Identification of the interleukin-6/oncostatin M response element in the rat tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter

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ABSTRACT

The rat tissue inhibitor of metalloproteinases 1 (TIMP-1) gene is expressed in rat hepatocytes, and this expression is up-regulated by interleukin 6 (IL-6). We report here the cloning of the 5' flanking region of the rat TIMP-1 gene and identification of an IL-6/oncostatin M (OSM) response element at –64 to –36 which functions in hepatic cells. Within this element we have identified two functional binding sites for transcription factors AP-1 (activatory protein-1) and STAT (signal transducer and activator of transcription). IL-6/OSM stimulation induces binding of a protein, identified as STAT3, to the IL-6/OSM response element, while binding of the AP-1 protein was constitutive. Binding sites for both AP-1 and STAT3 are necessary for full responsiveness of the TIMP-1 promoter to IL-6/OSM, as shown by deletion and mutation analysis. Furthermore, the entire IL-6/OSM response element conferred responsiveness onto a heterologous promoter, whereas this has not been observed when AP-1 and STAT elements were separately tested.

INTRODUCTION

Components of extracellular matrix, including proteoglycans and collagens, are continuously produced and secreted by cells. In many physiological processes such as embryonic development, bone and cartilage formation, wound healing and scar formation, angiogenesis, or implantation of ova, remodeling of the extracellular matrix is required (1,2). Formation and degradation of the extracellular matrix is also an important aspect of many pathological disorders, including tumor invasion and metastasis, rheumatoid arthritis, liver fibrosis or joint distraction (3,4). The extracellular matrix turnover is the effect of continuous synthesis and degradation of its components. This degradation is regulated on many levels, in particular through specific inhibition of the proteolytic activity of matrix metalloproteinases. A family of tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2 and,

recently, TIMP-3) has already been identified as major inhibitors of matrix metalloproteinases (5–7), and these are synthesized by a variety of different cells (including fibroblasts, monocytes, macrophages, chondrocytes, endothelial cells and synoviocytes) and regulated by a number of cytokines and growth and differentiation factors (2,8–12).

The liver (particularly hepatocytes) is the major source of plasma inhibitors, such as α_2 -macroglobulin, α_1 -proteinase inhibitor and α_1 -antichymotrypsin. The expression of these plasma inhibitors in the liver is up-regulated during the acute phase response, with interleukin-6 (IL-6) being a major activator (13). TIMP-1 is also produced in the liver, and its expression is enhanced after injection of lipopolysaccharide. In addition, human hepatoma HepG2 cells, rat hepatoma Fao cells and primary rat hepatocytes also synthesize TIMP-1, with IL-6 further activating its expression (14,15).

Here, we show that the activation of TIMP-1 gene expression in hepatic cells by IL-6 and by another IL-6 type cytokine, oncostatin M (OSM), is mediated by a specific IL-6/OSM response element present in the TIMP-1 promoter. The activation of the TIMP-1 gene correlates with the binding of an IL-6/OSM induced protein to the STAT binding site, and we have identified this protein as STAT3. However, full responsiveness of the promoter requires both the AP-1 and STAT binding sites, which are present within an identified IL-6/OSM responsive element.

MATERIALS AND METHODS

Screening of a genomic library, DNA subcloning and nucleotide sequencing

A rat genomic library (in Charon 4A) was a gift of Dr Z. Krawczyk (Gliwice, Poland). Screening was carried out in 5× Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA), 0.12 M phosphate buffer pH 6.8, 3× SSC (1× SSC: 150 mM NaCl and 15 mM sodium citrate) at 65°C using the mouse TIMP-1 cDNA as a probe (16). DNA fragments from recombinant phages were subcloned into plasmids using standard

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techniques (17). Sequencing was performed using a T7 DNA polymerase sequencing kit (Pharmacia) and plasmid dsDNA.

Cell culture and stimulation

Human hepatoma HepG2 cells and rat hepatoma Fao cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal calf serum and antibiotics. Cells were stimulated with 50 ng/ml (10^7 U/mg) IL-6 or 50 ng/ml (4.7×10^7 U/mg) OSM (both a gift from Immunex, Seattle, WA). Actinomycin D (5 μ g/ml) was added to the medium 20 min before either IL-6 or OSM treatment.

RNA preparation and Northern blot analysis

Total RNA was prepared using the phenol extraction method (18,19). Five μ g samples of RNA were subjected to formaldehyde gel electrophoresis using standard procedures (17) and transferred to Hybond-N membranes (Amersham), according to the manufacturer's instructions. The filters were prehybridized at 68°C for 3 h in 10% dextran sulfate, 1 M sodium chloride and 1% SDS, and hybridized in the same solution with cDNA fragments labeled by random priming (20). After the hybridization, non-specifically bound radioactivity was removed by washing in $2\times$ SSC at room temperature, followed by two washes in $2\times$ SSC/1% SDS at 68°C for 20 min.

Synthetic oligonucleotides

In order to obtain deletion mutants containing the regions (–61 to +55), (–51 to +55) and (–35 to +55) we used following PCR primers: 5'-AGTGTCTAGATGAGTAATGCGTC-3', 5'-GATGTCTAGAGCGTCCAGGAAGCCTGGAG-3', 5'-GAAGTCTAGAGGAGTATT-3' and a CAT primer annealing to the vector, 5'-ACGGTGGTATATCCAGTGAT-3'. Plasmids containing point mutations introduced into the AP-1 and STAT/Ets elements within TIMP-1 promoter were generated by PCR using as primers: mut(AP-1), 5'-AGTGTCTAGATGAGTCTGCGTCCAGGAAGC-3'; mut(halfSTAT)-1, 5'-AGTGTCTAGATGAGTAATGAGCTCAGGAAGCCTGGAG-3'; mut(halfSTAT)-2, 5'-AGTGTCTAGATGAGTAATCCCGGAGGAAGCCTGGAG-3'; mut(STAT/Ets), 5'-AGTGTCTAGATGAGTAATGCGTCCAGAGCCCTGGAGGAGTGA-3'; mut(AP-1+halfSTAT), 5'-AGTGTCTAGATGAGTCTGAGTCCAGGAAGCCTGGAG-3' and a CAT primer. All oligonucleotides used for gel retardation assays were designed to contain single-stranded 5' overhangs of four bases at both ends after annealing. The following double-stranded oligonucleotides were used as labeled probes or competitors in gel retardation assays (top strands are shown): rat TIMP-IL-6RE, 5'-GATCTGTGGATGAGTAATGCGTCCAGGAAGCCTG-3'; TIMP-IL-6REmut(halfSTAT), 5'-AGTGTCTAGATGATAATGAGCTCAGGAAGCCTGGAG-3'; TIMP-AP-1, 5'-GATCTGATGAGTAATGA-3'; TIMP-STAT, 5'-GATCTGTCCAGGAAGCA-3'; TIMP-Ets, 5'-GATCTAAGCAGGAAGCA-3'; rat SPI-3 STAT site (21), 5'-GATCTGTTCCCGAAA-3'; high affinity sis-inducible element of human fos promoter (SIE) (22), 5'-TCGACGT-CATTTCCCGTAAATCCCG-3'; and human collagenase AP-1 (TRE) site, 5'-TCGACTAGTATGAGTCAGCCG-3' (23).

Plasmid construction

Plasmid ptkCAT Δ EH is a derivative of pBLCAT2 (24) from which the *Eco*O109I–*Hind*III fragment (harboring the cryptic enhancer) was deleted. Plasmid prT-2400CAT was obtained by insertion of a 2.4 kb *Bam*HI–*Hinf*I(blunt) fragment containing the 5' flanking region of rat TIMP-1, into *Bam*HI/*Bgl*II(blunt) sites of ptkCAT Δ EH. Plasmids prT-1449CAT and prT-93CAT derive from prT-2400CAT from which *Sph*I–*Sph*I and *Xba*I–*Apa*I fragments were deleted respectively. Plasmid prT-88CAT and prT-45CAT were obtained by insertion of *Sty*I(blunt)–*Xho*I and *Bst*NI(blunt)–*Xho*I fragments from prT-93CAT into *Bam*HI(blunt)–*Xho*I sites of ptkCAT Δ EH respectively. Plasmids prT-61CAT, prT-51CAT, prT-35CAT, prTmut(AP-1)CAT, prTmut(halfSTAT)-1CAT, prTmut(halfSTAT)-2CAT, prTmut(STAT/Ets)CAT and prTmut(AP-1+halfSTAT)CAT were obtained by insertion of *Xba*I–*Xho*I digested PCR products into *Xba*I/*Xho*I sites of ptkCAT Δ EH. Plasmids p2x(rTIL-6RE)tkCAT, p5x(rTAP-1)tkCAT, p4x(rTSTAT)tkCAT and p6x(rTEts)tkCAT were generated by insertion of double stranded oligonucleotide(s) with *Bgl*II end into *Bam*HI site of ptkCAT Δ EH. All constructs were sequenced on both strands. The plasmid containing mouse TIMP-1 cDNA was kindly provided by Dr D. R. Edwards (University of Calgary, Calgary, Canada) (16). Plasmid pS3(4xSTAT)tkCAT, which contains four copies of SPI-3 STAT element fused to the *tk* promoter was used as a control (21).

Transient transfections

HepG2 cells were grown to 30% confluency and transfected in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, using calcium phosphate precipitates (25), with 5 μ g of plasmid DNA and 3 μ g of internal control plasmid pCH110 (Pharmacia). Cells were incubated with precipitate for 9 h, washed twice with PBS, and media changed. One day after transfection, cells were stimulated, then cultured another 24 h and harvested. Protein extracts were prepared by freeze-thawing (26), and protein concentration was determined by the BCA method (Sigma). Chloramphenicol acetyltransferase (CAT) and β -galactosidase assays were performed as described (27,28). CAT activity was normalized to the internal control β -galactosidase activity.

Extract preparation and gel retardation assays

Nuclear and cytosolic extracts were prepared as described previously (29,30). Double-stranded DNA fragments or oligonucleotides were labeled by filling in 5' protruding ends with Klenow enzyme using [α - 32 P]dCTP (3000 Ci/mmol). Gel retardation assays were carried out according to published procedures (31,32). Two to five μ g of nuclear extracts and ~10 fmol (10 000 c.p.m.) of probe were used. The polyclonal rabbit anti-mouse anti-STAT3 and anti-STAT1 antisera were kindly provided by Dr J. E. Darnell Jr (The Rockefeller University, New York, NY).

RESULTS

TIMP-1 is synthesized in rat liver, and its expression is enhanced by intraperitoneal injection of lipopolysaccharide (14). We have previously shown that IL-6 is capable of inducing TIMP-1 mRNA expression in primary rat hepatocytes (14,15). In the present report we stimulated rat hepatoma Fao cells with IL-6 and another IL-6 type cytokine, OSM, both in the presence and

Figure 1. Effect of Actinomycin D on the IL-6 and OSM activation of the rat TIMP-1 gene. Fao cells were treated with IL-6 (50 ng/ml), OSM (50 ng/ml) and (or) Actinomycin D (5 µg/ml). After the times indicated RNA was isolated and subjected to Northern blot analysis using mouse TIMP-1 cDNA as a probe. Lower panel shows RNA stained with ethidium bromide on a membrane.

absence of Actinomycin D, a known inhibitor of transcription. Figure 1 shows that IL-6 and OSM both activate TIMP-1 expression and that this activation is blocked by Actinomycin D. This result suggests a transcriptional mechanism for IL-6/OSM activation. In order to study the mechanism of IL-6/OSM induced transcription we cloned the rat TIMP-1 gene, together with the 5' flanking region. Approximately 4×10^5 recombinants of the rat genomic library were screened and 26 positive clones were obtained. Restriction mapping of the rat TIMP-1 gene and sequencing of the 5' flanking region is shown in Figure 2. In order to identify the regulatory element(s) responsible for IL-6/OSM activation, we cloned the 2.4 kb long 5' flanking region into the reporter plasmid encoding the bacterial CAT gene and transiently transfected HepG2 cells. As shown in Figure 3, transfected cells expressed the CAT gene, and treatment of these cells with IL-6 or OSM resulted in an increase in CAT activity. We then generated a series of 5' deletion mutants and transiently transfected these constructs into HepG2 cells. We stimulated the transfected cells with IL-6 or OSM and assayed each construct for basal promoter activity and IL-6/OSM responsiveness. As shown in Figure 3, the construct containing a 5' upstream region up to -93 had full basal activity and full IL-6/OSM responsiveness. There are two putative binding sites for the transcription factors Sp-1 and C/EBP at -93 to -87 and -75 to -67 respectively. The deletion of the Sp-1 site (to -87) had no effect. However, deletion from -87 to -61 severely reduced the basal promoter activity, although it did not abolish the IL-6/OSM responsiveness. This might result from the loss of the potential binding site for C/EBP. Further truncation to -51 and -41 led to further reduction of the basal activity and significant loss of the responsiveness to IL-6 and OSM. The shortest construct truncated to -35 had the lowest basal activity and barely responded to IL-6/OSM. These results indicate that the basal activity of the TIMP-1 promoter is probably determined by sequences present in the 87 bp long promoter whereas the IL-6/OSM responsive element(s) resides in the region between -61 and -35.

Further analysis of the IL-6/OSM responsive element was made by gel retardation assays using the (-64 to -36) promoter fragment. This fragment contains putative binding sites for transcription factors AP-1 (-59 to -53), STAT3 (-49 to -41) and Ets (-45 to -38). The results are shown in Figure 4. Binding of a constitutive and IL-6/OSM-induced protein to the (-64 to -36) fragment can readily be seen. The binding of the induced protein

Figure 2. Nucleotide sequence of the 5' flanking region of the rat TIMP-1 gene. (A) Restriction map of *Bam*HI (B), *Bgl*III (Bg), *Sph*I (S) and *Xba*I (X) sites in the rat TIMP-1 gene. Arrow indicates multiple transcription start sites according to the mouse and human genes. (B) Nucleotide sequence of the 5' flanking region of the rat TIMP-1 gene. EMBL accession no. X90486. Binding sites for AP-1, C/EBP, Sp-1 and STAT3 are boxed. The sequence representing TIMP-1 IL-6RE is underlined.

was competed for by an oligonucleotide containing the STAT binding site of the SPI-3 gene, whereas the constitutive band was competed by an oligonucleotide containing the AP-1 binding site of the collagenase gene. We also used shorter oligonucleotides containing TIMP-1 AP-1, STAT or Ets sites and again performed gel retardation assays. The TIMP-1 AP-1 oligonucleotide bound the constitutive protein, and IL-6 treatment of the cells had no effect on the gel shift pattern (Fig. 4C). In contrast, the TIMP-1 STAT oligonucleotide was bound by the IL-6 (or OSM) induced protein (Fig. 4B), whereas we did not observe any binding of protein present in our extracts to the TIMP-Ets oligonucleotide (Fig. 4D).

The IL-6/OSM induced protein has been identified by using polyclonal anti-STAT3 and anti-STAT1 antisera and performing supershift experiments. Nuclear extracts were preincubated with specific antiserum or preimmune serum and probes were then added. Figure 5A shows that the IL-6/OSM induced band completely disappeared when anti-STAT3 antiserum was used, but was not affected by preimmune serum and anti-STAT1 antiserum. When high affinity SIE probe was used as a control

Figure 3. Localization of the IL-6/OSM response element in the TIMP-1 promoter. HepG2 cells were transiently transfected with 5 μ g of plasmid prT-2400CAT, prT-1449CAT, prT-93CAT, prT-88CAT, prT-61CAT, prT-51CAT, prT-45CAT or prT-35CAT and 3 μ g of β -galactosidase expression vector pCH110 as an internal control for transfection efficiency. One day after transfection cells were stimulated with 50 ng/ml IL-6 or 50 ng/ml OSM, cultured for another 24 h and harvested. CAT activities were normalized to β -galactosidase activities [c.p.m./units].

IL-6-induced STAT3 or OSM-induced STAT1 and STAT3 were detected in the analyzed nuclear extracts (Fig. 5B).

The results from gel shift experiments showed that IL-6/OSM activation of the TIMP-1 gene can be correlated with the binding of STAT3 to the element located at -49 to -41. However, the deletion mutant containing this site but lacking the AP-1 element (-51 to +55) was not fully responsive to IL-6 or OSM. In order to evaluate the contribution of the AP-1 and STAT elements in the IL-6/OSM induced response of TIMP-1 gene, we mutated these elements and performed transient transfection experiments. As shown in Figure 6, mutation of the AP-1 element resulted in lower basal activity of the promoter and decreased responsiveness to IL-6/OSM by 60%. Thus, the mutation of this element results in the same effect as its deletion. The two mutations introduced into already mismatched half of the palindromic STAT binding site (consensus sequence of the overlapping Ets element was not changed) decreased the responsiveness by 25 and 60%. The mutation introduced into the second half of STAT element (and overlapping Ets element) decreased responsiveness to IL-6 or OSM by 60%. The mutation introduced into mismatched half of the palindromic STAT element also abolished the binding of STAT3 in the gel shift experiments (Fig. 5C). Finally, the promoter with the mutated AP-1 element and the mutated one half

of the palindromic STAT sequence showed only marginal response to IL-6/OSM. These experiments support the hypothesis that both the AP-1 and STAT elements are necessary for full responsiveness to IL-6 and OSM.

Next, we fused the TIMP-1 IL-6RE (-64 to -36), TIMP-STAT, TIMP-AP-1 and TIMP-Ets elements to the thymidine kinase promoter driving the transcription of the CAT gene, and again performed transient transfection experiments. Two copies of the TIMP-1 IL-6RE conferred a responsiveness to both IL-6 and OSM onto the *tk* promoter (Fig. 7). In contrast, CAT constructs containing multimers of either AP-1, STAT or Ets elements in front of the *tk* promoter did not respond to IL-6 or OSM. Thus, it seems that both adjacent elements (AP-1 and STAT) are necessary for full responsiveness of the TIMP promoter to IL-6 and OSM, and both elements are needed to confer responsiveness onto a heterologous promoter.

DISCUSSION

We report the cloning of the 5' flanking region of rat TIMP-1 gene and the identification of an IL-6/OSM responsive element at -64 to -36 (TIMP-1 IL-6RE). The deletion of this element resulted in the loss of responsiveness to both cytokines, and its fusion conferred a positive response onto a heterologous promoter. The identified element contains putative binding sites for the transcription factors AP-1, STAT and Ets with the distal part of this element binding AP-1 as shown by the gel retardation assay. AP-1 is rapidly activated by a variety of stimuli and contains the polypeptides that are encoded by the immediate-early response genes of the *fos* and *jun* families. In our experiments we did not observe any activation of AP-1 after treatment with IL-6 or OSM. This might be due to the already high basal expression of AP-1 in hepatoma cells. The proximal part of the IL-6/OSM response element contains overlapping binding sites for STAT and Ets proteins. The TIMP-1 IL-6RE binds an IL-6/OSM induced protein, and this protein binds to a shorter oligonucleotide containing the putative STAT binding site (Fig. 4). Using competitors and specific antibodies we have shown that this protein is STAT3 (Fig. 5). Recently, it was shown that STAT3 is a latent protein transiently phosphorylated in response to factors including the cytokines from the IL-6 family, upon which it forms dimers, translocates to the nucleus and binds to the regulatory elements of target genes (30,33-39). We did not observe any binding of proteins from HepG2 extracts to the oligonucleotide containing the Ets binding site although binding of recombinant Ets was reported to the mouse TIMP-1 promoter at -44 to -39 (40), and this element is conserved between mouse, rat and man.

The STAT element of TIMP-1 possesses one mismatch to the palindromic sequence proposed as a consensus binding site for STAT3 (30). Mutations introduced into the mismatched half of the palindrome reduced the IL-6/OSM responsiveness (by 25 and 60%) and STAT3 could no longer bind to the mutated element in the gel shift experiments. Mutation of the second part of the palindrome resulted in a similar loss of responsiveness to both cytokines by 60%. Since the mutation introduced into the mismatched half of palindrome did not change the overlapping Ets consensus we conclude that STAT proteins rather than Ets factors regulate the TIMP-1 expression.

In contrast to the entire IL-6 RE of TIMP-1, the separately tested AP-1, STAT and Ets elements identified within this element are not capable of conferring the IL-6/OSM responsive-

Figure 4. Gel retardation assay of nuclear extracts from HepG2 cells using fragments derived from TIMP-1 promoter. HepG2 cells were incubated with 50 ng/ml IL-6 or 50 ng/ml OSM for 15 min (A, B and D) or for 8 h (C). Cells were harvested and nuclear extracts prepared. Nuclear protein (2–5 μ g) was incubated with 32 P-labeled double-stranded fragments derived from the TIMP-1 promoter; TIMP-IL-6RE (A), TIMP-STAT (B), TIMP-AP-1 (C) and TIMP-Ets (D). As competitors, a 100-fold excess of each of the following unlabeled double-stranded oligonucleotides was added to the binding reaction: rat serine proteinase inhibitor 3 STAT site (STAT), human collagenase AP-1 site (AP-1). The formed DNA–protein complexes were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed to X-ray film. The arrow indicates the IL-6/OSM induced protein, a star marks the protein binding to the AP-1 element.

Figure 5. An IL-6/OSM induced protein binding to the IL-6RE of TIMP-1 gene is recognized by anti-STAT3 antibody. HepG2 cells were stimulated with 50 ng/ml IL-6 or 50 ng/ml OSM for 15 min and nuclear extracts were prepared. Nuclear proteins (5 μ g) were incubated in the gel retardation assay cocktail with the preimmune serum (PRE), anti-STAT1 or anti-STAT3 antiserum for 30 min (1:400 dilution). Binding to the TIMP-1 STAT element (A) or SIE (B) was then analyzed by gel retardation assay. The arrow indicates the IL-6/OSM induced protein binding to the TIMP-1 STAT element.

ness onto a heterologous promoter. The STAT elements of other genes (i.e. rat α 2-macroglobulin, serine proteinase inhibitor 3, ICAM) (21,30,41) do confer IL-6 responsiveness onto heterologous promoter. However, the TIMP-1 STAT element has a much lower affinity in comparison to these elements (data not shown), and simultaneous binding of an AP-1 is probably necessary to confer responsiveness onto the fused heterologous promoter. This correlates well with weaker IL-6/OSM stimulation of TIMP-1 gene expression when compared with the above genes.

The TIMP-1 IL-6RE has a very similar structure to the previously identified IL-6RE of *junB* gene (42). It was shown that the *junB* IL-6RE binds the AP-1 and Ets transcription factors. Furthermore, cotransfection of the Ets-expression plasmid resulted in the activation of this promoter (43). However, it has recently been shown (43,44) that after IL-6 stimulation, STAT3 binds to the Ets-like binding site of the *junB* IL-6RE, and this binding can be correlated with the transcriptional activity of *junB*. Moreover, the deletion or mutation of AP-1 binding site of the

Figure 6. Effect of point mutations in the AP-1 and STAT binding sites. Point mutations were introduced into the TIMP-1 promoter (B). HepG2 cells were transiently transfected with 5 μ g of plasmid prTmut(AP-1)CAT, prTmut(halfSTAT)1CAT, prTmut(halfSTAT)2CAT, prTmut(STAT/Ets)CAT or prTmut(AP-1+halfSTAT)CAT and 3 μ g of plasmid pCH110 as described in the legend to Figure 3. Cells were stimulated with 50 ng/ml IL-6 or 50 ng/ml OSM, and lysates were prepared 24 h later. CAT activities were normalized to β -galactosidase activities. The IL-6 and OSM responsiveness was expressed in fold induction, which was normalized by dividing the IL-6 (and OSM) response of each CAT gene construct by that of prT-35CAT (A). (C) HepG2 cells were incubated with 50 ng/ml IL-6 or 50 ng/ml OSM for 15 min, harvested and nuclear extracts were prepared. Nuclear protein (2 μ g) was incubated with the 32 P-labeled (-64 to -36) fragments derived from the wild-type TIMP-1 promoter or the promoter containing the mutation no 1 introduced into mismatched half of the palindromic STAT3 binding site. The formed DNA-protein complexes were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed to X-ray film. Arrow indicates binding of an IL-6/OSM induced protein.

junB promoter resulted in a loss of responsiveness to IL-6 (42). Similarly, the mutation introduced into the AP-1 site of the TIMP-1 promoter resulted in a drastic decrease of responsiveness to IL-6/OSM. The STAT element of *junB* promoter was shown not to bind the STAT1 protein (43). The stimulation of HepG2 cells with OSM leads to the activation of both STAT3 and STAT1 (Fig. 5B) but the protein binding to the TIMP-1 STAT site is recognized only by anti-STAT3 antibodies. Thus, it seems that similarly to *junB*, the TIMP-1 STAT element does not bind STAT1. The identified TIMP-1 element is an interesting example of IL-6/OSM responsive element which requires two internal binding sites for the transcription factors AP-1 and STAT3. This observation suggests that both AP-1 and STAT3 cooperate in the induction of TIMP-1 transcription. Possible interactions between these two factors need to be explored in the future.

Figure 7. The TIMP-1 IL-6RE element confers responsiveness to IL-6/OSM onto the *tk* promoter. The (-64 to -36), TIMP-AP-1, TIMP-STAT and TIMP-Ets elements were cloned in the front of the *tk* promoter. HepG2 cells were transfected with 5 μ g of obtained plasmids and 3 μ g of plasmid pCH110 as described in the legend to Figure 3. Cells were stimulated with 50 ng/ml IL-6 or 50 ng/ml OSM, and lysates were prepared 24 h later. CAT activities were normalized to β -galactosidase activities. The IL-6 and OSM responsiveness was expressed in fold induction.

NOTE ADDED IN PROOF

During the time this manuscript was being considered for publication the functional interaction between the AP-1 component c-jun and STAT3 β was demonstrated [Schaefer *et al.* (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 9097-9101].

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