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Combinatorial Interactions between AP-1 and ets Domain Proteins Contribute to the Developmental Regulation of the Macrophage Scavenger Receptor Gene

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Macrophage development is regulated by a complex set of hormone-like molecules and cell adhesion events that control the growth and differentiation of progenitor cells. The macrophage scavenger receptor (SR) gene becomes markedly upregulated during the final stages of monocyte-to-macrophage differentiation and provides a model for the identification and characterization of transcription factors that control this process. In this report, we have identified three genomic regulatory elements that are required for transactivation of the SR gene in the THP-1 monocytic leukemia cell line following induction of macrophage differentiation by tetradecanoyl phorbol acetate. Each of these regulatory elements contains a near-consensus binding site for members of the AP-1 gene family, while the two most quantitatively important elements also contain juxtaposed binding sites for ets domain transcription factors. We demonstrate that tetradecanoyl phorbol acetate treatment results in a marked and prolonged increase in AP-1 binding activity on these elements, which can be accounted for almost entirely by c-*jun* and *junB*. These proteins in turn form ternary complexes with additional factors that bind to the adjacent ets recognition motifs. Several indirect lines of evidence indicate that ets2 represents a component of this ternary complex. The combined expression of c-*jun*, ets2, and a constitutive form of *ras* result in synergistic increases in transcription from promoters containing the SR regulatory elements. These observations suggest that SR gene expression is regulated via a signal transduction pathway involving *ras*, AP-1, and ets domain proteins and imply that at least some of the signalling components involved in *ras*-dependent growth are also utilized to promote the expression of genes involved in terminal differentiation.

Macrophages are derived from circulating monocytes that have traversed the endothelium and migrated into tissues or body cavities. This transition from the circulation is temporally correlated with the unfolding of a program of terminal differentiation that involves the transcriptional activation of a diverse set of immediate/early and late genes. The precise pattern of gene expression that is evoked during this process is dependent on the particular cell-cell interactions and the milieu of cytokines and other regulatory molecules encountered by the nascent macrophage in the postcirculatory environment.

The type I and II macrophage scavenger receptors (SRs) represent late gene products that are highly restricted in their patterns of expression to macrophages and related cell types (36). These two forms of the SR are encoded by a single gene that gives rise to an alternatively spliced primary transcript (27, 42). Although their normal physiologic roles remain uncertain, biochemical studies have demonstrated that both forms of the SR are capable of binding and internalizing a relatively broad spectrum of polyanionic macromolecules. Ligands for SRs include proteins containing lysine residues that have been acetylated or otherwise derivatized by negatively charged lipid oxidation products (20). SRs have therefore been proposed to function in the uptake and degradation of oxidatively modified proteins at sites of inflammation. On the basis of their ability to take up and degrade oxidized low-density lipoproteins (16, 27), SRs appear to provide a mechanism for the development of cholesterol-engorged macrophages that are characteristic of early atherosclerotic lesions. More recently, SRs have been suggested to play a role in mediating adhesive interactions with endothelial cells (15).

Previous studies have suggested that SR expression is regulated by macrophage colony-stimulating factor (M-CSF), which enhances the proliferation of monocyte progenitors and is required for the survival and functional competence of mature macrophages (43). Treatment of primary monocytes with recombinant M-CSF has been shown to lead to increases in SR mRNA and protein levels (10, 24). The binding of M-CSF to its receptor activates the receptor's tyrosine kinase and elicits a pleiotropic response, which includes activation of the proto-oncogenes *src* and *ras* (6, 32) as well as stimulation of protein kinase C (PKC) (11, 22), phospholipase A_2 (38), and phosphatidylinositol 3-kinase (47). The mechanisms by which these events ultimately influence the expression of genes that become transcriptionally activated during the terminal phases of macrophage differentiation are not clear, however.

To identify transcription factors that could potentially function to mediate developmental activation of the SR gene, we have used the THP-1 monocytic leukemia cell line. THP-1 cells can be induced by tetradecanoyl phorbol acetate (TPA) to differentiate into macrophage-like cells (4) and to express SRs (21). TPA exerts its biological effects by altering gene expression through the activation of PKC. The induction of SR expression by TPA in THP-1 cells presumably reflects an event bypassing a signal that, in vivo, is initiated by ligand-receptor interactions occurring at the cell surface. At least one point of intersection between the receptor tyrosine kinases and PKC-dependent signalling pathway appears to be Raf-1 kinase,

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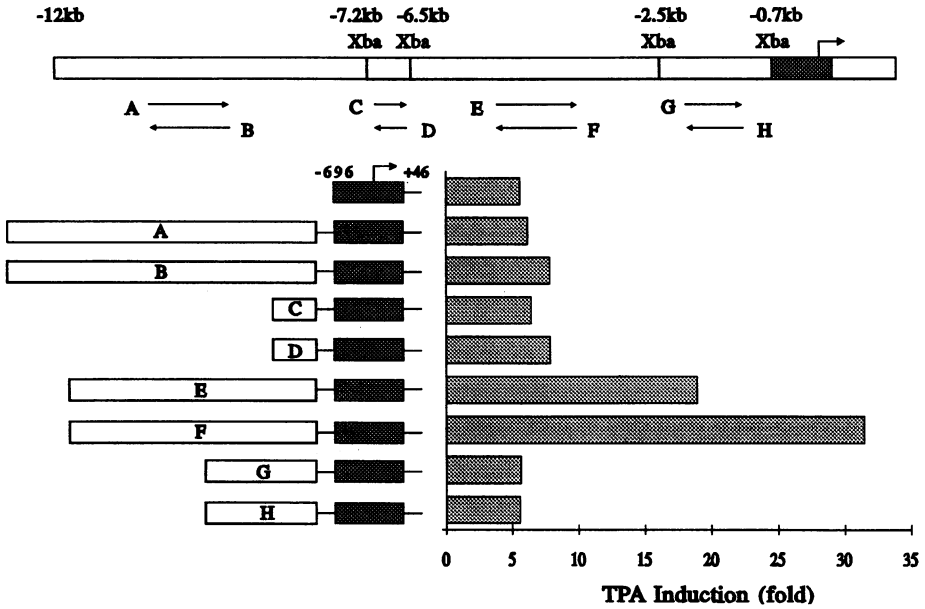

FIG. 1. Identification of genomic sequences mediating TPA-dependent transcriptional activation of the human SR gene. A diagram of the 5′ flanking region of the human SR gene is shown at the top. The positions relative to the transcriptional start site and the sizes of the *Xba*I fragments are shown. Each *Xba*I fragment was subcloned proximal to the human SR promoter region (−696 to +46 bp) in both orientations, as illustrated by the direction of arrows. Transfection experiments were performed by testing the TPA responsiveness of the SR promoter alone or fused to the indicated upstream fragments. THP-1 cells (2×10^7) were transfected with 4 μg of DNA and split equally into four 10-mm-diameter plates, two of which were treated with 100 nM TPA for 12 h. Luciferase activity was normalized to total protein and a β-actin luciferase external standard. The results are expressed as fold induction by TPA and are representative of three independent experiments.

which on the one hand can interact with GTP-bound *ras* (48, 49, 53) and on the other hand can be directly phosphorylated and activated by PKC-α (28).

We previously demonstrated that TPA maximally stimulated SR gene transcription between 8 and 16 h after TPA treatment (35). This induction was dependent on new protein synthesis and could be blocked by staurosporine, a potent PKC inhibitor. To determine the molecular mechanisms responsible for TPA-dependent transcriptional activation, we isolated genomic clones containing the promoter and upstream regulator elements of the bovine and human SR genes. The SR promoter was found to lack a conventional TATA box and initiated transcription from a cluster of closely spaced start sites (35). In this report, we have identified sequences within the SR gene that are essential for its transcriptional activation in THP-1 cells during the program of macrophage differentiation induced by TPA. These sequence motifs include three near-consensus AP-1 sites, two of which are juxtaposed with binding sites for members of the ets domain gene family. We demonstrate that members of the AP-1 family of transcription factors (particularly *jun* and *junB*) and ets2 function cooperatively on these binding sites. Furthermore, cooperative interactions between c-*jun* and ets2 are significantly increased by coexpression of a constitutively active form of H-*ras*. These observations suggest that transactivation of the SR gene in response to M-CSF proceeds, at least in part, through *ras*-dependent activation of *jun* and ets domain family members, transcription factors that have also been implicated in M-CSF-dependent proliferation.

MATERIALS AND METHODS

Plasmid constructs. The human SR promoter and flanking sequences from −696 to +46 were cloned upstream of the firefly luciferase DNA in the expression vector Δ5′ PSV 2 Luciferase (14). *Xba*I fragments corresponding to additional upstream sequences of human SR genomic DNA were subsequently subcloned proximal to the human SR promoter in both orientations (Fig. 1). Deletions of the region from −6.5 to −2.5 kb were generated either by restriction endonuclease digestion or by using the PCR to amplify specific internal sequences. Mutations were introduced into the AP-1, AP-4, and SP-1 sites of the TPA-responsive enhancer (−4.5 to −4.1 kb) by in vitro site-directed mutagenesis (31) using the following oligonucleotides: 5′Enh. AP-1 mut (GCCCAAAATAC GAATCTCGAGTTTCCTTGCAAGATC), 3′Enh. AP-1 mut (CACTAAAAGACTGAGATGTCGACAGAAGACTATA GAACAC), 3′Enh. AP-4 mut (CTCACCCCGCCCGTCGAC TTCACCCCTCCGTC), and Enh. SP-1 mut (ATAGAACAC CTCACCGTCGACCCACATCTCACCC).

In each case, the underlined sequence represents the nucleotides mutated from the wild-type sequence. All mutations were confirmed by dideoxy DNA sequence analysis. Transfection analyses of isolated AP-1 and ets binding sites were performed by subcloning oligonucleotides containing these sequences upstream of the TATA box of the rat prolactin gene promoter in the luciferase reporter plasmid P36B−. One and three copies of wild-type or mutated elements containing AP-1/ets sites were introduced in direct repeat orientations. The sense-strand sequences of the oligonucleotides used were as follows:

AP-1 ets

PrAP-1/ets, GATCCAGGAATGTGTCATTTCCTTTCTTCA

PmAP-1, GATCCAGGACGAATCCCTTTCCTTTCTTCA

Pmet, GATCCAGGAATGTGTCATTGAATTTCTTCA

The expression vector for rat c-*jun* (RSV-CJ) was a generous gift of M. Karin (2). A cDNA for human spi-1 was generously provided by F. Moreau-Gachelin. The cDNAs for human ets1 and ets2 were generously provided by D. Watson (52). The cDNA for H-*ras*$^{Val-12}$ was generously provided by J. Feramisco. Each cDNA was subcloned into the Rous sarcoma virus-based expression vector Rexp.

Cell culture and transient expression. THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gemini), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. F9 cells (American Type Culture Collection) were maintained in Dulbecco modified Eagle medium containing heat-inactivated 10% fetal calf serum and penicillin-streptomycin. For transfection, THP-1 cells were plated at 5×10^5 cells per ml the day before transfection; 2×10^7 cells were transfected with 4 μg of reporter plasmid by the DEAE-dextran method as described previously (17). The transfected cells were then equally divided into four 10-mm-diameter plates. Duplicate plates were treated with solvent or TPA at final concentration of 100 nM 24 to 36 h after transfection. Cells were harvested 12 h after TPA treatment. F9 cells were plated at a density of 10^5 cells per ml at least 5 h before transfection by the $CaPO_4$ (8) method. Typically, 1 μg of reporter plasmid, 0.5 μg of each expression plasmid, and 6 μg of salmon sperm DNA (as carrier) were added per 3 ml of medium. All transfection groups received equal final plasmid concentrations. Differences in the amount of expression plasmid used between points were balanced by using the appropriate empty vector plasmid. The medium containing the precipitate was removed 16 to 24 h after transfection, and the cells were cultured in 3 ml of fresh medium for another 24 h before harvesting. Luciferase activity was determined as previously described (14) and normalized to total protein and an external standard provided by a plasmid containing the β-actin promoter linked to luciferase. In some cases, an internal standard consisting of β-actin–β-galactosidase was used. Normalization of luciferase activity to the β-galactosidase standard had little effect on the fold or variability of TPA induction and was therefore not used in all assays. All transfections were done at least twice with duplicate points, and in most cases, two independent preparations of DNA were tested.

Preparation of nuclear extracts. Nuclear extracts were prepared from mouse peritoneal macrophages and exponentially growing THP-1 cells treated with either TPA (100 nM) or ethanol for 16 h. The cells were then washed with ice-cold phosphate-buffered saline and incubated in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol) on ice for 10 min. After centrifugation, the cells were resuspended in buffer A plus 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 4 mM benzamidine, 10 mg of leupeptin per ml, and 10 mg of aprotinin per ml and placed on ice for 5 to 10 min to allow lysis of the plasma membrane. The nuclei were isolated by centrifugation and resuspended in buffer C (20 mM HEPES [pH 7.9], 20% glycerol, 400 mM KCl, 0.2 M EDTA, 1 mM dithiothreitol, and proteinase inhibitors) and incubated at 4°C for 30 min. After centrifugation at 65,000 rpm for 20 min in a Beckman TLA-100 rotor at 4°C, aliquots of nuclear protein were frozen at −80°C, and protein concentration was measured by the Bradford assay.

DNA binding assays. Double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, filled with deoxynucleoside triphosphates by using the Klenow fragment of DNA polymerase, and purified on 10% nondenaturing polyacrylamide gels. The oligonucleotides used for DNA binding assays were identical to those used for transfection analysis of the isolated AP-1/ets-responsive elements described above. The sequences of the sense strands of the collagenase AP-1 and retinoic acid response element (RARE) oligonucleotides were AAGGGGATCCATAAAG CATGAGTCAGACACCTCTG and CGATCTAGATCTA AGGGTTCACCAAAAGGTCATCGAGCTCAGATCTC GT, respectively.

DNA binding experiments were initiated by preincubation of 2 to 12 μg of nuclear extract protein with 1 μg of double-stranded poly(dI-dC) in 150 mM KCl–20 mM HEPES (pH 7.5)–2 mM dithiothreitol–7.5% glycerol–0.1% Nonidet P-40 for 20 min on ice in a volume of 18 μl. The radiolabeled DNA probe (50,000 cpm in 2 μl) was then added, and the mixture was incubated on ice for another 20 min. For antibody studies, 1 μl of antiserum was added during the preincubation, and the preincubation without probe was extended to 1 h. DNA-protein complexes were fractionated by electrophoresis at room temperature on a nondenaturing 5% polyacrylamide gel at 200 V for 3 h in 0.3× Tris-borate-EDTA buffer. Gels were dried and visualized by autoradiography. Methylation interference experiments were performed as described previously (19). Specific antibodies against c-*fos*, *fra*, *junB*, and *junD* were kindly provided by R. Bravo (29). Anti-c-*jun* was provided by S. E. Adunyah (1). Antibodies for ets1 and ets2 were from Santa Cruz Biotechnology. Antiserum against PU.1 was raised against a recombinant protein containing the PU.1 DNA binding domain. This antisera was found to be capable of supershifting endogenous PU.1 bound to a consensus PU.1 recognition motif.

RESULTS

Identification of proximal and distal upstream regions required for TPA induction. To identify *cis*-active elements responsible for transcriptional activation of the SR gene in response to TPA, the SR promoter and 5′ flanking genomic sequences were used to direct the expression of a luciferase reporter gene (Fig. 1). Transient expression assays indicated that the 742-bp region corresponding to −696 to +46 from the major transcriptional start site was sufficient for basal expression in THP-1 cells. However, this sequence conferred only a two- to fourfold induction in response to TPA (Fig. 1). Our previous studies of TPA-dependent induction of the SR gene suggested an approximate 10-fold increase in transcription in THP-1 cells as measured by nuclear run-on assays. These results suggested that the proximal region from −696 to +46 did not contain sufficient information to confer the level of transcriptional activation observed for the endogenous gene. A screening assay was therefore performed to search for a TPA-dependent enhancer in genomic sequences located further upstream of the promoter. As illustrated in Fig. 1, *Xba*I fragments of genomic DNA located from −12 to −0.7 kb were introduced in both orientations proximal to the region from −696 to +46 bp containing the SR promoter. A 18- to 20-fold increase in luciferase activity was observed in response to TPA when constructs E and F were tested, suggesting that additional TPA-responsive elements were present in the region from −2.5 to −6.5 kb from the transcriptional start site (Fig. 1). Deletions of this upstream region were tested for their effects on basal and TPA-induced activity (Fig. 2A). Two qualitatively different types of regulatory elements were present in the fragments from −6.5 to −2.5 kb. Deletion studies demonstrated that the minimal sequences required for enhancement of TPA responsiveness resided from −4.5 to −4.1 kb from the transcriptional start site (Fig. 2A). These

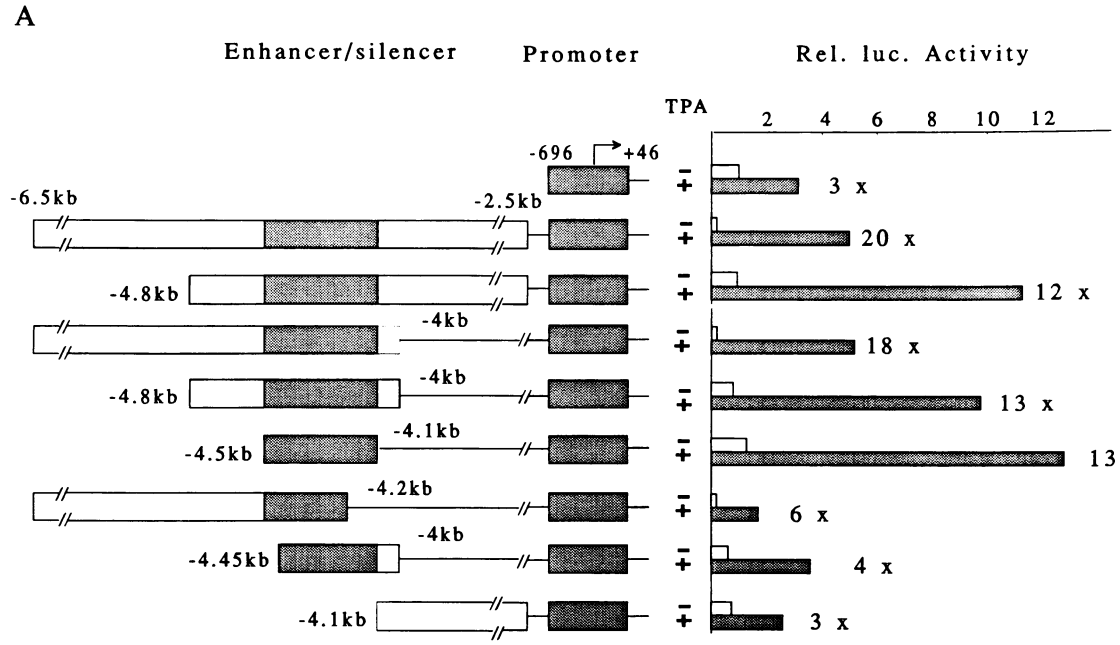

FIG. 2. Definition of the minimal upstream sequences required for TPA-dependent enhancement of SR promoter activity. (A) Deletion analysis of the human SR enhancer/silencer. Deletions of the human SR gene upstream region from −6.5 to −2.5 kb were subcloned proximal to the −696 SR promoter, as shown diagrammatically. The constructs were then transfected into THP-1 cells and analyzed as described for Fig. 1. The basal activity of the promoter was set at 1, and relative luciferase (Rel. luc.) activities of the enhancer/silencer constructs under basal and TPA-treated conditions are shown. The results are representative of three independent experiments. (B) Nucleotide sequence of the minimal upstream region required for full TPA-dependent enhancement of the SR promoter. This sequence is located between −4.5 and −4.1 kb relative the major transcriptional start site. Two potential AP-1 binding sites, an ets recognition motif, two near-consensus AP-4 sites, and an SP-1 site are indicated.

studies also suggested that the upstream sequences from −6.5 to −4.8 kb contained a silencing element, because transfection of genomic sequences including this region consistently inhibited basal SR promoter activity by 70 to 80% (Fig. 2A). In constructs containing the TPA-dependent enhancer, the suppressive effect of the upstream sequences was relieved following TPA treatment, resulting in an 18- to 20-fold induction. In the absence of the upstream inhibitory sequences, absolute levels of expression were higher, but the fold induction in response to TPA was somewhat less (12-fold). The upstream sequences therefore appear to tighten the regulation of the SR gene in response to TPA by decreasing basal expression.

These observations suggested that the response to TPA was mediated by elements located near the promoter and between −4.1 to −4.5 kb upstream. Transfer of the fragment from −4.1 to −4.5 kb to the herpes simplex virus thymidine kinase promoter resulted in relatively little enhancement in response to TPA, indicating that this element functioned preferentially in the context of its own promoter (data not shown). The sequence of the minimal enhancer is shown in Fig. 2B. Several potential transcription factor binding motifs were observed, including juxtaposed binding sites for members of the AP-1 and ets domain gene families at the 5′ end of the enhancer (5′ AP-1/ets), an AP-1-like site at the 3′ end of the enhancer (3′ AP-1), two AP-4-like sites, and one SP-1-like site. A strong similarity was observed between the AP-1/ets sequence of the enhancer and a region from −48 to −72 in the promoter (Fig. 3A). To determine the functional importance of these potential transcription factor binding sites, the activities of promoter and enhancer constructs containing point mutations in these sites were evaluated (Fig. 3). Mutation of either the AP-1 motif or the ets motif in the SR promoter abolished its ability to respond to TPA. Mutation of the AP-1 site in the 5′ AP-1/ets element of the enhancer reduced the TPA responsiveness by

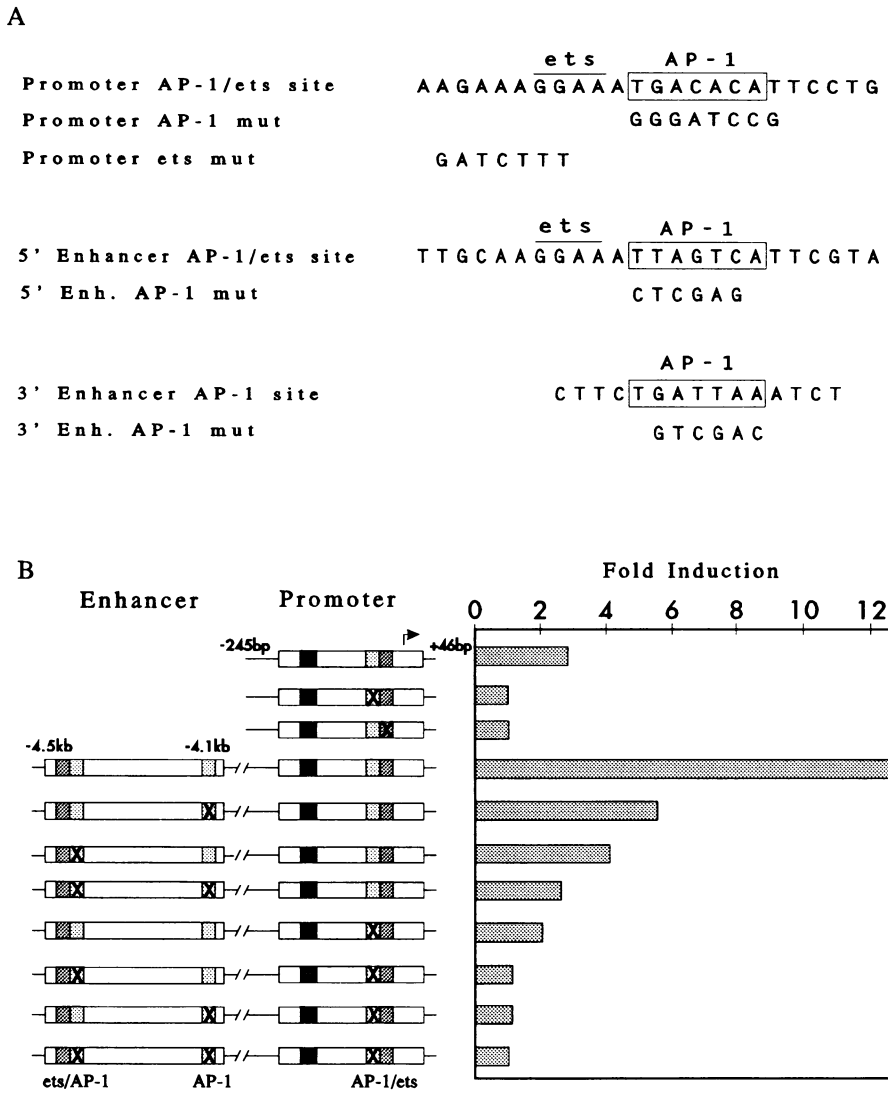

FIG. 3. Mutational analysis of the AP-1 and ets motifs in the proximal and upstream SR regulatory regions. (A) Nucleotide sequences of the putative ets and AP-1 binding motifs present within the SR promoter and enhancer. Mutations introduced into these motifs are shown below the wild-type response elements. The antisense strand of the AP-1/ets element present in the SR promoter is illustrated to facilitate comparison with the enhancer AP-1/ets sequence. (B) Functional analysis of SR regulatory regions containing point mutations in putative AP-1 and ets binding motifs. Plasmids containing the indicated mutations in AP-1 or ets sites within the SR promoter and enhancer were transfected into THP-1 cells and treated with TPA as described in the text. The effects of TPA are expressed as fold induction of reporter gene activity.

60%, and mutation of the 3′ AP-1 site reduced the TPA responsiveness by 50%. If both of the AP-1 sites were mutated, the ability of this upstream element to enhance TPA-dependent transcription was abolished. In contrast, mutations in the AP-4 or SP-1 site did not affect the activity of the enhancer (data not shown). It was also observed that the activity of the enhancer was dependent on the AP-1 site of the promoter, since the TPA activities of the enhancer and its mutants were greatly reduced when they were linked to the promoter with the AP-1 site mutated (Fig. 3B). These results further support the interpretation that the AP-1 sites in the SR promoter and enhancer function cooperatively.

To further examine the relative roles of the AP-1 and ets motifs in mediating TPA-dependent transcriptional activation, we produced luciferase reporter plasmids in which one and three copies of the promoter AP-1/ets sequence or the corresponding sequences containing mutations in the AP-1 or ets binding site were introduced upstream of a minimal promoter derived from the rat prolactin gene (Fig. 4). The minimal prolactin promoter exhibited very little activity before or after TPA treatment. Introduction of one copy of the wild-type promoter TPA response element (PrAP-1/ets) increased basal expression and conferred a two- to threefold response to TPA. Three copies of the PrAP-1/ets dramatically increased basal activity and conferred an approximate sevenfold response to TPA. Mutation of the AP-1 site completely abolished basal and TPA-induced activity for both one and three copies of PmAP-1. Mutation of the ets site reduced basal activity and abolished the TPA response when tested as a single copy. When tested in the context of three copies, mutation of the ets site reduced basal expression but did not abolish the response to TPA (Fig. 4).

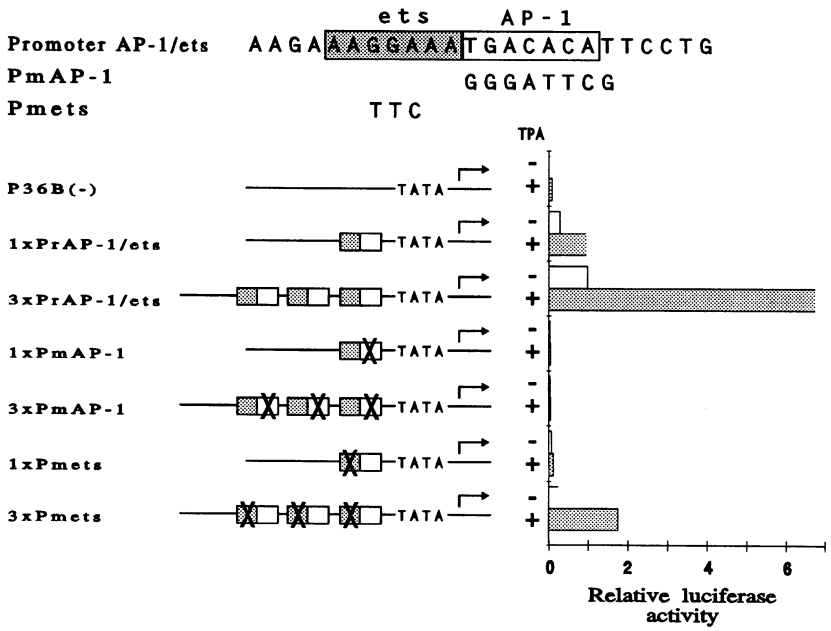

FIG. 4. Functional analysis of the SR promoter AP-1/ets site in the context of a minimal heterologous promoter. The nucleotide sequences of the wild-type SR promoter AP-1/ets site (PrAP-1/ets) and corresponding sequences containing mutations in the AP-1 motif (PmAP-1) or ets motif (Pmets) are shown at the top. One or three copies of the wild-type or mutated elements were introduced upstream of the minimal prolactin gene promoter as shown schematically at the left. The resultant plasmids were transfected into THP-1 cells, and promoter activity was determined in the presence and absence of TPA.

***jun* and *junB* interact with an additional factor to form ternary complexes on the proximal and distal AP-1/ets sites.** The transient transfection analysis of sequences mediating TPA-dependent transcription suggested that maximal responsiveness of the scavenger receptor gene required both AP-1 and ets domain family members. To determine whether the putative AP-1 and ets binding sites actually bound members of the AP-1 and/or ets gene families, we performed in vitro protein-DNA binding assays (Fig. 5). Nuclear extracts were prepared from THP-1 cells before and 16 h after TPA treatment. Electrophoretic mobility shift assays were performed with a probe containing the 5′AP-1/ets element present in the SR enhancer. An inducible DNA binding activity was observed following treatment of THP-1 cells with TPA. This activity was competed for by the 5′ AP-1/ets site itself, the AP-1/ets site present in the SR promoter, and the AP-1 binding site present in the collagenase gene. An unrelated sequence mediating retinoic acid responsiveness (RARE) did not compete (Fig. 5A). Similar results were also observed when the promoter AP-1/ets element was used as a probe (data not shown).

To determine the identities of these factors, nuclear extracts from TPA-treated cells were incubated with antibodies against members of the AP-1 family (anti-*fos*, anti-*fra*, anti-*jun*, anti-*junB*, and anti-*junD*) prior to addition of oligonucleotide probes. Individual antibodies against *jun* and *junB* were observed to partially block the binding of the TPA-inducible complex. However, the presence of both antibodies produced a total block of binding (Fig. 5B). In contrast, control immunoglobulin G or antibodies against other members of the AP-1 family did not affect the binding. These experiments indicated that *jun* and *junB* binding activities were induced in THP-1 cells following TPA treatment and are likely to be involved in regulating the expression of the SR gene in response to activation of PKC.

Methylation interference analysis of the TPA-inducible complex demonstrated that the major groove contacts with G residues were restricted to the AP-1 binding motif, suggesting that this complex contained only *jun* and *junB* homodimers or heterodimers (Fig. 6). However, by increasing the amount of nuclear protein used in the DNA binding reaction from 2 to 10 μg, a new and more slowly migrating complex was observed. Methylation interference analysis of this complex demonstrated that the major groove contacts included both the AP-1 binding motif and the adjacent ets domain binding motif. This upper complex did not form when either the AP-1 or the ets binding site was mutated (data not shown). These experiments suggested that *jun* and *junB* first associate with the AP-1 site and facilitate the binding of an additional factor to the adjacent ets site, resulting in the formation of a ternary complex. To determine whether these complexes were also present in terminally differentiated macrophages that express the SR gene, nuclear extracts were prepared from primary mouse peritoneal macrophages. These extracts contained proteins that associated with the AP-1/ets motif in a manner that was indistinguishable from that of the proteins present in THP-1 cells (Fig. 6). In an attempt to determine the identity of the protein or proteins binding to the ets site, binding experiments were performed in the presence of an antiserum to the ets domain proteins, ets1, ets2, and PU.1. Experiments using antisera to ets1 and ets2 were not informative, however, as the antisera were unable to abolish or perturb the binding patterns of control proteins (data not shown). Experiments using antisera to PU.1, which was capable of supershifting endogenous PU.1 on a consensus PU.1 recognition motif, had no effect on the ternary complex (data not shown).

Functional evidence for cooperative interactions between c-*jun*, *junB*, and ets2. Because it was not possible to clearly identify the factor(s) interacting with *jun* and *junB* by using available antisera, the ets domain proteins ets1, ets2, and PU.1/spi-1 were tested for their ability to activate transcription

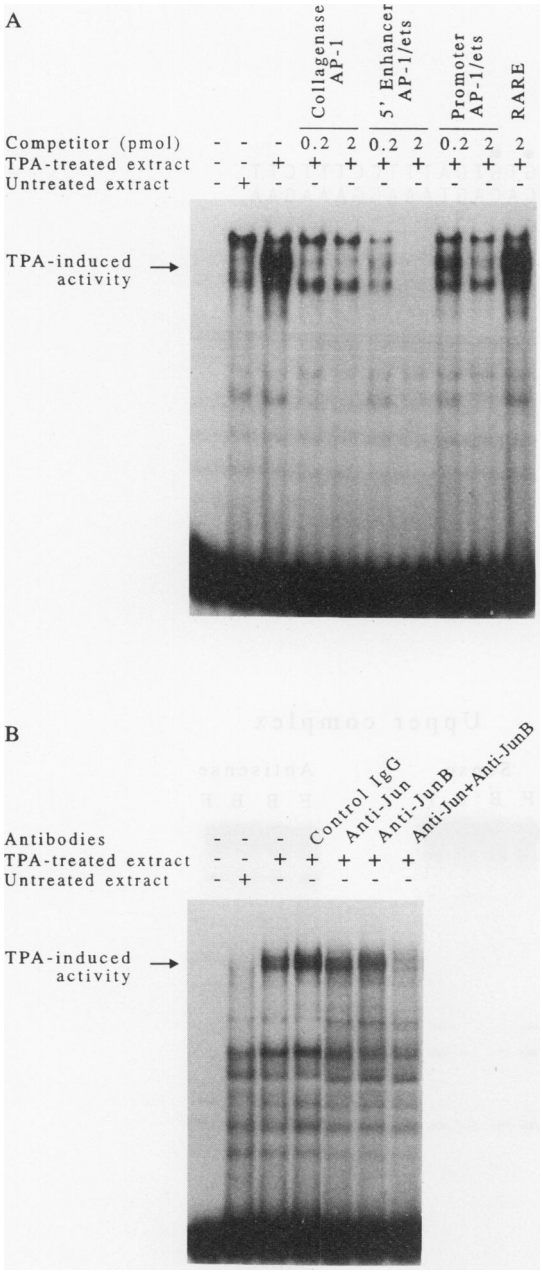

FIG. 5. *jun* and *junB* binding activities are induced in THP-1 cells following treatment with TPA. (A) TPA induces a DNA binding activity in THP-1 cells that recognizes the SR AP-1 elements. Nuclear extracts were prepared from THP-1 cells before and 16 h following treatment with TPA. Equal amounts of nuclear protein (3 μg) were incubated with a radiolabeled, double-stranded oligonucleotide corresponding to the AP-1/ets motif present in the SR TPA-responsive enhancer. Competition was performed with unlabeled oligonucleotides corresponding to the enhancer AP-1/ets site itself, the SR promoter AP-1/ets site, the collagenase AP-1 site, and the RARE present in the RARβ2 promoter. (B) Antibodies to c-*jun* and *junB* block the binding of the TPA-induced AP-1 activity. Antibodies were preincubated alone or together with nuclear extract prepared from TPA-treated THP-1 cells. DNA binding reactions were then carried out as described for panel A. IgG, immunoglobulin G.

and cooperate with *jun* and *junB* in transient cotransfection assays. ets2 and spi-1 were chosen because they are known to be expressed in primary macrophages and THP-1 cells (26, 39). The F9 embryonic carcinoma cell line was used for coexpression experiments because of its low levels of endogenous AP-1 and ets activities (9, 30). When evaluated individually, expression of c-*jun* and ets2 were observed to increase expression of the AP-1/ets-containing promoter by 30- and 5-fold, respectively. Expression of *junB* enhanced expression slightly, while ets1 and spi-1 had almost no effect. Coexpression of c-*jun* and *junB* led to a 15-fold increase in expression over the control, which was intermediate between the levels obtained for c-*jun* or *junB* alone. Coexpression of c-*jun* and ets2 led to a 100-fold increase in expression over the control. Cotransfection of PU.1 had little or no effect on the expression of the AP-1/ets-containing promoter (Fig. 7) but was able to stimulate transcription of a promoter containing a consensus PU.1 binding site (data not shown). These results are consistent with our failure to detect PU.1 as a component of the AP-1/ets ternary complex by using a PU.1-specific antiserum. Because the M-CSF receptor couples to the *ras*-dependent signal transduction pathway, the effects of coexpression of a constitutively active form of H-*ras*, $ras^{Val\text{-}12}$ (45, 46), on *jun*- and ets2-dependent transcription were examined. Coexpression of $ras^{Val\text{-}12}$ enhanced *jun*-dependent transcription by a factor of 3 and ets2-dependent transcription by a factor of 12. Coexpression of c-*jun*, ets2, and $ras^{Val\text{-}12}$ led to a fourfold increase in activity over that observed for the combination of c-*jun* and ets2 alone. Similar results were observed for the SR promoter sequence from −245 to +45 bp of the transcriptional start site that contains the proximal AP-1/ets motif (data not shown). This finding is consistent with the hypothesis that cooperative interactions between *jun* and ets2 are important for the developmental regulation of the SR gene, and that these factors are targets of the *ras*-dependent signal transduction pathway.

DISCUSSION

The macrophage SR gene provides a model for the identification and characterization of transcription factors that control the terminal differentiation of the macrophage. In the present studies, we have identified genomic sequences that mediate transcriptional activation of the SR gene in THP-1 cells in response to TPA. Three distinct elements that work in concert to generate a maximal transcriptional response were identified. All three of these elements contained consensus or near-consensus DNA binding sites for members of the AP-1 family of transcriptional activators. In addition, two of these elements contained juxtaposed binding sites for members of the ets domain gene family.

The AP-1 gene family consists of a large number of transcription factors that are related by the presence of conserved dimerization and DNA binding domains. All members of this family bind to DNA recognition elements that correspond to the consensus sequence TGA(G/C)TCA as obligate dimers or heterodimers. AP-1 proteins regulate the expression of a diverse set of immediate/early genes that exhibit very rapid and transient transcriptional responses to signals transmitted from cell surface receptors (3). These immediate responses reflect posttranslational modifications of preformed AP-1 proteins, particularly well studied in the case of c-*jun* (40, 44), that result in increased transcriptional activities independent of new protein synthesis.

In contrast to immediate/early genes, transcriptional activation of the SR gene in response to TPA is dependent on new

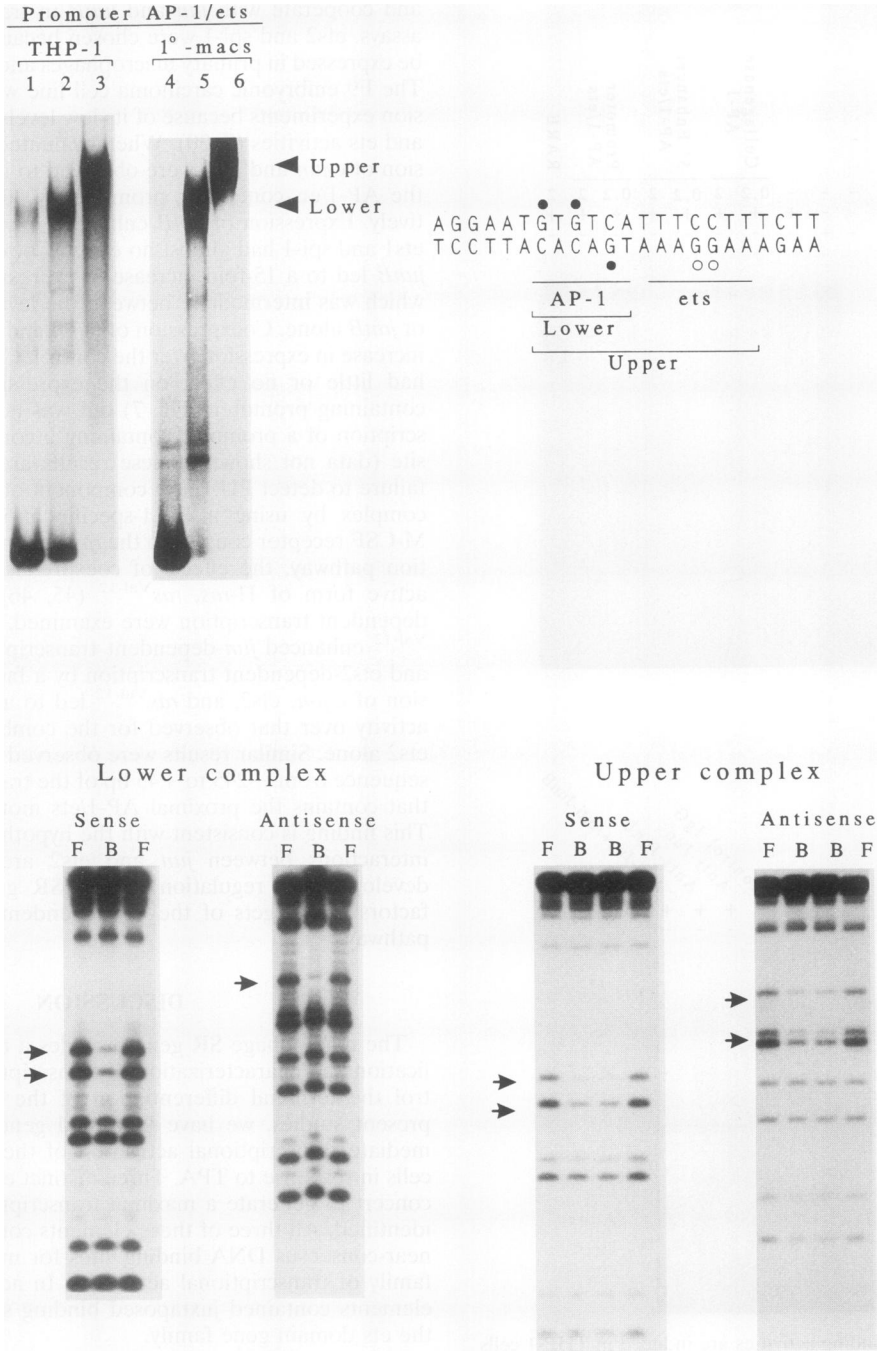

FIG. 6. *jun* and *junB* form a ternary complex with a factor that binds to the adjacent ets recognition motif. Increasing amounts of nuclear extract from THP-1 cells (lanes 1 to 3) or mouse peritoneal macrophages (lanes 4 to 6) were incubated with an oligonucleotide corresponding to the AP-1/ets motif present in the SR promoter. At lower protein concentrations, a single, lower complex formed that corresponds to the complex that was blocked by antibodies to *jun* and *junB*, as illustrated in Fig. 5B. At increasing protein concentrations, an upper complex formed (lanes 3 and 6). The AP-1/ets oligonucleotides were selectively labeled at either end and used for methylation interference analysis of both the lower (lower left) and upper (lower right) complexes obtained from THP-1 nuclear extracts. G residues at which N-7 methylation inhibited DNA binding are indicated by arrows. The results are summarized at the upper right. Methylation interference was observed exclusively within the AP-1 recognition motif for the lower complex (filled circles). In contrast, methylation interference was observed for G residues within both the AP-1 and ets recognition motifs (open circles) for the upper complex.

protein synthesis and does not become maximal until 12 to 16 h following treatment (35). Consistent with these observations, we found that TPA treatment of THP-1 cells resulted in the induction of an AP-1 binding activity that was maximal at approximately 16 h following treatment. By using specific antisera, nearly all of this activity could be accounted for by c-*jun* and *junB*. One potential mechanism to account for the increase in AP-1 binding activity would be dephosphorylation

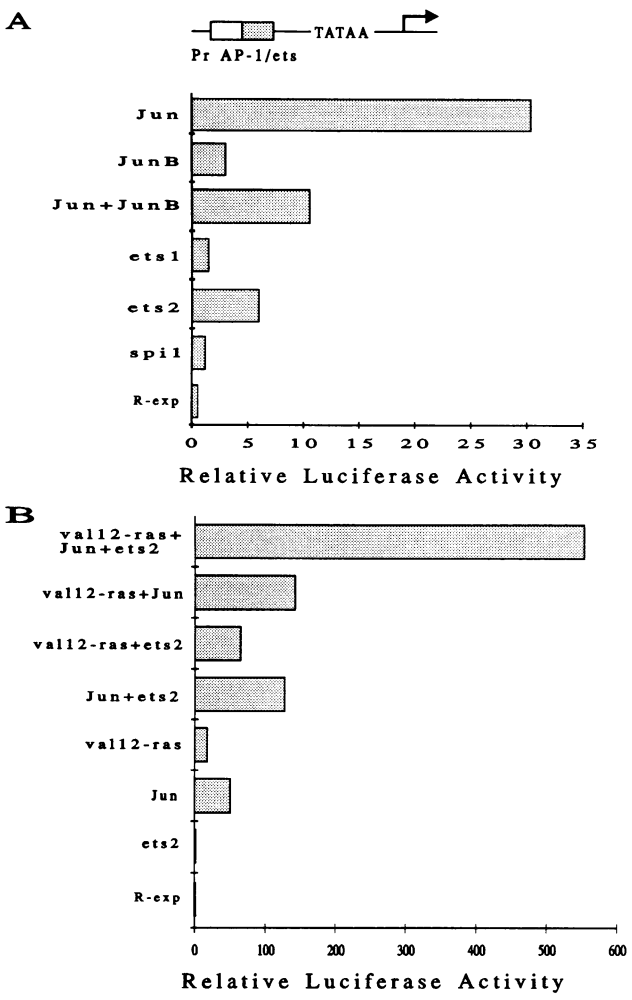

FIG. 7. *jun*, ets2, and *ras* act synergistically to enhance transcription from the SR promoter AP-1/ets site. (A) Relative transcriptional activation of the SR AP-1/ets site by AP-1 and ets domain proteins. The indicated cDNAs encoding c-*jun*, *junB*, ets1, ets2, and PU.1/spi-1 were introduced into the Rous sarcoma virus-based expression plasmid Rexp. F9 teratocarcinoma cells were cotransfected with 0.5 mg of the indicated expression vectors and 1 mg of a reporter gene containing the SR promoter AP-1/ets site placed proximal to the minimal rat prolactin promoter. (B) Interaction between c-*jun*, ets2, and ras^{Val-12}. The indicated combinations of expression plasmids were cotransfected with 1 mg of the AP-1/ets reporter plasmid into F9 teratocarcinoma cells; 0.5 mg of expression plasmid was used for each cDNA. The total amount of expression plasmid used for each point was balanced with the empty vector (Rexp) to maintain a constant level of transfected DNA. In both panels A and B, the results are derived from averages of triplicate points and are representative of four separate experiments.

of phosphoserine and phosphothreonine residues adjacent to the DNA binding domain of c-*jun*. These residues are targets for casein kinase II and, when phosphorylated, inhibit the ability of c-*jun* to bind to DNA (33). One consequence of activation of PKC appears to be the regulation of a *jun* phosphatase that dephosphorylates these residues, thereby stimulating c-*jun* binding and transcriptional activity. The increase in c-*jun* and *junB* DNA binding activities is also likely to reflect new protein synthesis, however, because it is temporally correlated with increases in c-*jun* and *junB* mRNA levels (12, 52a). Transient cotransfection experiments indicated that c-*jun* was significantly more effective than *junB* in stimulating expression from the SR AP-1 sites. Coexpression of c-*jun* and *junB* led to a level of expression intermediate between that obtained for c-*jun* and *junB* alone. These observations are consistent with studies of other AP-1-responsive genes that have suggested that *junB* modulates the activity of c-*jun* (13).

c-*jun* and/or *junB* were found to form ternary complexes with a factor that interacted with an adjacent ets domain binding motif present in both the 5′ enhancer and promoter TPA response elements. This interaction appears to be functionally important, because selective mutation of the ets domain binding motif abolished or impaired the transcriptional response to TPA. Similar juxtapositions of AP-1 and ets binding motifs have been recognized to represent a functional unit involved in mediating transcriptional responses to receptor-dependent signalling pathways in several other genes (5, 41, 51). A recurring feature of the ets family of transcription factors is the ability of various members to interact with adjacent DNA-binding proteins to form ternary complexes that increase the specificity and magnitude of transcriptional responses (5, 18, 25). Several indirect lines of evidence implicate ets2 as a candidate protein involved in ternary complex formation on the SR promoter and enhancer AP-1/ets elements. First, ets2 mRNA is expressed in THP-1 cells and is upregulated following TPA treatment (52a). Second, the sequences corresponding to the core and flanking sequences of the ets binding motif in both the 5′ enhancer and promoter TPA response elements compare favorably with other ets2-responsive genes (50) as well as the optimum binding sites selected from randomized oligonucleotides by the ets2 DNA binding domain (33a). Finally, ets2, but not spi-1 or ets1, led to synergistic increases in the transcriptional activity of promoters containing these sites when coexpressed with c-*jun* in F9 cells. We have as yet been unable to unequivocally identify the ets domain proteins present in the ternary complexes derived from THP-1 cell extracts. It remains possible that additional ets domain proteins contribute to these complexes. Specific identification of these proteins could be of potential therapeutic significance with respect to altering the levels of SR expression in atherosclerosis.

Both c-*jun* and ets2 are widely expressed, and these proteins do not account for the preferential expression of the SR gene in cells of the macrophage lineage (34). However, our findings suggest that c-*jun*, *junB*, and ets2 function as downstream targets of the M-CSF receptor and mediate at least some of the effects of M-CSF on SR expression. c-*jun* and *junB* mRNA levels increase following stimulation of primary human monocytes with M-CSF (37). Furthermore, we have demonstrated that primary mouse peritoneal macrophages contain factors that form ternary complexes on the SR AP-1/ets motif that are indistinguishable from the proteins present in THP-1 cells (Fig. 6). Although the M-CSF receptor does not couple directly to phospholipase Cγ, M-CSF treatment of monocytes has been reported to activate PKC (11, 22). This would presumably set in motion a series of events analogous to those resulting from the treatment of THP-1 cells with TPA. In addition, the M-CSF receptor directly activates the *ras*-dependent signal transduction pathway (6). This pathway has been demonstrated to enhance transcription from several promoters containing combined AP-1/ets motifs (7, 23, 41). Activation of the c-*jun* component of AP-1 has been demonstrated to proceed via a *ras*-dependent kinase cascade involving Raf and mitogen-activated protein kinases that ultimately result in the N-terminal phosphorylation of c-*jun* (40, 44, 48). In the present studies, coexpression of a constitutively active form of *ras* was

observed to markedly enhance the synergistic activities of c-*jun* and ets2 on the SR AP-1/ets elements, consistent with a role of the *ras*-dependent signal transduction pathway in regulating SR expression in macrophages.

It is intriguing that elements mediating transcriptional activation of the SR gene during the process of terminal differentiation correspond to downstream targets of cytoplasmic and nuclear proto-oncogenes, whose activities are normally considered to correlate with cellular growth. For example, a dominant negative form of ets2 has been demonstrated to be capable of inhibiting the mitogenic actions of the M-CSF receptor (32), a result that implies that at least some of the target genes of ets2 in cells of the monocyte-macrophage lineage are required for M-CSF-dependent growth. In contrast to growth-promoting genes, the SR does not become maximally expressed until after monocytes have become postmitotic. Similarly, treatment of THP-1 cells with TPA to induce macrophage differentiation also results in growth arrest. It will be of considerable interest to determine how the signals controlling proliferation are distinguished from signals controlling differentiation in order to coordinate the transition from growth to functional competence.

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