cells. *Lanes 6a* and *20a,* nuclear protein extract from cells treated for 6 and 24 h, respectively, with 20 ng/ml IL-6 incubated with radiolabeled AP1 probe in the presence of 200-fold molar excess of unlabeled AP1 oligonucleotide. *Lane 6b,* supershift after incubation of the reaction mix with specific phosphorylated c-Jun antibody prior to the gel retardation assay. *Lane Ca,* nuclear protein extract from control cells incubated with radiolabeled AP1 probe. *Lane Cb,* nuclear protein extract from control cells in the presence of 200-fold molar excess of unlabeled C/EBP oligonucleotide. *Lane Cc,* supershift after incubation of the reaction mix with specific C/EBP antibody (*αebp*). The results presented are representative of four separate experiments. *NE,* nuclear protein extract; *comp.,* competitor; *Ab.,* antibody; *αpJ,* anti-phosphorylated c-Jun antibody; *AP,* unlabeled AP1 oligonucleotide, *ebp,* C/EBP oligonucleotide; *probe,* radiolabeled probe.

phatase activity, which was particularly marked at 6 h (44%). At 12 and 24 h, this activity was higher than at 6 h (50 and 53%, respectively, of control) (Fig. 11*B*).

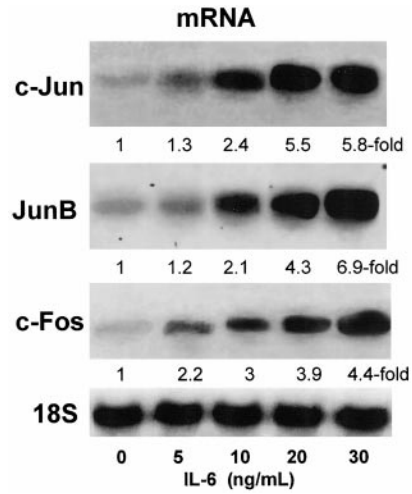

FIG. 7. **c-Jun, JunB, and c-Fos mRNA are induced by IL-6.** Total RNA was isolated from Rat-1 fibroblasts cultured with 0–30 ng/ml IL-6 for 24 h and analyzed by Northern blotting as described under "Experimental Procedures." The blot was hybridized with ^{32}P-labeled probes specific for c-Jun, JunB, c-Fos, and 18 S RNA, which served as a control for sample loading. Autoradiograms were quantitated by scanning laser densitometry. The level of mRNA in each sample was normalized to the level of 18 S RNA. Blots are representative of at least three separate experiments.

DISCUSSION

We show that IL-6 increased the steady-state levels of MMP-13 mRNA in a dose- and time-dependent manner (Fig. 1) and that this effect mediated within the gene promoter (Fig. 3). This effect was evident after 6 h of treatment but was particularly marked after 24 h. This prolonged incubation time required by IL-6 to stimulate MMP-13 gene expression and MMP-13 mRNA levels suggests that *de novo* synthesis of a protein may be required for this effect. This requirement is supported by the fact that the IL-6-induced increase in MMP-13 mRNA levels was blocked by inhibiting protein synthesis with cycloheximide (Fig. 1*D*). As expected, the enhanced MMP-13 gene expression was associated with a striking increase in the immunoreactive MMP-13 protein (Fig. 2*A*). These results agree with those reported by Franchimont *et al.* (25) and Kusano *et al.* (45), who demonstrated that IL-6, in the presence of its soluble receptor, increased MMP-13 mRNA levels (25, 45), immunoreactive MMP-13 (25), and its biological activity (25). Our study demonstrated that the effect of IL-6 on MMP-13 secreted into the culture medium was much higher than that induced on the steady-state levels of MMP-13 mRNA. We speculate that this difference might be ascribed to an effect of IL-6 on the extracellular metabolism of secreted MMP-13. In fact, Sehgal and Thompson (46) recently showed that transforming growth factor β1 induced a marked increase in stability of MMP-2 protein, resulting in a significantly enhanced MMP-2 protein level in culture medium, despite an unchanged steady-state level of MMP-2 mRNA. Although some authors have shown that IL-6 significantly enhanced TIMP-1 production and TIMP-1 mRNA expression in human fibroblasts and other cell lines (17, 22, 25–28), we found that IL-6 increased immunoreactive TIMP-1 only slightly (Fig. 2*B*).

Rat MMP-13 gene displays a general organization similar to that of other members of the MMP family (47, 48), particularly to human (49) and rabbit MMP-1 genes (50). All share a common 10-exon organization (48, 49, 51) and contain a typical TATA box in addition to TRE and polyomavirus enhancer activator 3 (PEA-3) consensus sites in their promoter region (15, 48, 52–57), suggesting a common regulatory mechanism of gene transcription. Our study showed that sequences upstream

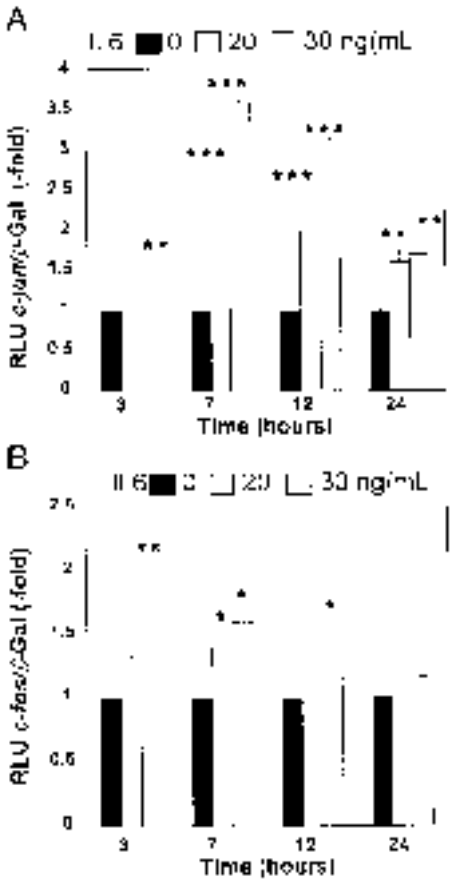

FIG. 8. **Effect of IL-6 on c-*jun* and c-*fos* promoter activity.** Rat-1 fibroblasts were transfected as indicated in Fig. 2 with 0.5 μg pJun-Luc (*A*) or pFos-Luc (*B*) as reporter plasmids plus 0.5 μg of pRSV-β-galactosidase and 1.1 μg of pUC19 plasmids. Transfected cells were incubated with 0–30 ng/ml IL-6 for 3–24 h. After the indicated time, cells were harvested and luciferase, and β-galactosidase activities were measured in homogenates. Luciferase values were normalized for differences in transfection efficiencies. Fold represents luciferase/β-galactosidase ratio in the presence of IL-6 divided by that obtained in the absence of IL-6. Data are the means ± SD of one experiment with triplicate determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, as compared with the control cells.

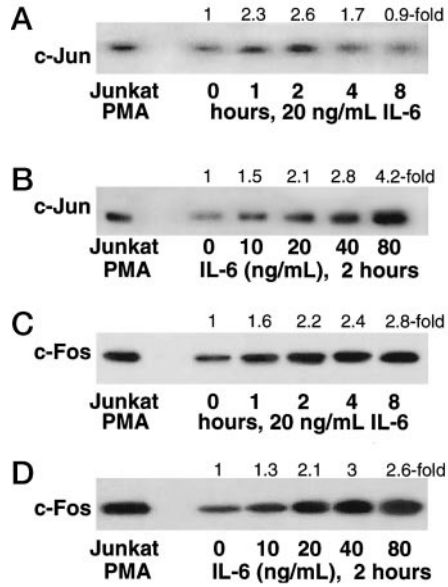

FIG. 9. **Kinetic of induction of c-Jun and c-Fos proteins by IL-6.** Twenty-five micrograms of whole cell protein extracts from cells incubated in the absence or presence of increasing concentrations of IL-6 for 1–8 h were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to membrane for immunoblot analysis as described under "Experimental Procedures." Ponceau S staining was used to confirm equal protein loading. Immunoblots were then probed with either specific anti-c-Jun (*A* and *B*) or anti-c-Fos (*C* and *D*) antibodies and detected by enhanced chemiluminescence. Whole cell protein extract from J-Jhan cells treated with 25 ng/ml PMA was used as positive control of c-Jun and c-Fos (*Junkat PMA*). Blots are representative of at least three separate experiments.

of base pair −79 are dispensable in IL-6-mediated stimulation of MMP-13 gene expression, whereas integrity of the TRE site appears to be essential for the IL-6 induced response (Fig. 4). The TRE has been implicated in the expression of many of the MMP genes (58, 59), including MMP-13 (59). However, some of these genes (60, 61) require the cooperative action of the TRE with the PEA-3 element to obtain maximal inductability by a number of stimuli. The present study shows that PEA-3 was not required to obtain a response to IL-6 (Fig. 4). These results concur with those reported by Pendás *et al.* (48), who showed that PEA-3 was not significant in the response of the human MMP-13 to 12-*O*-tetradecanoylphorbol-13-acetate. These discrepancies between the role played by the PEA-3 site in human and rat MMP-13 and its role in other members of the MMP family may be ascribed to the distance existing between TRE and PEA-3 elements (48). Whereas human and rat MMP-13 contain 20 nucleotides between TRE and PEA-3 sites, the distance between these two elements is only 9 nucleotides in other MMP genes.

The stimulatory effect of IL-6 on the TRE site was also confirmed in cells transfected with a luciferase construct containing two copies of the TRE upstream of a minimal promoter (Fig. 5). Very little information exists about the effect of IL-6 on the activation of genes with a TRE. Daffada *et al.* (62) found that IL-6 had no effect on the expression of pTRE-CAT in transient transfected cells, suggesting that AP1 is not induced by IL-6 treatment. On the contrary, Melamed *et al.* (63) showed that IL-6 induced a TRE-binding complex, which was abolished by anti-Jun specific antibodies.

To confirm that IL-6 promotes the binding of nuclear proteins to the TRE, gel retardation experiments were performed (Fig. 6). Treatment of cells with IL-6 induced a dose- and time-related increase in the formation of a TRE-protein complex (Fig. 6) that contained phosphorylated c-Jun. These results support the role played by TRE and AP1 in mediating the effect of IL-6 on rat MMP-13 gene expression.

The AP1 transcription factor actually represents a heterogeneous group composed of members of the Jun and Fos families. These proteins form a variety of homo- and heterodimers that bind to a common DNA recognition site (25, 64). In this study we showed that IL-6 induced c-Jun, JunB and c-Fos mRNA (Fig. 7), c-*jun* and c-*fos* promoters (Fig. 8*A*) and c-Jun and c-Fos proteins in Rat-1 fibroblasts (Fig. 9). Other authors have also shown that IL-6 stimulated *junB* gene expression in a variety of cells (65–74) by acting on a region containing an ETS and a STAT3 binding site (67, 69, 72). More recently, Cressman *et al.* (75) have shown that the expression of *junB* and STAT3 are markedly reduced in the liver of IL-6-deficient mice.

The increase in c-Fos mRNA seems to be only partially due to enhanced transcriptional activity, because luciferase activity in cells transiently transfected with the plasmid pFos-Luc increased only slightly after IL-6 treatment. There are few studies concerning the effect of IL-6 on c-*fos* gene expression or Fos protein. However, a number of authors have found the induction of c-*fos* gene in a variety of cells (71, 76, 77) and Cressman *et al.* (75) reported that hepatectomy induced the expression of c-Fos protein in the liver of control mice but was reduced or absent in the livers of IL-6-knockout mice. Nevertheless, other

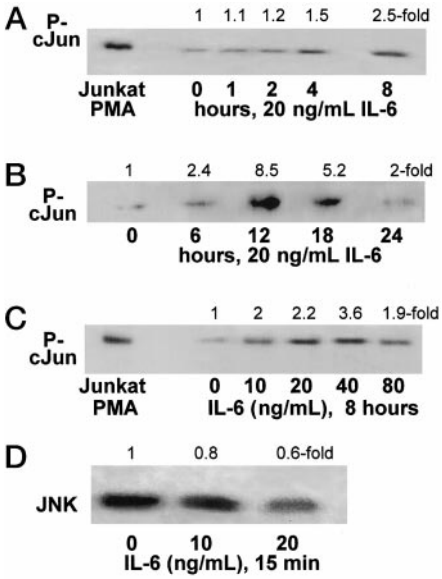

FIG. 10. **Kinetic of IL-6-induced phosphorylation of c-Jun.** Whole cell protein extracts from experiments described in Fig. 8. Immunoblots were probed with specific anti-phosphoserine-63 c-Jun antibody (*A–C*). Whole cell protein extract from J-Jhan cells treated with 25 ng/ml PMA was used as positive control of phosphorylated c-Jun (*Junkat PMA*). *D,* effect of IL-6 on JNK activity in Rat-1 fibroblasts. Recombinant GST-c-Jun was incubated with 25 μg of whole cell protein extracts from Rat-1 fibroblasts treated without or with 10 or 20 ng/ml IL-6 for 15 min. JNK-mediated phosphorylation of GST-c-Jun was assessed by incorporation of [γ-^{32}P]ATP, followed by SDS-polyacrylamide gel electrophoresis. Autoradiograms were quantitated by scanning laser densitometry. The *single band* represents GST-c-Jun. *P-cJun,* phosphorylated c-Jun. Blots are representative of at least three separate experiments.

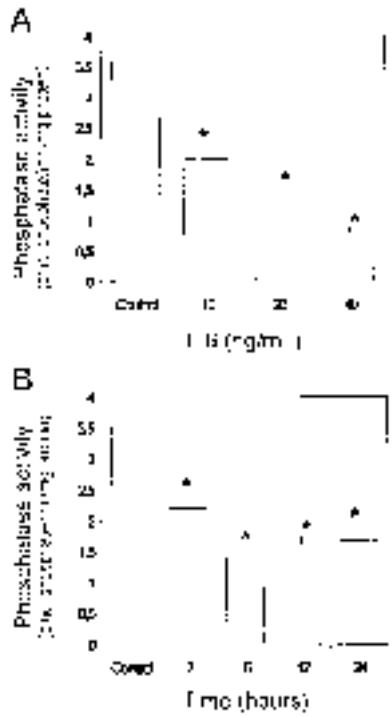

FIG. 11. **Effects of IL-6 on serine/threonine phosphatase activity in cultured Rat-1 fibroblasts.** *A,* cell cultures were incubated without or with 10–40 ng/ml IL-6 for 6 h. Cell lysate was passed once through a Sephadex G-25 column to remove free phosphate and incubated for 30 min with or without substrate (serine/threonine phosphopeptide) in a protein phosphatase-2A buffer. Phosphatase activity was assessed by the free phosphates released from the substrate during the reaction. *B,* phosphatase activity in lysates from cells incubated with 20 ng/ml IL-6 for 3–24 h. Data are mean values of triplicate samples and are expressed as pmol of phosphate/min/μg of protein. Similar results were obtained in two separate experiments. *, $p <$ 0.001, as compared with the control cells.

authors, working on a variety of cells lines, could not demonstrate any effect of IL-6 on c-*fos*. These studies showed that only some early response genes, such as the *jun* family, but not c-*myc* or c-*fos*, were stimulated by the addition of IL-6 (66–68).

Transcriptional activity of AP1 depends not only on the abundance of AP1 components and their ability to bind DNA but also on the degree of phosphorylation of these proteins (64). Phosphorylation of c-Jun in its activation domain at serine 63 and 73 prolongs its half-life and potentiates the ability of c-Jun to activate transcription as either a homodimer or as a heterodimer with c-Fos (64). Western blots using a specific monoclonal antibody for serine 63 phosphorylated c-Jun demonstrated that IL-6 induces an increase in this form of c-Jun, which was particularly marked after 12 h of treatment (Fig. 10). This result concurred with the study of Lütticken *et al.* (70), who showed that IL-6 triggers a delayed phosphorylation of STAT3 at serine residues. A variety of protein kinases, including pp42, pp54, and pp44 mitogen-activated protein kinases, p34cdc2, protein kinase C, casein kinase II, efficiently phosphorylates c-Jun (78). JNK, also known as stress-activated protein kinase, is a member of the mitogen-activated protein kinase family that phosphorylates serines 63 and 73 of c-Jun and potentiates its transactivation function (42). However, our study indicates that IL-6 does not induce the phosphorylation of c-Jun by stimulating JNK activity (Fig. 10*D*). Therefore, we have to consider that the increase in phosphorylated c-Jun is the result of either a decrease in protein phosphatase activity or an activation of another protein kinase involved in the phosphorylation of c-Jun (78). Thus, a number of studies have clearly demonstrated that inhibition of protein phosphatases 1 and 2A by okadaic acid results in an induction of collagenase, JunB, and c-Fos mRNA and a potent activation of AP1, through serine/threonine phosphorylation (79–81). The results of our study concur with these reports by demonstrating that treatment of cells with IL-6 decreased serine/threonine phosphatase activity in a dose-dependent manner and that this effect was particularly marked at 6 h of treatment (Fig. 11). Despite that, we cannot exclude the participation of another protein kinase. In fact, Belka *et al.* (82) found that IL-6-mediated phosphorylation of the small heat shock protein 27 was the result from activation of the mitogen-activated protein-kinase-activated protein kinase 2, a serine/threonine kinase that is activated by mitogen-activated protein kinase.

In conclusion, this study shows that treatment of Rat 1 fibroblasts with IL-6 stimulated MMP-13 gene expression in a time- and dose-dependent manner. This effect was associated with an enhanced expression of *jun* and *fos* genes, an increase in the DNA-binding activity of AP1, and an elevation of phosphorylated c-Jun. The latter increase was not mediated by enhanced JNK activity but was associated with decreased serine/threonine phosphatase activity.

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