Tissue inhibitor of metalloproteinases-1 (TIMP-1) can be regulated by gp130 cytokines such as IL-6 and oncostatin M (OSM). Polymere chain reaction deletion analysis of the murine TIMP-1 proximal promoter in chloramphenicol acetyltransferase reporter gene constructs identified an AP-1 element (−59−53) that allows maximal responsiveness to OSM in HepG2 cells. Fos and Jun nuclear factors bound constitutively to this site as identified by supershift analysis in electrophoretic mobility shift assays, and oncostatin M (but not IL-6) induced an additional “complex 2” that contained c-Fos and JunD. OSM stimulation a rapid and transient increase in c-Fos mRNA and nuclear protein that coincided with complex 2 formation. Phorbol 13-myristate 12-acetate could also induce c-Fos but could not regulate the TIMP-1 reporter gene constructs. Transfection studies also showed that 3′-deletion of sequences downstream of the transcriptional start site (+1/+47) markedly reduced OSM -fold induction. Nuclear factors bound to SP1 and Ets sequences were detected, but were not altered upon OSM stimulation. Although OSM and IL-6 induced STAT (signal transducers and activators of transcription) factors to bind a high affinity Sis-inducible element DNA probe, binding to homologous TIMP-1 promoter sequences was not detected. Thus, OSM (but not IL-6) stimulates c-Fos, which participates in maximal activation of TIMP-1 transcription, likely in cooperation with other factors such as SP1 or as yet unidentified mechanisms involving the +1 to +47 region of the promoter.

Cytokines play a pivotal role as soluble growth factors acting on immune and non-immune cells to coordinate the progression of and resolution from an inflammatory response. The interleukin-6 (IL-6)¹ family of cytokines which include IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, ciliary neurotropic factor, and cardiotrophin-1 possess both unique and shared biological activities and utilize a common signal transducing receptor subunit gp130 (1, 2). In addition, the polypeptide primary structures of OSM, LIF, and, to a lesser extent IL-6, ciliary neurotropic factor, and granulocyte colony-stimulating factor display sequence homology, suggesting a functional relationship for these cytokines (3). Biochemical studies on signaling by gp130 have demonstrated tyrosine phosphorylation of a variety of components of the Ras-MAP kinase cascade (4, 5) and activation of the recently characterized family of STATs (signal transducers and activators of transcription) (6, 7). Upon activation, cytosolic STAT proteins homo- or heterodimerize and translocate to the nucleus where they bind DNA elements termed γ-interferon activation sequence (GAS). One target gene of STAT proteins is c-fos, which harbors a GAS-like DNA element, the Sis-inducible element (SIE), within its promoter.

OSM is secreted as a 28-kDa polypeptide by mitogen-activated T cells and endotoxin-stimulated macrophages (8). It was first characterized by its ability to specifically inhibit the growth of the A375 melanoma cell line (9). Subsequently, OSM was demonstrated to stimulate growth of fibroblasts (10), aortic endothelial cells (11), and hematopoietic cells (12), as well as promote leukemic cell differentiation (3), while suppressing embryonic stem cell differentiation (13) and several tumor cell lines including the HTB10 lung carcinoma (14). OSM elicits acute phase protein production by hepatocytes and HepG2 hepatoma cells (15). In addition, unlike other IL-6-cytokines, OSM also specifically up-regulates low density lipoprotein receptor (16) in HepG2 cells. The transduction of signals that lead to biological activities unique to OSM but not other IL-6 family members is not yet defined.

Extracellular zinc-dependent endopeptidases (the matrix metalloproteinases) can be inhibited by a family of proteins called tissue inhibitors of metalloproteinases (includes TIMP-1, -2, -3, and -4), which act to modulate extracellular matrix metabolism by matrix metalloproteinases (17–21). TIMP-1 expression is up-regulated in fibroblasts by a variety of soluble factors including IL-1 (22), tumor necrosis factor (23), epidermal growth factor (23), transforming growth factor-β (23), phorbol esters (24), and retinoic acid (25). We have shown that OSM potently induces mRNA expression of the TIMP-1 gene in hepatocyte cell lines and primary fibroblasts (26). Work by Edwards et al. (27) has suggested that the −95 to +47 region of the murine TIMP-1 promoter is sufficient to confer serum responsiveness in mouse cells and this region contains several putative regulatory motifs including SP1, AP-1, and Ets DNA elements. Here, we examine OSM regulation of the −95 to +47 TIMP-1 promoter in HepG2 cells and characterize the partici-
pation of c-Fos in the binding of nuclear factors to an AP-1 site is between −62 and −53 that is necessary for marked OSM (but not IL-6) up-regulation of promoter/CAT reporter gene expression. We examined IL-6- and phorbol 13-myristate 12-acetate (PMA)-induced responses to identify OSM-specific effects on this promoter region and the participation of SP1, Ets, and other promoter sequences in regulation of TIMP-1 expression.

MATERIALS AND METHODS

Cell Culture and Reagents—The human hepatoma cell line HepG2 (purchased from ATCC) cultured and passaged by standard techniques in Eagle’s minimal essential medium (supplemented with 10% fetal bovine serum (FBS)). Cytokines used were: purified human recombinant OSM, expressed in Chinese hamster ovary cells, provided by M. Hanson (Bristol-Myers Squibb Research Institute, Seattle, WA); purified recombinant human IL-6, IL-1β, and LIF, kindly provided by Dr. M. Widmer (Immunex Corp., Seattle, WA); interferon-γ, purchased from Genzyme Corp. (Cambridge, MA). Rabbit polyclonal antibodies against Fos/Jun (anti-pan-Fos, anti-c-Fos, anti-c-Jun, anti-JunB, anti-JunD), SP1, STAT3 and Ets-1/Ets-2 nuclear factors, and specific peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-STAT1 (ISGF3) was from Transduction Laboratories (Lexington, KY). Phenylmethylsulfonyl fluoride, pepstatin A, PMA, puramycin, and emetine were purchased from Sigma. When added, puramycin and emetine were used at a concentration of 20 μg/ml. Leupeptin was purchased from Boehringer Mannheim.

Northern Blots—Total RNA was from HepG2 cells according to Chomczynski and Sacchi (28). Subconfluent HepG2 cultures were washed and replenished in medium containing 2% FBS. Cytokines (at indicated concentrations) were then added, and cultures were incubated for the indicated times before RNA isolation. Northern blots were prepared by standard techniques and probed with human TIMP-1 cDNA (gift of Dr. A. J. P. Docherty, Celltech, Slough, United Kingdom) and rat c-Fos cDNA (kindly provided by Dr. Tony Cruz, Mount Sinai, Toronto, Canada). The intensity of ethidium bromide-stained 18 S and 28 S bands on the blots was used to estimate loading of RNA.

Polymerase Chain Reaction (PCR) Deletion of the TIMP-1 Promoter and Cloning—Deletions of the TIMP-1 promoter were carried out by generating truncated PCR products within −95 to +47. PCR reactions consisted of Vent polymerase (5 units; New England Biolabs), 100 ng of TIMP-1 (−223 to +47) bPBLCAT3 (27) template DNA, 150 μM sense and antisense oligonucleotide primers, 5 mM dNTPs, and 1 × Vent polymerase New England Biolabs buffer in a 50-μl final reaction volume. Reaction mixtures were overlaid with 20 μl of mineral oil and denatured for 1 min at 95 °C, followed by annealing of primers at 55 °C for 2 min and primer extension at 72 °C for 2 min in a Perkin-Elmer PCR thermal cycler for 35 cycles.

Primers were used to generate 5′ deletions of the TIMP-1 promoter included: −62/−38 for construct B, which contains AP-1 and Ets sites (5′-GACCTAAAGTTGGTGACCTTGACGAGACCCGG-3′) and −51/−38 for construct C, which contains only an AP-1 site (5′-GAGCTTAC-TTGGCTTCAGGAAAGCCTGGAGG-3′); and −39/-/−16 for construct D (5′-GAGCTTACTCTGTGGAGGAACTGGTCTCCGGCC-3′). 3′ PCR primers (for constructs B–D) included a sequence 3′ to the pBLCAT3 multiple cloning site (−470/+47) (5′-CGATAAAATTCCTGAAGGGCGG-3′). Restriction sites for HindIII in 5′ promoters (AGGCTT) and BglII in 3′ promoters (AGATCT), as well as 6 nuclear residues 5′ to each site (for efficient restriction enzyme digestion of DNA termini), were included in the designed primers. PCR products were purified from an 8% polyacrylamide gel, restriction-digested with HindIII/BglII, re-purified, and ligated into linearized pBLCAT3 vector (HindIII/BglII-digested). Colony hybridization (Colonies/Plaque Screen, NEN Research Products) with end-labeled PCR primers as probes were then used to screen for DH5α bacterial transformants. DNA sequencing was used to verify the correct constructs.

Chloramphenicol Acetyltransferase (CAT) Assays—HepG2 cells in 100-mm dishes were transfected with 10 μg of CAT reporter plasmid DNA (co-transfected with 1.4 μg of pSV-βGal plasmid, Promega) by the calcium phosphate coprecipitation method. Cells were allowed to recover overnight and then replated into six-well tissue culture plates. Prior to cytokine stimulation, cells were serum-starved in serum-free α-minimal essential medium for 6 h. Cytokines were then added for 18 h. CAT assays were carried out using cell lysates according to standard protocols and 3H-labeled chloramphenicol products quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. Values were normalized to β-galactosidase activity in lysates (29).

Preparation of Nuclear Extracts—HepG2 cells were stimulated with the indicated cytokines for various time periods. Nuclear extracts were prepared according to Andrews et al. (30) with the addition of modifications. Buffers A and C contained 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 2 μg/ml pepstatin A and leupeptin. Cells were resuspended in 400 μl of buffer A, and nuclear proteins were extracted in 100 μl of buffer C, frozen in liquid nitrogen, and stored at −70 °C.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extract (15 μg) was incubated with 2 μg of poly(dI-dC) and 5 μg of calf thymus DNA in a binding buffer (5 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 1 mM spermidine, and 5 mM glycerol) for 15 min on ice. A total of 106 cpm of 32P-labeled probe was added, and the binding reaction (20 μl) was incubated at room temperature for 20 min. Excess of a unlabeled oligonucleotide (50–100-fold) was added to the reaction for competition assays. Following the reaction, samples were electrophoresed on 5% polyacrylamide gels (40:1) containing 1.25% glycerol in 0.25 × TBE (1 × TBE: 89 mM Tris borate, 2 mM EDTA) at 95 V for 3.5 h and dried prior to autoradiography. For supershift analysis, antibodies were added following the binding reaction for 1 h at 4 °C. Sequences of oligonucleotides used for mobility shift assays are shown in Table 1, and their location in the TIMP-1 promoter is illustrated in Fig. 2. AP-1 and SP1 consensus oligonucleotides were purchased from Santa Cruz Biotechnology Inc. Oligonucleotides were annealed by heating to 100 °C for 5 min in 100 mM MgCl2, and 400 μl Tris-Cl (pH 8), followed by gradual cooling to 20 °C. TIMP-1 DNA probes were end-labeled using polynucleotide kinase and the high affinity Sis-inducible element (hSIE) labeled by the fill-in reaction using 32P-dCTP and the Klenow enzyme. Probes were then gel-purified.

Western Blots—Nuclear extracts were electrophoresed on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel prior to transfer of proteins onto an Immobilon nitrocellulose membrane. Membranes were then blocked overnight in 1 × PBS and 5% milk. Following a 10-min incubation in wash buffer (1 × PBS, 0.5% Tween 20), membranes were incubated with primary rabbit polyclonal antibody (anti-c-Fos, anti-fos, anti-c-Jun, anti-JunB, anti-JunD, anti-pan-Fos, anti-c-Jun, anti-JunB, or anti-JunD; Santa Cruz Biotechnology Inc.) at a 1:2500 dilution for 1 h at room temperature. To assess specificity of antibody binding, 2 μg of peptide was preincubated with antibody for 1 h at 4 °C (as recommended by Santa Cruz Biotechnology Inc.). Membranes were then washed three times for 10 min each. Goat anti-rabbit horseradish peroxidase (Sigma) was added for an additional (1:7500 dilution) 1 h and Immobilon filters were developed by Enhanced Chemiluminescence Renaissance (NEN Life Science Products).

RESULTS

Oncostatin M Induces TIMP-1 and c-Fos Expression—We have found previously that OSM elevates TIMP-1 mRNA levels after overnight stimulation in a variety of cell types including HepG2 (26). Examination of TIMP-1 mRNA expression in HepG2 cells over a time course of OSM stimulation (Fig. 1) revealed a 5-fold increase in TIMP-1 message by 1 h, near maximal at 2 h (40-fold), and maximal mRNA levels at 6 h (60-fold). OSM also transiently stimulated mRNA levels of the AP-1 immediate-early gene c-fos (Fig. 1). As demonstrated previously in response to other stimuli (31), the up-regulation of c-fos mRNA occurred early (maximal induction at 30 min (20-fold)) and decreased thereafter. Thus, c-Fos mRNA levels peaked slightly before the marked increase in TIMP-1 mRNA.

OSM Regulates the TIMP-1 Proximal Promoter—The proximal promoter of the TIMP-1 gene (−95/−47 sequence) can regulate downstream CAT gene expression in pBLCAT3 chimeric reporter constructs (27). When transiently transfected into HepG2 cells, the expression of −95/−47CAT could be elevated upon OSM (5.2-fold) or IL-6 (3.8-fold) stimulation (Fig. 2, construct A). Several putative DNA elements can be identified within −95 to +47 region of TIMP-1, including those for SP1, AP-1 (Fos/Jun), and Ets proteins. To examine the potential role of these sites in transcription, we generated 5′ deletions of this region and cotransfected HepG2 cells with constructs A–F (Fig. 2) and pSV-βGal to normalize for transcription efficiency. Basal levels of CAT transcription were reduced in the plasmid lack-
OSM Stimulation of c-Fos in HepG2 Cells

Oligonucleotides were synthesized, annealed, and purified as indicated under "Materials and Methods." The sequences correspond to the TIMP-1 promoter regions as indicated. Defined nuclear factor binding sites for SP1, AP-1, and Ets have been underlined. Probe 3A contains a mutated AP-1 site within this region (construct C). The AP-1 site within this region (construct C) markedly reduced IL-6 responsiveness to OSM (from 11.4- to 2.7-fold), whereas repression of the AP-1 site within this region (construct C) markedly reduced IL-6 responsiveness to OSM (from 11.4- to 2.7-fold), whereas responsiveness to OSM (from 11.4- to 3.7-fold) and also reduced IL-6 responses (from 4.1- to 2.3-fold). Thus, sequences within +1/+47 may cooperate with the TIMP-1 AP-1 element within −62/−53 for maximal responsiveness to OSM. AP-1 elements are common within TATA-less promoters and have been demonstrated to participate in basal transcription (32). A chimeric plasmid with mutation of the AP-1 site (construct F) showed reduced basal transcription (4-fold) but was still inducible by OSM (construct E versus F).

**Nuclear Factors Bound to the TIMP-1 Promoter**—We have examined a variety of overlapping oligonucleotide probes (probes 1–6; location shown in Fig. 2) spanning −95 to +47 of the TIMP-1 promoter in EMSA analysis to identify elements that bind nuclear factors in HepG2 cells. A specific bandshift in unstimulated cell nuclear extracts that we termed “complex 1” containing −95 to −63 sequences (construct B), and further reduced in the plasmid that also lacked the −62 to −53 sequences, which contained the AP-1 site (construct C). The −62/+47CAT chimeric plasmid (construct B) demonstrated maximal responsiveness to OSM with an 11.4-fold increase in CAT activity over unstimulated cells, whereas IL-6-induced 4.1-fold increases. Deletion of the AP-1 site within this region (construct C) markedly reduced responsiveness to OSM (from 11.4- to 2.7-fold), whereas responses to IL-6 decreased from 4.1- to 2-fold. Thus, a promoter region from −62 to −53 of TIMP-1, containing a putative AP-1 binding site (at −59/−53), contributes to basal transcription and to induction of transcription by OSM. Deletion of this sequence removed any significant difference between OSM and IL-6 activity in this assay. Interestingly, deletion of sequences from +1 to +47 (construct E versus B) dramatically abrogated the responsiveness to OSM (from 11.4- to 3.7-fold) and also reduced IL-6 responses (from 4.1- to 2.3-fold). Thus, sequences within +1/+47 may cooperate with the TIMP-1 AP-1 element within −62/−53 for maximal responsiveness to OSM. AP-1 elements are common within TATA-less promoters and have been demonstrated to participate in basal transcription (32). A chimeric plasmid with mutation of the AP-1 site (construct F) showed reduced basal transcription (4-fold) but was still inducible by OSM (construct E versus F).

**FIG. 2. Deletion analysis of the TIMP-1 promoter.** Deletions of the TIMP-1 promoter spanning −95 to +47 were generated by PCR and cloned into pBLCAT3 to examine their capacity to regulate CAT reporter gene expression. HepG2 cells were co-transfected with each of the constructs (schematically illustrated at left) and pSV-βGal and treated with either OSM (50 ng/ml) or IL-6 (100 ng/ml) for 18 h. Cellular extracts were then prepared, and CAT activity was measured by standard methods and normalized to β-galactosidase activity. Results are shown as percent conversion and fold induction upon cytokine stimulation. Values represent the mean of three separate experiments (standard deviation in parentheses), each experiment done in duplicate.

**TABLE I**

**Oligonucleotides used in EMSA**

| Oligonucleotide | Sequence (5′→3′) |
|-----------------|-----------------|
| Probe 1 (−95/−66) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 2 (1−66/−52) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 3 (AP-1/ets (−61/−34)) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 4A (uAP-1) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 4 (Ets) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 3A (−61/−34) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 5 (TIMP-1) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| Probe 6 (SP1) | 5′-AGCTGCAACCCCGCCCTAGTCCTGTTACCCAGGCTGTG-3′ |
| hSIE | 3′-GCGGTTGAGGCGGGAAGCGTACCTAG-5′ |
| AP-1 consensus | 3′-GCGGTTGAGGCGGGAAGCGTACCTAG-5′ |
| SP1 consensus | 3′-GCGGTTGAGGCGGGAAGCGTACCTAG-5′ |

**FIG. 1.** OSM up-regulates TIMP-1 and c-Fos mRNA levels. HepG2 cells were treated with OSM from 15 min to 18 h and RNA extracted and probed by Northern analysis for TIMP-1 or c-Fos mRNA. Ethidium bromide (EtBr) staining of 28 S and 18 S ribosomal RNA is shown in the bottom panel to show equivalent loading of RNA samples.
OSM Stimulation of c-Fos in HepG2 Cells

Fig. 3. OSM induces binding of TIMP-1 AP-1 probe complexes in a time-dependent fashion. Binding of nuclear factors to oligonucleotides (Table I) spanning the TIMP-1 promoter were tested by EMSA. The probes were as follows: A, AP-1 (probe 2); B, AP-1-Ets (probe 3); C, Ets (probe 4); and hSIE (D). HepG2 cells were treated with OSM from 0 to 12 h, and nuclear extracts were prepared and stored at −70 °C until analysis. EMSA gels were dried and subjected to autoradiography. Longer gel profiles (as seen in Fig. 4) showed no other specific bands; thus, only the tops of the gels are shown here.

of a second complex after 30 min of OSM stimulation with decreased mobility (“complex 2”) that persisted for several hours. The mobility of complex 2 appeared to increase following a mobility decrease (“complex 2”) that persisted for several minutes after 30 min of OSM stimulation with OSM from 0 to 12 h, and nuclear extracts were prepared and stored at −70 °C until analysis. EMSA gels were dried and subjected to autoradiography. Longer gel profiles (as seen in Fig. 4) showed no other specific bands; thus, only the tops of the gels are shown here.

Fig. 4. Cold competition for AP-1 and STAT nuclear factor binding. Nuclear extracts from HepG2 cells stimulated for 0.25 (15 min) or 1 h were probed with AP-1/Ets (probe 3) or the hSIE probe in EMSA analysis. A 50-fold excess of unlabeled cold competitor-oligonucleotide was added as indicated. Complexes 1 and 2 were competed by cold probe 3 but not by hSIE, whereas cold hSIE competed for labeled hSIE. The open circle (○) indicates the position of nonspecific complexes.

Supershift analysis of TIMP-1 AP-1 bands were carried out to identify Fos and Jun components of these complexes. Nuclear extracts were prepared from HepG2 cells treated with OSM for 0, 1, and 12 h and incubated with polyclonal antibodies specific for AP-1 proteins prior to EMSA analysis. Using an antibody reactive against all Fos-related antigens (anti-pan-Fos), complex 1 was obliterated at 0, 1, and 12 h, and a Fos supershift was clearly visible at 1- and 12-h time points (Fig. 5). The anti-pan-Fos antibody also supershifted complex 2 from HepG2 cells treated with OSM for 1 h (Fig. 6A). However, when an anti-c-Fos specific antibody was used, only complex 2 was supershifted and complex 1 remained unaffected, suggesting that c-Fos is a prerequisite for the OSM-stimulated assembly of complex 2. Of the anti-Jun antibodies used in EMSA binding reactions, anti-JunD and anti-JunB supershifted TIMP-1 AP-1 probe complexes (Fig. 6B). JunD was detected at time 0, 1, and 12 h of OSM treatment. JunB could also be detected in these complexes, whereas c-Jun could not be detected.
ing probe 2 is indicated by nonspecific binding to probe. The mobility of nonspecific proteins contains c-Fos, which likely complexes with JunD or JunB.

complex 1 consists of Fos and Jun, complex 2 specifically contained at any time. Together, these data suggest that, whereas Timp-1 AP-1 element by supershift analysis.

HepG2 cell nuclear extracts prepared from cells treated for 0, 0.25, 1, and 12 h were probed with either a wild type AP-1/Ets probe (probe 3) or mutAP-1/Ets probe containing mutations in the AP-1 site (probe 3A). The open circle (○) represents nonspecific binding. Mutation of the AP-1 site removed any detectable binding of OSM-inducible nuclear factors to this probe. STAT factor activation in nuclear extracts was confirmed using the hSIE probe at 0 and 0.25 h time points after OSM stimulation.

OSM stimulation of c-Fos in HepG2 Cells

OSM, but Not Other Cytokines, Induces Complex 2—Given the overlapping biological functions of OSM and IL-6, these and other cytokines were examined together for their ability to induce DNA binding of complexes to the TIMP-1 AP-1 probes in EMSAs. Interestingly, OSM was the only cytokine examined that strongly induced complex 2 (Fig. 8A), whereas both OSM and IL-6 markedly induced activation of STAT binding to the hSIE probe (Fig. 8B). Thus, within the same assay, OSM and IL-6 utilize shared (STAT) and distinct (AP-1-complex 2) nuclear signaling pathways leading to protein-DNA interactions.

PMA has been shown to activate AP-1 (34) and stimulate TIMP-1 expression (24). When tested in HepG2 cells, PMA potently stimulated complex 2 formation (AP-1 probe 2) and to a greater extent than OSM (Fig. 8A). To examine the activity of PMA on transcription, we compared the effectiveness of PMA to OSM or IL-6 in stimulating CAT activity of the TIMP-1 promoter. Table II shows that PMA alone (20% FBS) was unable to up-regulate CAT activity through the TIMP-1 −62/+47 promoter element whereas OSM induced 10-fold increases and IL-6 induced 4-fold changes. When used in combination with IL-6, PMA also failed to stimulate CAT activity beyond that of IL-6 alone. Thus, despite equivalent STAT activation and c-Fos induction as in OSM-treated cells, the combination of IL-6 and PMA was not sufficient to induce similar levels of transcriptional activation.

FIG. 6. Detection of c-Fos and Jun nuclear factors bound to the TIMP-1 AP-1 element by supershift analysis. Cells were stimulated with OSM for 0, 1, or 12 h and nuclear extracts prepared. A, anti-pan-Fos (reactive against all Fos-related antigens) or anti-c-Fos antibody was used in supershift analysis of EMSA using the TIMP-1 AP-1 (probe 2) in binding reactions. EMSAs were then carried out to resolve gel-shifted and supershifted complexes on a 5% polyacrylamide nondenaturating gel. B, supershift analysis was carried out using antibodies to c-Jun, JunB, and JunD in binding reactions with nuclear extract of 1-h OSM-stimulated cells and the AP-1 probe 2. Antibody was added to the reactions without extract (right three lanes, no extract) to show no nonspecific binding to probe. The mobility of nonspecific proteins binding probe 2 is indicated by an open circle (○).

Because sequences within −95 to +1 contained two putative SP1 binding sites, both of these were examined for the binding of HepG2 nuclear factors. Oligonucleotides spanning −95 to −66 (probe 1) or −19 to +2 (probe 6) constitutively bound two specific complexes, which remained unchanged in response to OSM treatment (Fig. 7, A and B). Both complexes were competed by cold SP1 probe (Fig. 7A) and are consistent with the mobility of SP1 nuclear factor binding to a consensus SP1 DNA element (data not shown). Anti-SP1 antibody supershifted the slower migrating complex but did not appear to affect the faster migrating specific complex (Fig. 7C). This may indicate the binding of other SP1 family members (34). Binding of SP1 to the −95/−66 oligonucleotide was only detectable following a one week exposure of EMSA gels (data not shown), although weak binding to this probe may be due to insufficient sequence flanking the 5′ end of the SP1 site. Thus, SP1 nuclear factors constitutively occupy binding sites within the TIMP-1 promoter and OSM does not appear to affect the SP1 DNA binding.

FIG. 7. SP1 nuclear factor binding to the TIMP-1 promoter. A, an excess of cold specific unlabeled competitor oligonucleotides (cold SP1) or irrelevant competitor (cold AP-1/Ets, probe 3) were used to identify specific binding of SP1 (probe 6) gel-shifted complexes. Free probe migrates at the bottom of the gel. B, nuclear extracts from cells stimulated with OSM for 1, 0.25, 1, or 12 h were incubated with a TIMP-1 putative SP1 DNA binding element (probe 6) binding examined by EMSA analysis. C, anti-SP1 antibody was used to supershift SP1 binding to probe 6. Supershifts were inhibited by preincubation of anti-SP1 antibody with SP1 peptide but not by an irrelevant peptide (Fos peptide). The open circle (○) represents the position of nonspecific complexes.
**Oncostatin M Stimulates c-Fos Protein Nuclear Accumulation**—Because the data implicate c-Fos in gel-shifted complex 2, and OSM markedly stimulated early transient expression of c-Fos mRNA (Fig. 1), we examined c-Fos protein levels in nuclear extracts from HepG2 cells by Western blots (Fig. 8C). Expression of c-Fos protein was abrogated in the presence of protein synthesis inhibitors (puromycin and emetine) (Fig. 8C). Western blot analysis indicated that OSM (50 ng/ml) stimulated, in a time-dependent manner, c-Fos protein accumulation in the HepG2 cells (Fig. 8B). We observed that by OSM and requires additional regulatory elements for the pronounced induction of the TIMP-1 gene beyond that induced by IL-6.

**TABLE II**

| Stimulus          | -Fold change over control |
|-------------------|---------------------------|
| OSM (20 ng/ml)    | 10.5 ± 3.6                |
| IL-6 (20 ng/ml)   | 4.2 ± 1.0                 |
| PMA (50 nM)       | 0.9 ± 0.2                 |
| 20% FBS           | 0.5 ± 0.2                 |
| IL-6 (20 ng/ml) + PMA (50 nM) | 3.6 ± 0.6 |

Accumulation of c-Fos by 1 h, which persisted to 12 h of OSM treatment (Fig. 9B). Interestingly, the electrophoretic mobility of c-Fos was reduced at 12 h when compared with 1 h of OSM stimulation. This difference in mobility is first observed after 2 h of OSM treatment (data not shown), persists for up to 12 h, and may represent a change in the phosphorylation status of c-Fos.

In contrast to c-Fos, and consistent with their presence in AP-1 gel-shifted complexes, both JunB and JunD were constitutively present and JunD was moderately up-regulated at the 1 h time point of OSM treatment (Fig. 9, C and D). Puromycin and emetine had no effect on JunB at early time points of OSM stimulation and only affected JunD levels after 12 h of treatment. Detection of c-Fos, JunB, and JunD in Western blots was confirmed by specific competition of relevant peptides for each of the antibodies used, and c-Jun was undetectable from the same nuclear protein extracts (data not shown). Thus, OSM stimulates the nuclear accumulation of c-Fos protein, whereas JunB and JunD nuclear factors are constitutively resident within the nucleus and appear largely unaffected by OSM at the level of new protein synthesis.

**DISCUSSION**

The transcription factor c-Fos is one of several participants in various Fos/Jun complexes that bind AP-1 sites of gene promoters to regulate transcription (31). We have shown here that, upon stimulation by OSM in HepG2 cells, c-Fos is highly induced at the mRNA and protein level and takes part in an accumulation of c-Fos by 1 h, which persisted to 12 h of OSM treatment (Fig. 9B). Interestingly, the electrophoretic mobility of c-Fos was reduced at 12 h when compared with 1 h of OSM stimulation. This difference in mobility is first observed after 2 h of OSM treatment (data not shown), persists for up to 12 h, and may represent a change in the phosphorylation status of c-Fos.

In contrast to c-Fos, and consistent with their presence in AP-1 gel-shifted complexes, both JunB and JunD were constitutively present and JunD was moderately up-regulated at the 1 h time point of OSM treatment (Fig. 9, C and D). Puromycin and emetine had no effect on JunB at early time points of OSM stimulation and only affected JunD levels after 12 h of treatment. Detection of c-Fos, JunB, and JunD in Western blots was confirmed by specific competition of relevant peptides for each of the antibodies used, and c-Jun was undetectable from the same nuclear protein extracts (data not shown). Thus, OSM stimulates the nuclear accumulation of c-Fos protein, whereas JunB and JunD nuclear factors are constitutively resident within the nucleus and appear largely unaffected by OSM at the level of new protein synthesis.
activates cells through the LIF receptor (type I OSM receptor) units unique to individual receptor complexes may contribute receptor complexes on different cells. Alternatively, the sub-phin-1), which could be attributed to differential expression of

mRNA expression of the TIMP-1 gene in HepG2 cells, and (38). We have here confirmed that OSM potently induces

phosphatidylinositol 3

ating signals transduced by OSM, including those affecting

Ras-MAP kinase pathway, such as Ras, Raf-1, Grb2, Shc, and

related family members, OSM stimulates components of the

complex 2 formation, and its nuclear accumulation in response

OSM is dependent upon new protein synthesis. Such AP-1 activation may contribute to effects of OSM on growth regulation or expression of other genes. Previous work has shown OSM-mediated induction of other immediate early genes such as egr-1, c-jun, and c-myc in fibroblasts (35), which also suggests a broad range of gene products could in turn be regulated by OSM stimulation.

Although functional redundancy within the IL-6-type family of cytokines is a common observation, OSM manifests both shared and distinct biological activities with other family members (IL-6, LIF, IL-11, ciliary neurotropic factor, and cardiotrophin-1), which could be attributed to differential expression of receptor complexes on different cells. Alternatively, the sub-units unique to individual receptor complexes may contribute to the biological activities of these cytokines. OSM binds and activates cells through the LIF receptor (type I OSM receptor) and a newly identified complex of gp130 and OSM receptor β chain (type II OSM receptor) (36) in human cells. Like other related family members, OSM stimulates components of the Ras-MAP kinase pathway, such as Ras, Raf-1, Grb2, Shc, and the p42 MAP kinase (4, 37), and the JAK-STAT pathway (6, 7). Other signaling pathways have also been implicated in mediating signals transduced by OSM, including those affecting phosphatidylinositol 3'-kinase and the canonical Src kinase (38). We have here confirmed that OSM potently induces mRNA expression of the TIMP-1 gene in HepG2 cells, and shown that OSM strongly enhances transcription of a CAT reporter gene flanked by −62 to +47 of TIMP-1 promoter construct. Up-regulation of CAT activity was most strongly induced by OSM treatment, less so by IL-6, and not at all by 20% FBS, PMA, or the combination of IL-6 and PMA. Taken together, this implicates OSM in activating a signaling event(s) distinct from IL-6 or serum factors that likely act on target DNA elements within the TIMP-1 promoter for up-regulating its transcription and expression. Interleukin-6 also regulates the TIMP-1 promoter (Fig. 2) but appears not to activate c-Fos or form complex 2; nor did IL-6 regulate AP-1-containing promoter/CAT (Fig. 2) constructs to as great a degree as OSM. This is consistent with previous work, which showed a similar effect on the regulation of the rat TIMP-1 promoter, as well as an AP-1 site requirement for maximal responses (39), although AP-1 activation was not identified. A role for AP-1 in TIMP-1 promoter regulation has also been implicated in F9 cells over-expressing AP-1 genes (40). Because OSM is more effective at stimulating acute phase protein production by HepG2 cells than other gp130 cytokines (26), and regulates low density lipoprotein receptors on these cells (16), we suggest that c-Fos activation is an additional component of OSM signaling which contributes to differential effects. Alternatively, OSM or IL-6 may confer differences in Fos/Jun complexes through post-translational modifications such as phosphorylation or alter the composition of associated dimers of AP-1.

In addition to the AP-1 site (27, 40, 41), the proximal TIMP-1 promoter also contains putative regulatory motifs including an Ets binding sequence (27, 40) with homology to STAT elements, and SP1 elements (27). Both the Ets site (putative STAT site) and SP1 site appeared to contribute somewhat to basal transcription levels based on deletion analysis (Fig. 2); however, binding of factors to these sites was not altered upon OSM stimulation. Previous studies have shown that Ets can cooperate with AP-1 in regulating transcription in other cells (42, 43). Others have shown that STATs can bind AP-1/Ets sequences of the rat TIMP-1 in HepG2 cells (39), and human TIMP-1 promoter in astrocytes (44) and that this site also contributes to transcription by OSM. Our results in HepG2 cells did not detect binding of STATs to this sequence despite the presence of activated STAT-1 and STAT-3 (hSIE binding) in the nuclear extracts and long exposures of gels. In addition, mutation of the AP-1 site in the AP-1-Ets probe completely eliminated detection of any OSM-inducible nuclear factors capable of binding this probe (Fig. 5). Differences between the levels of STAT proteins expressed in HepG2 cells and astrocytes could account for this result. The prominence of AP-1 nuclear factor binding to this sequence that we observe is consistent with previous studies (44), wherein AP-1 binding appeared dramatically greater than STAT binding in EMSA assays with an equivalent of an AP-1/Ets probe. We suggest that this abundance of AP-1 binding reflects a physiological importance among other factors participating in the regulation of this proximal TIMP-1 promoter.

Although deletion of the AP-1 motif (−59/−52) markedly affected OSM activation, this AP-1 element was not sufficient for full OSM-responsiveness because deletion of sequences between +1 to +47 also reduced transcription (Fig. 2). The TIMP-1 gene may utilize SP1 binding sites and a putative initiator element downstream of the transcription initiation site as described for other TATA-less promoters. We have observed a putative pyrimidine-rich initiator element immediately 3′ to +1 of the TIMP-1 gene that may be necessary for the assembly of a competent transcriptional machinery apparatus for transcription initiation (45). Alternatively, additional responsive elements 3′ to the initiation start site may cooperate with the TIMP-1 AP-1 element at −59/−53 in response to OSM. Interestingly, Logan et al. (40) have characterized a weak AP-1 binding site within +1/+47, which may also be a target for OSM signals. We are currently examining this aspect.

Although c-Fos and its participation in complex 2 could also be stimulated by PMA (as assessed by supershifts; data not shown), PMA alone was unable to up-regulate TIMP-1 promoter activity as assayed by CAT reporter gene expression (Table II). In addition, we found that the co-stimulation of HepG2 cells with IL-6 (STAT induction) and PMA (c-Fos induction) could not appreciably up-regulate the CAT activity driven by the −62 to +47 TIMP promoter. This also suggests that OSM induces an additional signal that regulates TIMP-1 in this system. STAT proteins may interact with SP1 (46) and Jun (47) proteins to cooperate in elevating transcription in other systems; thus, a similar mechanism could occur in HepG2 cells. Alternatively, OSM and PMA may differ qualitatively in affecting posttranslational modification of AP-1 nuclear factors, such as phosphorylation, that influence the transactivation potential of these transcription factors.

In summary, maximal expression of TIMP-1 by OSM (but not IL-6) may, at least in part, be attributed to the induction of c-Fos and its complex with AP-1 sites in the proximal promoter. This may also involve posttranslational modifications such as phosphorylation to these factors. Cooperation of nuclear proteins binding to SP1 and Ets DNA elements is also needed for maximal expression, and OSM signaling may recruit additional factors that interact with sequences downstream from +1 to +47. Our results also support the existence of shared and distinct signaling pathways by OSM and IL-6 in HepG2 cells.
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