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A Novel *cis*-Acting Element Is Essential for Cytokine-Mediated Transcriptional Induction of the Serum Amyloid A Gene in Nonhepatic Cells

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Serum amyloid A (SAA) is a plasma protein which has been associated with several diseases, including amyloidosis, arthritis, and atherosclerosis, and its abnormal expression, particularly in nonhepatic cells, is implicated in the pathogenesis of these diseases. Transfection and DNA-binding studies were performed to investigate the mechanism controlling cytokine-induced, nonhepatic expression of the SAA gene. We have identified a novel promoter, located between positions −280 and −224, that confers interleukin-6 (IL-6) inducibility to an SAA-chloramphenicol acetyltransferase reporter gene in both nonhepatic and hepatic cells. DNase I protection assays revealed, within this region, three homologous highly pyrimidine rich octanucleotide sequence motifs, termed SAA-activating sequences (SAS). Specific mutations within these three SAS motifs severely reduced IL-6-mediated induction of the reporter gene in transfected nonhepatic cells but not in liver cells. A nuclear factor activated by IL-6 in both hepatic and nonhepatic cells efficiently interacts with the SAS. The induction kinetics and cycloheximide sensitivity of this SAS-binding factor (SAF) suggested that de novo synthesis of this factor itself or an activator protein is essential. Loss of DNA-binding ability as a result of in vitro dephosphorylation, induction of SAA-chloramphenicol acetyltransferase reporter gene activity in the presence of a protein phosphatase inhibitor, and loss of IL-6-mediated inducible DNA-binding activity and reporter gene activation in the presence of genistein, a protein kinase inhibitor, further indicate that a phosphorylation step is necessary for the activation of SAF. Our results suggest that SAF is a key regulator of cytokine-mediated SAA gene expression in some nonhepatic cells.

Serum amyloid A (SAA) is the precursor of the amyloid A protein, a chief constituent of amyloid fibrils in secondary or reactive amyloidosis associated with chronic inflammatory disorders, such as rheumatoid arthritis and juvenile chronic arthritis (21). How the fragmented SAA becomes deposited as fibril AA protein is not well known. It is suggested that at high concentrations, collagenase may degrade SAA and allow its deposition as amyloid fibrils (34). Under normal conditions, a trace amount of SAA is present, but during inflammation, its level is increased 1,000-fold within 24 h as part of a response to various injuries, including trauma or infection (25). The serum level of SAA rapidly returns to normal with the cessation of inflammation, and such a transient induction of SAA in serum does not result in pathogenesis. The strong phylogenetic conservation of SAA together with the dramatic upregulation during physiological alterations suggests that this protein has beneficial roles in (i) maintenance of cellular homeostasis, (ii) lipid transport or metabolism (4, 24), (iii) inhibition of neutrophil oxidative burst (28), and (iv) suppression of immune response (2). However, sustained higher than normal serum levels of SAA are seen in patients with arthritis at concentrations as high as 1,000 μg/ml or more (5, 11, 29). SAA is also known to be an inducer of collagenase activity (8, 34) in synovial cells that can initiate breakdown of the interstitial collagens, a factor crucial to the pathogenesis of articular destruction in rheumatoid arthritis (22). Recent studies have shown that SAA can alter cholesterol efflux by binding to cholesterol (3, 10, 20, 27). This type of remodeling event has been suggested to have an effect on the development of atherosclerosis during chronic inflammation (3). Indeed, SAA mRNA has been detected in the atherosclerotic lesions of coronary and carotid arteries (33), and induced expression of SAA is seen in cultured monocytes/macrophages by cytokines (51). These studies strongly suggest a link between abnormal SAA expression and several pathophysiological events. Studies on the regulatory mechanism of SAA, therefore, may provide a molecular basis of therapeutic measures. Although SAA is transcribed at a high level in hepatocytes, it is also synthesized in several nonhepatic cells, including lung, kidney, and spleen cells, synoviocytes, and monocytes/macrophages. In fact, considerable debate has existed about the source of SAA associated with pathogenesis. Several studies have suggested that local production of SAA (43, 49, 51), in addition to its synthesis in the liver during chronic inflammation, may have a contributory role in amyloidosis, rheumatoid arthritis, and atherosclerosis. Understanding the mechanism of SAA induction in nonhepatic cells thus becomes important for efficient control of SAA overproduction.

Characterization of the rabbit SAA promoter demonstrated the presence of two adjacent functional C/EBP-binding elements and one NF-κB-binding element at the proximal promoter region (37, 39). We have shown that induction and interaction of C/EBP-β, C/EBP-δ, and the RelA subunit of the NF-κB family are involved in controlling the transcriptional induction of the rabbit SAA gene in the liver in response to cytokines produced during inflammation (36, 38). In human and rat SAA genes, similar C/EBP and NF-κB DNA-binding elements have been identified (6, 26). Although the mechanism of SAA gene induction in the liver is well studied, very little is known regarding the molecular details of SAA induction in nonhepatic cells. In this study, we have addressed this aspect and identified a novel *cis*-acting enhancer element that

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is crucial for the induction of SAA expression in nonhepatic cells. Mutation or deletion of this element severely reduces the cytokine responsiveness of the SAA promoter in nonhepatic cells but not in liver cells. A family of interleukin-6 (IL-6)-induced nuclear factors that interact with this promoter region has been detected in several cell types.

MATERIALS AND METHODS

Plasmids. Reporter chloramphenicol acetyltransferase (CAT) plasmids, pSAA-0.4CAT and pSAA-0.19CAT, were constructed by ligating SAA genomic DNA sequences from −401 to +63 and −193 to +63, respectively, into plasmid vector pBLCAT3 (31). Plasmids pSAA (−401/−193) CAT, pSAA (−314/−193) CAT, and pSAA (−280/−193) CAT were constructed by ligating SAA DNA sequences from −401 to −193, −314 to −193, and −280 to −193, respectively, into plasmid vector pBLCAT2 (31). A mutant derivative of pSAA-0.4CAT, pSAA-0.4mtSASCAT, was constructed by site-directed mutagenesis of the wild-type SAA DNA sequence between −274 and −230, using the Promega Altered Sites II in vitro mutagenesis system. The sequence of the mutagenesis oligonucleotide was 5′-CAATACTGTTACTGGGCTCCCCCTTCCTCTCTACTCGAATTCCTC-3′ (underlined nucleotides represent mutated bases). All constructs were verified by DNA sequence analysis to determine their authenticity and orientation. In transfection assays, we used the pBLCAT2 and pBLCAT3 vectors as controls.

Antisera. Antiserum to Ets1 and Ets2 was obtained from Santa Cruz Biotechnology, Inc. Antisera to Stat proteins were obtained from Transduction Laboratories. Antisera to NF-κB p50 and NF-κB p65 were a generous gift of M. Hannink. Antisera to C/EBP-α, C/EBP-β, and C/EBP-δ were a generous gift of S. L. McKnight.

Cell cultures. HepG2 liver cells, BNL CL.2 mouse liver cells, rabbit synoviocytes (HIG82), and rabbit lung fibroblasts (R9ab) were obtained from the American Type Culture Collection. R9ab fibroblasts were isolated from lung tissue of a normal, female New Zealand White rabbit. HIG82 synoviocytes were derived from the interarticular soft tissue of the knee joint of a normal, female New Zealand White rabbit. These rabbit cell lines have retained many of the features of normal rabbit lung and synoviocytes with similar differentiation properties. Synoviocytes, similar to the tissue of origin, are activated by phorbol myristic acid and IL-1 and express genes coding for enzymes such as collagenase, gelatinase, and caseinase. All of these cells were grown in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/liter) supplemented with 7% fetal calf serum (FCS).

Transient transfection assays. The cells were seeded in 35-mm-diameter tissue culture dishes at a density of 10^4 cells per plate, and transfections were carried out by using the calcium phosphate method (19) with a mixture of DNAs containing 5 μg of reporter CAT plasmid, 2 μg of plasmid pSV-β-gal (Promega) as a control for measuring transfection efficiency, and carrier plasmid DNA so that the total amount of DNA in each transfection assay remained constant at 10 μg. The cells were washed with phosphate-buffered saline (PBS) after overnight incubation with calcium phosphate-DNA mixture and then shocked for 1 min with 15% glycerol in PBS and refed with fresh medium containing 2.5% FCS and supplemented with 500 U of IL-6 (obtained from Immunex Corporation) per ml for cytokine stimulation. Cells were harvested 24 h later, and cell extracts were prepared. Extracts were assayed for β-galactosidase activity, and then appropriate amounts of extract normalized for β-galactosidase activity were used in the CAT assay as described earlier (37). All transfection experiments were performed in triplicate.

Nuclear extracts and EMSA. Nuclear extracts were prepared from cultured cells grown in Dulbecco's modified Eagle medium supplemented with 7% FCS, which was then replaced by medium containing 2.5% FCS; the cells were grown overnight, and then the medium was supplemented with 500 U of IL-6 per ml for different lengths of time, as noted in the figure legends. In some experiments, prior to IL-6 treatment, the cells were treated for 1 h with cycloheximide at a concentration of 10 μg/ml. For genistein treatment, the cells were incubated with this protein kinase inhibitor at a concentration of 200 μg/ml for 4 h. A combination of IL-6 (500 U/ml) and genistein was used. For orthovanadate treatment, the cells were incubated for 4 h with this phosphatase inhibitor (100 μM). The cells were harvested and washed in PBS, and nuclear extracts were prepared as described by Dignam et al. (12). Protein concentration was determined by the method of Bradford (7). Electrophoretic mobility shift assay (EMSA) was performed as described earlier (38) with different ^{32}P-labeled double-stranded DNA probes described in the text and figure legends. The probes were labeled by filling in the overhangs at the termini with the Klenow fragment of DNA polymerase, incorporating [α-^{32}P]dATP. Competitor oligonucleotides, described in the text and in figure legends, were included in some EMSAs. For antibody interaction studies, antisera specific to several transcription factors, described in the figure legends, were included in the binding reaction during a 60-min preincubation on ice.

DNase I protection assay. A single 5′-end ^{32}P-labeled SAA DNA fragment (−314 to −193) was incubated with nuclear extracts from HepG2 and lung cells according to the procedure for the binding assay (38), and DNase I treatment was carried out as described previously (37). The DNA fragments were resolved in a 12% polyacrylamide–urea gel.

UV cross-linking assay. A synthetic template oligonucleotide containing SAA DNA sequence from −274 to −230 was annealed to a 15-mer oligonucleotide complementary from one end of the template and extended with the Klenow fragment of DNA polymerase in the presence of 5′-bromodoxyuridine and [α-^{32}P]dCTP. The labeled DNA was purified from a 6% polyacrylamide gel and then used as the probe in binding assays. Following the binding reaction, which was carried out by using a standard protocol (38), the reaction mixtures were exposed to UV light (300 nm) for 15 min and then incubated with 0.5 U of DNase I (Promega) for 15 min at 37°C. The reaction was stopped by adding an equal volume of a buffer containing 0.125 M Tris HCl (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) β-mercaptoethanol, 6 M urea, and 10% (vol/vol) glycerol, the mixture was heated at 95°C for 12 min, and the samples were then fractionated in a SDS–11% polyacrylamide gel.

Dephosphorylation of nuclear extracts. Dephosphorylation was carried out by using calf intestinal alkaline phosphatase (CIP) as described earlier (38). As a control, some reaction mixtures contained, in addition to CIP, a combination of phosphatase inhibitors (50 mM NaF, 1 mM sodium orthovanadate, and 5 μM okadaic acid). These treated nuclear extracts were subsequently used in the EMSA as described above.

Oligonucleotides. The sequences of double-stranded oligonucleotides synthesized for use as competitors were as follows: C/EBP, 5′-TGCAGATTGCGCAATCTGCA-3′; NF-κB, 5′-GATCCATGGGGAATTCCCCATG-3′; Sp1, 5′-TCGACTGGGCGGAGTCTCGA-3′; AP1, 5′-CGCTTGATGACTCAGCCGGAA-3′; Oct1, 5′-TGTCGAATGCAAATCACTAGAA-3′; APRF/Stat, 5′-CTTCTGGGAATTCC-3′; AP2, 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′; Egr, 5′-GGATCCAGCGGGGGCGAGCGGGGGCGA-3′; SAS-wt, 5′-CTTCCTCTCCACCCACAGCCCCC-3′; and SAS-mt, 5′-CTTCCTCTCTACTCGAATTCCTC-3′. Underlined nucleotides represent mutated bases. SAS-wt and SAS-mt oligonucleotides used in the DNA binding assays contain the two adjacent SAA-activating sequence (SAS) motifs only.

Western blot (immunoblot) assay. Nuclear extracts (20 μg of protein) were fractionated in an SDS–11% polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting with anti-C/EBP-α, -β, and -δ antisera was performed as described earlier (38).

RESULTS

Identification of a novel *cis*-acting enhancer element that is essential for inducible expression of the SAA gene in some nonhepatic cells. We previously showed that in liver cells, two C/EBP-binding elements and one NF-κB-binding element control the inducible expression of SAA gene in response to inflammation (36–39). Furthermore, we have shown that in the liver, when both C/EBP and NF-κB factors are induced and activated, expression of SAA is synergistically induced by a heteromeric complex of NF-κB and C/EBP factors (36). To understand whether these two groups of factors also regulate the inducibility of SAA in nonliver cells, we examined the activities of two SAA-CAT reporter genes (Fig. 1A) in these cells. We chose synovial and lung cells because SAA is known to be induced in these cells during inflammation (32, 34). The reporter gene pSAA-0.4CAT, which contains the SAA promoter DNA from positions −401 to +63, was induced by IL-6 in all cell types (Fig. 1B). In contrast, activity of the reporter gene pSAA-0.19CAT (−193/+63), which contains the C/EBP- and NF-κB-binding elements of the SAA promoter (37, 39), was induced by IL-6 in liver cells but remained less responsive in lung cells and synoviocytes.

Absence or no induction of C/EBP and NF-κB may explain the unresponsiveness of the pSAA-0.19CAT reporter gene to IL-6 in lung cells and synoviocytes. We therefore addressed the question of whether C/EBP and NF-κB are present and activated by IL-6 in these cells. Nuclear extracts prepared from control and IL-6-treated liver cells, lung cells, and synoviocytes were analyzed in a DNA binding assay for C/EBP- and NF-κB-specific DNA-binding activity, using SAA DNA (−193 to −79) containing both C/EBP and NF-κB elements as a probe (Fig. 2A). Treatment with IL-6 induced the appearance of some DNA-protein complexes in HepG2 liver cells (lane 2) but not in lung cells and synoviocytes (lanes 3 to 6). Further characterization of these complexes in HepG2 cells was performed

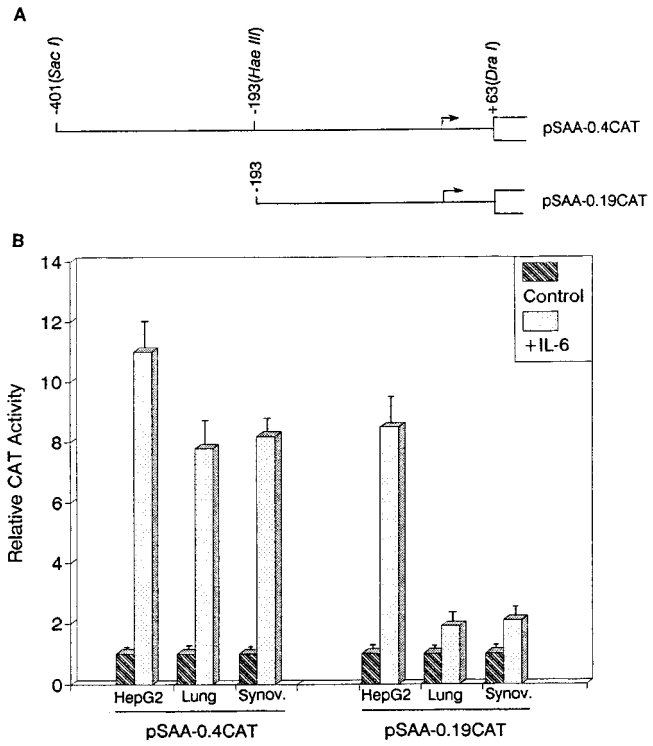


FIG. 1. Analysis of IL-6-inducible promoter controlling nonhepatic SAA induction. (A) Physical maps of the reporter plasmids, pSAA-0.4CAT and pSAA-0.19CAT, containing proximal promoter region of the rabbit SAA gene. The arrows indicate the transcription start site and the direction of transcription. (B) HepG2 liver cells, R9ab lung cells, and HIG82 synoviocytes (Synov.) were transfected with 5 μg of DNA of the two reporter plasmids, and the CAT gene was expressed in the presence and absence of IL-6 (500 U/ml). Details of the transfection and CAT assay are described in Materials and Methods. Transfections were performed in triplicate, and fold induction of the CAT activity in the IL-6-induced transfected cells relative to that of the uninduced transfected cells was determined and plotted as relative CAT activity.

with C/EBP- and NF-κB-specific competitor oligonucleotides and antibodies (Fig. 2B). The NF-κB-specific oligonucleotide had no effect (lane 6), but the C/EBP-specific oligonucleotide efficiently competed for DNA-protein complex formation (lane 2). This finding demonstrated the induction of only C/EBP nuclear factors by IL-6 in HepG2 cells. Furthermore, antibody interaction studies suggested that these complexes are heterodimers of C/EBP-α and C/EBP-β (lanes 3 and 4). Interestingly, we did not see activation of C/EBP-δ in IL-6-treated HepG2 cells (lane 5), although C/EBP-δ has been found to be highly induced in both lipopolysaccharide- and turpentine-treated liver tissues (38, 41). Antibodies specific to p65 and p50 subunits of NF-κB had no effect (lanes 7 and 8), which further confirmed their absence in IL-6-induced HepG2 cells.

To test whether absence of the C/EBP-specific DNA-binding activity in IL-6-treated lung cells and synoviocytes was due to the lack of these proteins, we performed a Western immunoblot analysis (Fig. 2C). In HepG2 liver cells, both C/EBP-α and C/EBP-β isoforms were detected, and although IL-6 treatment increased the DNA-binding activity of C/EBPs, the total amount of these factors was not changed considerably (lanes 1 and 2). This result supports the notion that IL-6-mediated activation of C/EBP-β is largely dependent on its posttranslational modification (35, 50). Interestingly, constitutive moderate levels of C/EBP isoforms were seen in both control and IL-6-treated lung cells and synoviocytes (Fig. 2C, lanes 3 to 6), although no considerable induction of C/EBP-specific DNA-binding activity was detected in these nuclear extracts (Fig. 2A, lanes 3 to 6). The presence of C/EBP-β and C/EBP-δ mRNAs in lung tissue has also been reported earlier (9, 23). The presence of a constitutive level of proteins yet no DNA-binding activity (compare Fig. 2A, lanes 3 to 6, and Fig. 2C, lanes 3 to 6) suggests the presence of an inhibitor molecule that can interfere with the DNA-binding ability of C/EBPs. Indeed, a recent report provided evidence of such a negative regulator, called CHOP-10, in many tissues, including the lung, that by forming a heterodimeric complex inhibits the DNA-binding and transactivating abilities of C/EBP-α and C/EBP-β (44). Whether such an inhibitor is present in lung cell and synoviocyte nuclear extracts remains to be seen. Nonetheless, collectively the results presented in Fig. 1 and 2 indicated that at least in some nonliver cells, IL-6 inducibility of the SAA gene is controlled not by C/EBP and NF-κB elements but by regulatory sequences present upstream between positions −401 and −193.

To better localize a nonhepatic IL-6-responsive promoter, we prepared a set of deletion promoter constructs by ligating various lengths of DNA fragments spanning the region between −401 and −193 of the SAA promoter upstream of the pBLCAT2 vector (31). These deletion constructs, shown in Fig. 3, were transfected in lung cells and synoviocytes and the reporter CAT gene was expressed in the transiently transfected cells in the absence and presence of IL-6. Results of this experiment indicated that deletion of sequences up to −280 had no effect on the inducibility of the reporter gene and suggested that a nonhepatic IL-6-responsive element is present between nucleotide positions −280 and −193 of the SAA promoter. Similar results were obtained when two additional nonliver cells, RAB-9 skin and LLC-RK1 kidney cells, were used in the transfection assay (data not shown).

IL-6 activates a nuclear factor that binds to the cytokine-responsive promoter region of the SAA gene. To determine whether any nuclear factor(s) interacts with the specific cytokine-responsive SAA promoter in nonhepatic cells, nuclear extracts prepared from uninduced and IL-6-induced cells were analyzed by EMSA. The bp −280 to −193 DNA fragment of the SAA promoter was used as a probe (Fig. 4A). A faint DNA-protein complex was visible in nuclear extracts prepared from uninduced cells (lanes 1, 3, 5, and 7). The intensity of the same complex was highly increased when same protein amount of nuclear extracts of IL-6-induced cells was used (lanes 2, 4, 6, and 8). This result demonstrated the activation of a nuclear factor, SAS-binding factor (SAF), that can interact with the SAA promoter (−280 to −193). The induced DNA-protein complex was competed for by the homologous DNA (Fig. 4B, lane 2). To further localize the DNA-binding region, three subfragments of this 88-bp region (−280 to −193) were generated and used as competitors in EMSA (Fig. 4B, lanes 3 to 5). Inhibition of the DNA-protein complex by fragments B (−280 to −250) and C (−249 to −224) but not by fragment D (−223 to −193) led us to conclude that the DNA-binding region is localized between positions −280 and −224. In a reciprocal experiment using these DNA fragments as probes, a similar result was obtained (Fig. 4C). Fragments A (−280 to −224), B (−280 to −250), and C (−249 to −224) interacted with the IL-6-induced nuclear factor. Detection of nuclear factor interaction with both fragments B and C indicated the presence of at least two similar DNA-binding elements within this region (−280 to −224). Inhibition of the DNA-protein complex by both fragments B and C (Fig. 4B, lanes 3 and 4) is consistent with this interpretation. Interestingly, the binding

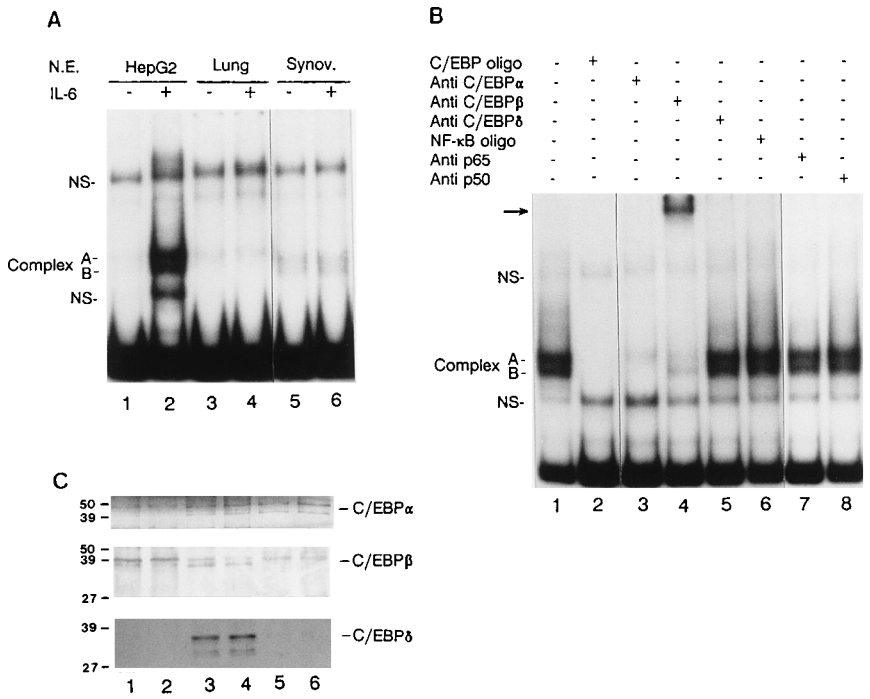


FIG. 2. Binding and characterization of nuclear factors to the SAA proximal promoter region containing the C/EBP and NF-κB binding sites. (A) Nuclear extracts (N.E.; 10 μg of protein) prepared from uninduced and 4-h IL-6-induced HepG2 cells, lung cells, and synoviocytes were incubated with ^{32}P-labeled SAA DNA (−193 to −79) containing the C/EBP and NF-κB binding sites. The DNA-protein complexes were resolved in a native 6% polyacrylamide gel. The migration positions of two complexes A and B are shown. Two nonspecific bands (NS) were routinely detected in various amounts. (B) The complexes formed by HepG2 cell nuclear extract were characterized by using C/EBP and NF-κB specific competitor oligonucleotides and antisera. The supershifted complex in lane 4 is indicated by an arrow. (C) Western blot analysis of C/EBP levels in HepG2 cell (lanes 1 and 2), lung cell (lanes 3 and 4), and synoviocyte (lanes 5 and 6) nuclear extracts. Twenty-microgram aliquots of nuclear proteins were electrophoretically separated and further probed with specific antisera. Lanes 1, 3, and 5 contain uninduced nuclear extracts; lanes 2, 4, and 6 contain IL-6-induced nuclear extracts. Migration positions of the molecular weight markers are indicated in kilodaltons.

efficiency of fragment B seemed significantly lower than that of fragment C. In addition to being induced by IL-6 in liver cells, lung cells, and synoviocytes, SAF has been found to be induced by IL-6 in human carcinoma cell HeLa S3, human monocyte cell U-937, rabbit skin fibroblast RAB-9, and kidney epithelial LLC-RK1 cells (data not shown).

DNase I footprint analysis. DNase I footprinting was performed to further characterize the binding sites within the 56 bp (−280 to −224) region of the SAA promoter. Figure 5A shows the DNase I-protected region detected when IL-6-induced lung and HepG2 cell nuclear extracts were used as protein sources. Two protected regions, one from −272 to −264 and the other from −244 to −228, were identified under these conditions. The footprint result supported the presence of at least two binding elements, as concluded earlier from EMSA (Fig. 4B and C). Essentially identical DNase I footprints were obtained with nuclear extracts from IL-6-induced synoviocytes and skin cells (data not shown). Careful examination of the DNase I-protected sequence identified three similar sequence motifs, CACCGTCA, CACCCACA, and CA GCCCCC, one located between −280 and −250 and two located between −249 and −224. These octanucleotide motifs are termed SAS elements. As evident from Fig. 5B, the three SAS motifs of the rabbit SAA promoter are highly conserved in the human and mouse SAA genes. It is quite likely that the higher binding efficiency of fragment C is due to the presence of two motifs, as opposed to a single one present in fragment B (Fig. 4C, lanes 2 and 3).

Mutation of the SAF-binding elements severely reduces IL-6-mediated SAA gene induction in lung cells and synoviocytes but not in liver cells. The effect of SAF-binding-element mutation on the transcriptional induction of the extended SAA promoter region (up to nucleotide position −401) was examined by introducing specific mutations into the three SAF-binding elements of the SAA promoter. In vitro site-directed mutagenesis was used to mutate the wild-type SAA sequence (Fig. 6A), and the efficacy of the mutant SAA DNA was verified by EMSA (Fig. 6B). Incubation of radioactive mutant SAS DNA with IL-6-induced lung nuclear extract showed no SAF-specific binding (Fig. 6B, lane 1). In a reciprocal experiment, an excess of unlabeled mutant SAS oligonucleotide could not prevent (lane 4) the formation of an SAF-specific complex with the wild-type radioactive SAS oligonucleotide. These results demonstrated that mutations introduced in the SAF-binding elements are adequate for preventing the interaction of SAF. The effect of this mutation on the functionality of the SAA promoter was also tested. We linked a DNA fragment of the SAA promoter from positions −401 to +63 containing mutations only at the three SAF-binding elements to the promoterless pBLCAT3 vector. The wild-type reporter pSAA-0.4CAT and the mutant derivative pSAA-0.4mtSAS-CAT plasmid DNAs were transfected in lung cells, synoviocytes, and liver cells. After transfection, cells were grown either in medium alone or in the presence of IL-6 for 24 h. As seen in Fig. 6C, mutation in the three SAF-binding elements severely reduced the inducibility of the SAA promoter in lung cells and synoviocytes. In contrast, this mutation had a much less inhibitory effect in liver cells. This finding is in agreement with the results shown in Fig. 1. Together, the data indicated that intact SAF-binding elements are essential for induction of the SAA gene in nonhepatic cell types such as lung cells and synoviocytes. In liver cells, although the SAF-binding elements

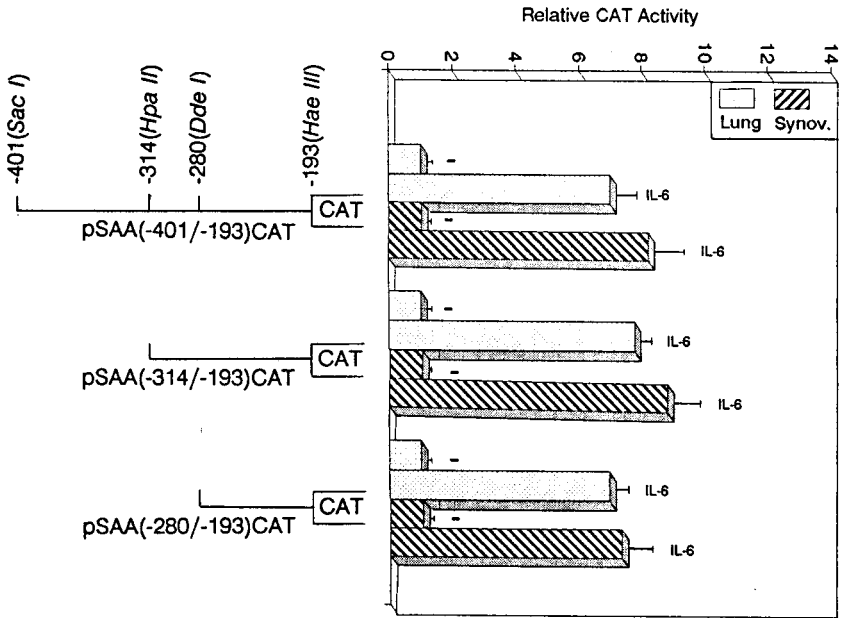


FIG. 3. Deletion analysis of the SAA proximal promoter region for inducible promoter function in two nonhepatic cell types. Three reporter plasmids (5 μg of DNA) containing progressively deleted SAA promoter regions were used to transfect lung cells and synoviocytes (Synov.) in the absence (−) or presence of IL-6 (500 U/ml). Details of the transfection and CAT assay are described in Materials and Methods. The results represent averages of three independent experiments.

did not appear to be crucial for IL-6-mediated transcriptional induction, their mutation nevertheless lowered the overall level of induction.

SAF is a novel transcription factor. An initial search of the National Institutes of Health TFD database (15) revealed no known sequences homologous to the SAF binding site. Since some transcription factors can interact with divergent sequences, several oligonucleotides of known transcription factor recognition sequences were used to compete with the labeled SAS oligonucleotide probe in EMSA. None of these competitor oligonucleotides significantly reduced or abolished the formation of an SAF-specific complex in the DNA binding assay (Fig. 7A). We also used polyclonal antibodies raised against some known cytokine-inducible transcription factors such as Ets (16), Stat91 (48), Stat3 (1, 56), Stat5 (52), NF-κB (47), and C/EBP (9) in EMSA. All of these antibodies also had no effect on the binding of SAF (Fig. 7B), suggesting that SAF is distinct from these known inflammation-responsive transcription factors. This finding also indicates that these cytokine-inducible factors do not form any protein-protein complex with SAF, a phenomenon often observed in some transcription factor families such as complexes between C/EBP and NF-κB (36).

UV cross-linking of the SAF-specific complex. To begin the characterization of SAF, UV cross-linking was performed (Fig.

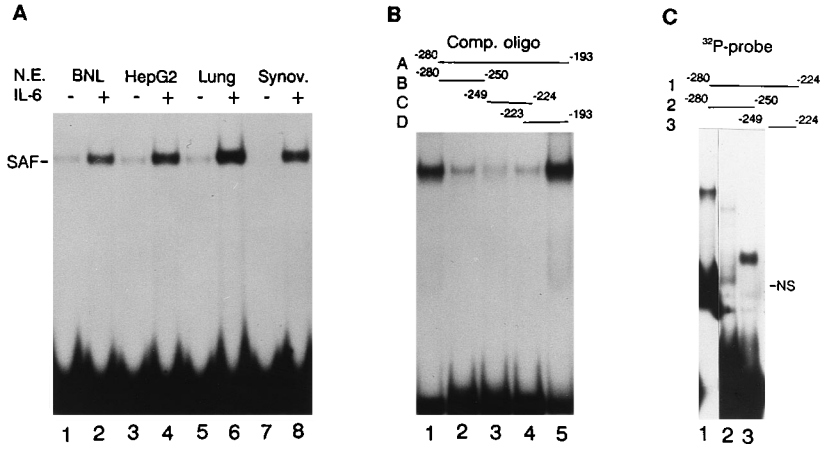
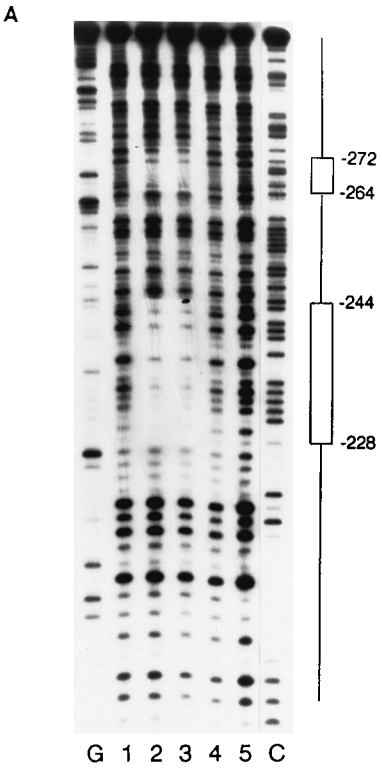


FIG. 4. Binding of nuclear factors to the IL-6-inducible promoter located between bp −280 and −193. (A) Nuclear extracts (N.E.; 10 μg of protein) prepared from uninduced and 4-h IL-6-induced BNL liver cells, HepG2 liver cells, lung cells, and synoviocytes (Synov.) were incubated with a ^{32}P-labeled SAA DNA probe containing sequences from −280 to −193. The migration position of the IL-6-inducible DNA-protein complex is shown. (B) The ^{32}P-labeled SAA DNA (−280 to −193) probe was incubated with 4-h IL-6-induced lung cell nuclear extract (10 μg of protein). In some binding reactions, nonradioactive DNA fragments (50 pmol per assay) of the SAA gene, as indicated, were included to assess their abilities to compete for binding to nuclear factor. Lane 1 contains no competitor oligonucleotide (Comp. oligo) DNA; lanes 2 to 5 contain DNA fragments A, B, C, and D, respectively. (C) Three DNA fragments carrying different regions of the SAA promoter were radiolabeled and incubated with 4-h IL-6 induced lung cell nuclear extract (10 μg of protein). A nonspecific DNA-protein complex (NS) was detected in some assays.



B

	Motif 1	Motif 2	Motif 3
Rabbit	CACCGTCA (-271/-264)	CACCCACA (-243/-236)	CAGCCCCC (-237/-230)
Human	CGCCATCA (-302/-295)	CAGCTGCA (-271/-264)	CTTCCCCC (-263/-256)
Mouse	CACTGCCT (552/559)	CACCCTCT (569/576)	CTCTCCCC (578/585)

FIG. 5. DNase I footprint analysis. (A) A single 5′-end-labeled DNA fragment (−314 to −193) was incubated under conditions for EMSA with IL-6-treated nuclear extracts (30 μg of protein) from lung (lanes 2 and 4) and HepG2 (lanes 3 and 5) cells in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of competitor SAA DNA (−280 to −193). DNA-protein complexes were incubated with DNase I, and the resultant DNA fragments were fractionated in a 12% sequencing gel containing 8 M urea. In lane 1, the probe was incubated without any added nuclear extract. G- and C-specific reactions were performed as markers. (B) DNA sequences of three motifs, two of which are overlapping, in the rabbit SAA gene detected in the DNase I protection assay. Similar sequence motifs in human (14) and mouse (30) SAA genes are aligned with the rabbit sequence. The nucleotide sequence number designation of the mouse SAA2 gene is as reported by Lowell et al. (30).

8). An oligonucleotide probe containing SAF-binding elements (−274 to −230) was synthesized by using bromodeoxyuridine to substitute thymidine residues. Nuclear extracts from IL-6-induced liver cells, lung cells, and synoviocytes were incubated with the probe, UV cross-linked, and separated in an SDS-polyacrylamide gel. A prominent band migrating at about 55 kDa was seen with three nuclear extracts, although the size of the band seen in liver nuclear extract appeared to be slightly smaller (lane 8). These bands did not appear in the presence of excess unmodified, unlabeled competitor oligonucleotide (lanes 3, 6, and 9). Some faint bands of lower molecular weight were also visible; these could have arisen as a result of the in vitro degradation of nuclear proteins, because they were not seen in all experiments.

Time course of IL-6-mediated induction of SAF and effect of inhibition of protein synthesis. To determine the time course of induction of SAF, we prepared nuclear extract from lung cells incubated in the presence of IL-6 for various lengths of time (Fig. 9A). An SAF-specific complex was detected at 60 min, and the activity seemed to reach maximum at about 3 to 4 h. The radioactive spot in lane 2 seems to be artifactual. Similar results were seen with both liver cell and synoviocyte nuclear extracts (not shown). The induction of SAF activity in synoviocytes occurred with parallel kinetics as the transcriptional activation of SAA in rabbit synovial cells by phorbol myristic acid (34).

To determine whether SAF is synthesized de novo, protein synthesis was blocked by 1 h of cycloheximide preincubation prior to treatment of the cells with IL-6 for an additional 4 h (Fig. 9B). The SAF-specific DNA-protein complex was induced by IL-6 under control conditions (lane 2). The induction was inhibited when cells were treated with cycloheximide (lane 3). To ensure that cycloheximide treatment did not generally abolish the ability of all DNA-binding proteins in the extract to interact with their target sequences, we monitored the Sp1 DNA-binding activity in these nuclear extracts. Sp1 is present constitutively at a high level in lung cells, and it is fairly stable in nature (45). Because of relatively long half-life of Sp1, functional Sp1 activity can be detected for at least several hours following protein synthesis block (44a). As seen in Fig. 9C, the Sp1-specific DNA-binding activity remained unaffected by cycloheximide treatment, indicating that Sp1 activity is not dependent on new protein synthesis. In contrary, loss of SAF

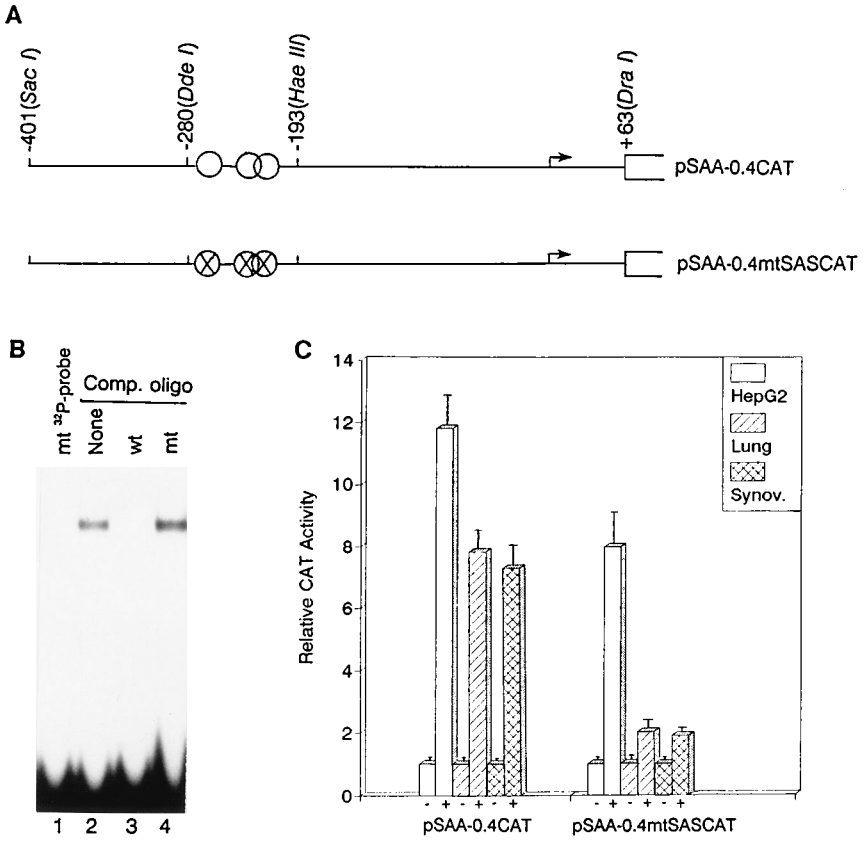


FIG. 6. Mutation analysis of the SAA promoter, SAS. (A) Maps of the reporter plasmid pSAA-0.4CAT and a mutant derivative, pSAA-0.4mtSASCAT, containing the SAA gene proximal promoter. The three SAS motifs of the SAA promoter (−401 to +63) were mutagenized by using the Promega in vitro mutagenesis system as described in Materials and Methods. The SAA DNA sequences between positions −274 and −230 were altered. Three motifs are indicated by the circles. (B) EMSA using mutant and wild-type SAS probes. Nuclear extract (10 μg of protein) prepared from 4-h IL-6-treated lung cells was incubated with a ^{32}P-labeled SAA probe (−280 to −193) containing mutant (mt) sequence (lane 1) or wild-type (wt) sequence (lanes 2 to 4). Unlabeled SAS-wt and SAS-mt oligonucleotides (described in Materials and Methods; 50 pmol per assay) were added as competitor oligonucleotides (Comp. oligo) in some binding reactions (lanes 3 and 4). (C) Results of transfection assays using plasmids pSAA-0.4CAT and pSAA-0.4mtSASCAT (5 μg) in three cell types in the absence (−) or presence (+) of IL-6 (500 U/ml). Transfections were performed in triplicate, and the average values of relative CAT activity are presented. Synov., synoviocytes.

activity suggests that ongoing protein synthesis is required for the activation of SAF.

Dephosphorylation reduces the DNA-binding ability of SAF in vitro, and protein phosphatase inhibitor stimulates the promoter activity of a reporter gene containing SAF-binding elements. Since the activities of many transcription factors are regulated by phosphorylation, we tested whether the DNA-binding activity of SAF is dependent on a phosphorylation step. IL-6-induced lung nuclear extract was first incubated with increasing concentrations of CIP and tested for its DNA-binding ability by using a ^{32}P-labeled SAA (−280 to −193) probe. As seen in Fig. 10A, CIP treatment reduced the intensity of the SAF-specific DNA-protein complex in a dose-dependent manner (lanes 1 to 4). In control experiments (lanes 5 and 6) in which CIP treatment of the nuclear extract was performed in the presence of phosphatase inhibitors, SAF-specific DNA-binding activity was not affected. This result confirmed that reduction of SAF-specific complex formation in lanes 1 to 4 is not due to degradation of SAF nuclear factor by any contaminating proteases present in the CIP. Also, to ensure that the proteins before and after phosphatase treatment are similar in quality, untreated and CIP-treated nuclear extracts were separated by SDS-PAGE and stained with Coomassie blue dye for visualization (data not shown). Next, nuclear extracts were prepared from lung cells grown in the presence of either IL-6,

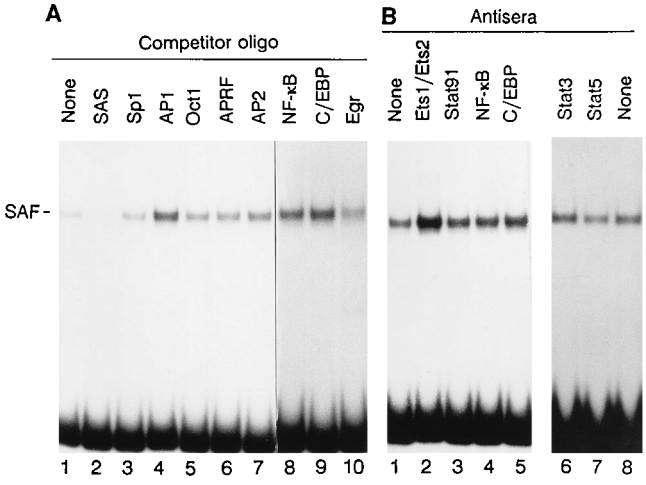


FIG. 7. Characterization of SAF activity. Nuclear extract (10 μg of protein) prepared from 4-h IL-6-treated lung cells was incubated with a ^{32}P-labeled SAA probe (−280 to −193). (A) In some binding assays, unlabeled competitor oligonucleotides, whose sequences are given in Materials and Methods, were added at a concentration of 50 pmol per assay. (B) Nuclear extract was preincubated with antiserum (2 μl of a 1:10 dilution of stock antibody) for 1 h on ice prior to addition of the ^{32}P-labeled SAA probe (−280 to −193). Following the binding reaction, the mixtures were fractionated in a 6% native polyacrylamide gel.

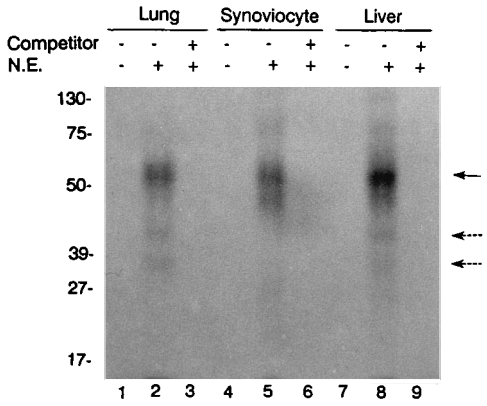


FIG. 8. UV cross-linking assay. Nuclear extracts (N.E.) prepared from 4-h IL-6-induced lung cells, synoviocytes, and HepG2 liver cells (10 μg of protein) were incubated with a bromodeoxyuridine-containing ^{32}P-labeled SAA probe. Some reactions contained unlabeled wild-type SAS-wt oligonucleotide (50 pmol per assay) as a competitor. The reaction mixtures were fractionated in an SDS–11% polyacrylamide gel. Molecular masses of standard protein markers are indicated in kilodaltons. The major protein that bound to the SAA probe is indicated by an arrow. Several minor proteins are also detected (indicated by broken arrows).

IL-6 plus genistein, or vanadate (Fig. 10B). Genistein severely inhibited the stimulatory effect of IL-6 (lane 3). In contrast, the SAF-specific DNA-binding activity was stimulated by vanadate (lane 2). These results also indicated that the DNA-binding activity of SAF is regulated by a phosphorylation event.

For evaluating the role of phosphorylation in vivo, we used protein phosphatase and protein kinase inhibitors in the transfection assay. A reporter gene containing bp −280 to −193 of the SAA 5′ promoter region was transfected in lung cells and incubated in the presence of various agents (Fig. 10C). Sodium orthovanadate, a protein phosphatase inhibitor, stimulated the reporter gene activity, while genistein, a protein kinase inhibitor, prevented IL-6-mediated stimulation of reporter gene activity. Taken together, these results indicated that a protein phosphorylation step is involved in the full activation of SAF.

DISCUSSION

Changes in the circulating serum levels of SAA are a consequence of the changes in the SAA gene expression mediated by several proinflammatory cytokines released during acute inflammation. Although the liver has been found to be the major organ that responds to these cytokines to produce and secrete SAA, recent evidence has demonstrated that many nonhepatic organs also produce SAA in response to these proinflammatory cytokines and that this local production of SAA may be responsible for the pathology associated with the inflammatory episodes of rheumatoid arthritis, reactive amyloidosis, and possibly also atherosclerosis (8, 18, 33, 34). Normally, SAA expression in response to acute inflammation is considered to be beneficial for maintaining cellular homeostasis, and the induction is always transient, allowing only temporary production of this protein. However, chronic inflammation and associated local persistent expression of SAA seem to be harmful to the host, leading to the pathophysiological conditions. In an effort to develop a control measure, we have examined how the nonhepatic expression of SAA is induced. We have shown that the mechanism of SAA induction follows a distinct pathway in some nonhepatic cells in which it is less dependent on C/EBP and NF-κB elements, the two major regulators of inducible hepatic SAA expression. In this study, we have demonstrated that induction of SAA in some nonhepatic cells is primarily dependent on a novel cytokine-responsive promoter element designated SAS. We have provided evidence that interaction of SAF, a cytokine-inducible factor detected in several cell types, with this promoter is crucial for the induction of SAA in some nonhepatic cells.

The importance of SAS sequence motifs in nonhepatic inducible expression of SAA was strengthened by site-directed mutagenesis experiments (Fig. 6). The role of these motifs in the context of an intact SAA promoter was demonstrated by introducing specific mutations that prevented the high-affinity binding of the transcription factor present in the IL-6-induced nuclear extracts (Fig. 6B). These mutations in the SAS motifs severely reduced the inducibility of the reporter gene conferred by the SAA promoter in lung cells and synoviocytes but not in liver cells (Fig. 6C). Severity of the loss of the reporter gene inducibility in two representative nonhepatic cells established the significance of SAS motifs and also supported the earlier results (Fig. 1) which indicated that inducible hepatic expression of SAA is primarily dependent on the C/EBP and NF-κB elements. The importance of the SAS and the associated factor SAF is strengthened by (i) its inducibility in response to proinflammatory cytokine IL-6 and (ii) the relative abundance of SAF in several nonhepatic cell types that also express SAA. Another significant feature is the multiplicity of SAS motifs present within a 44-bp sequence of the SAA promoter, although the presence of multiple copies of an element as a cluster or sometimes in tandem within a short domain of a promoter is not very uncommon. Numerous genes with tandem copies of Sp1 element have been reported (13, 42). Several acute-phase genes including SAA contain two adjacent C/EBP binding sites (37, 40, 55). In SAS motifs, we noted a high degree of variation in binding efficiency, with the upstream element being less potent than the downstream SAS (Fig. 3C, lanes 3 and 4). Similar variable affinity of multiple C/EBP sites in the SAA gene was also observed (37). The significance of multiple binding sites for a transcription factor is not well understood. However, the presence of these elements and their ability to actively bind factors may indicate that a combined effect may influence expression of the gene. It

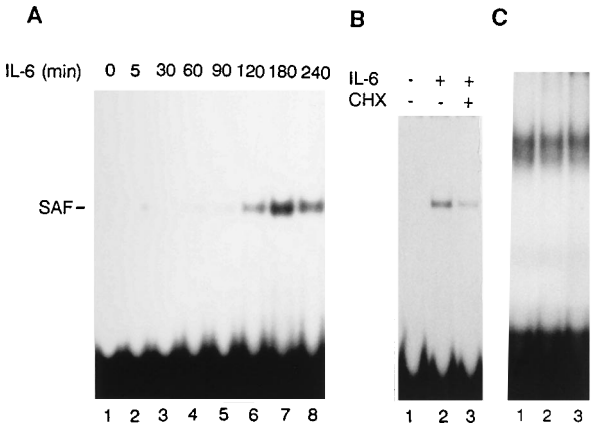


FIG. 9. Kinetics of SAF activation. (A) Nuclear extracts (10 μg of protein) prepared from lung cells treated with IL-6 (500 U/ml) for different lengths of time were incubated with a ^{32}P-labeled SAA probe (−280 to −193). (B) Lung cells were exposed to IL-6 (500 U/ml) for 4 h without (lane 2) or with (lane 3) cycloheximide (CHX) pretreatment. Nuclear extracts (10 μg of protein) prepared from these cells were incubated with a ^{32}P-labeled SAA probe (−280 to −193). (C) The nuclear extracts used for panel B were incubated with a ^{32}P-labeled Sp1-specific DNA probe, whose sequence is given in Materials and Methods.

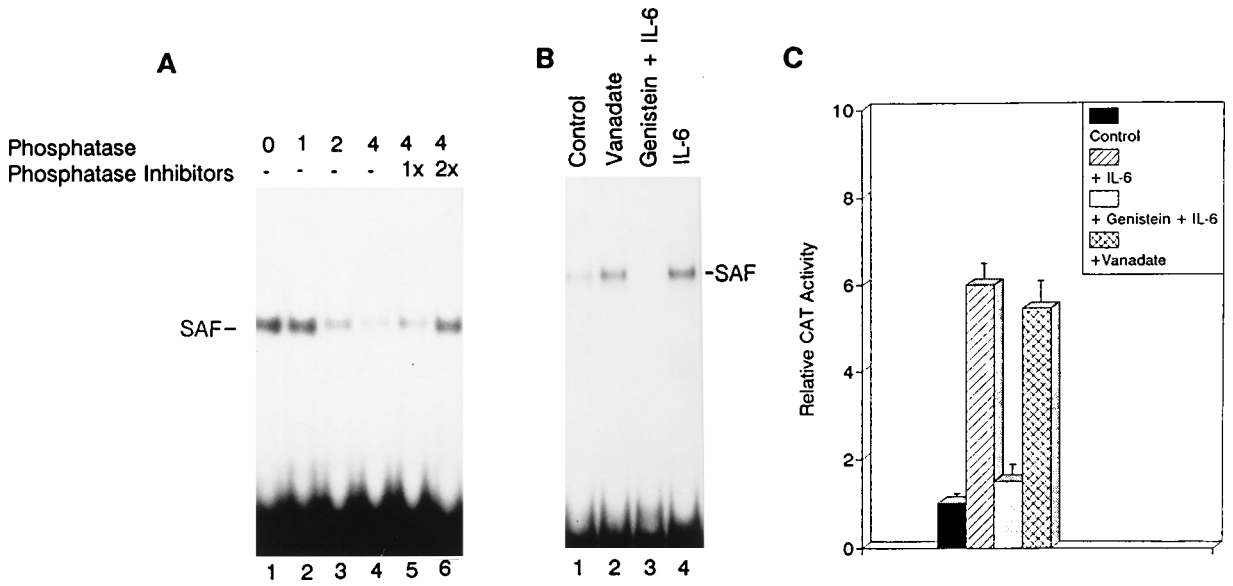


FIG. 10. Evidence that phosphorylation regulates the activity of SAF. (A) Nuclear extracts (10 μg of protein) prepared from 4-h IL-6-treated lung cells were incubated for 30 min with increasing amounts of CIP ranging from 0 to 4 U per reaction. The incubation mixture in lane 5 also contained phosphatase inhibitors (NaF [50 mM], okadaic acid [5 μM], and sodium orthovanadate [1 mM]) during CIP treatment. The phosphatase inhibitor concentration in lane 6 is twice that in lane 5. Phosphatase-treated nuclear extracts were subsequently used in DNA binding assays with a ^{32}P-labeled SAA probe (−280 to −193). (B) Nuclear extracts (10 μg of protein) prepared from lung cells treated with vanadate (100 μM), genistein (200 μg/ml) plus IL-6 (500 U/ml), and IL-6 alone (500 U/ml) were incubated with a ^{32}P-labeled SAA probe (−280 to −193). (C) Activity of the reporter gene pSAA (−280/−193) CAT in transfected lung cells in the presence of IL-6 (500 U/ml), genistein (200 μg/ml) plus IL-6 (500 U/ml), or vanadate (100 μM). The CAT assay was performed as described in Materials and Methods. Results represent averages of three separate transfection experiments.

is tempting to speculate that multiple elements may be occupied by the cognate transcription factors, giving rise to a optimal stereospecific complex of the promoter suitable for transcriptional induction. In the case of the SAS motifs studied here, it is possible that a concerted involvement of the three motifs of SAS give rise to the inducibility of this cytokine-responsive element.

Analysis of a transcription factor database failed to reveal an identifiable match for the SAF target sequence. This lack of a match is further substantiated by using antibodies and competitor oligonucleotides representing the consensus binding sites for several known transcription factors (Fig. 7). Estimation of the size of this factor, determined by UV cross-linking assay, indicated that the major component of SAF is a protein of about 55 kDa. Several minor proteins with different molecular masses were also detected. Presently it is not known whether these minor proteins are the result of degradation of the 55-kDa polypeptide. It is also possible that the minor proteins represent members of a family of proteins which bind to the SAS probe. This possibility is not totally unrealistic in view of the fact that many transcription factors belong to families, namely, NF-κB and C/EBP, which include many members with various sizes, and these proteins recognize and bind to same or similar DNA sequences. More extensive analysis of the SAF should reveal whether it too belongs to a family.

The delayed appearance of SAF activity (Fig. 9) and inhibition of its induction by cycloheximide strongly suggest that ongoing protein synthesis is required for the induction of this protein. Also, activation of SAF seems to be dependent on a phosphorylation step because (i) genistein, a protein kinase inhibitor, can block the IL-6-mediated induction of a SAA-CAT reporter gene and the DNA-binding activity of SAF, (ii) sodium orthovanadate, a protein phosphatase inhibitor, can induce the reporter gene activity, and (iii) in vitro dephosphorylation of the nuclear extracts reduces the SAF-specific DNA-binding activity. However, from these data it is not clear whether cycloheximide inhibits the de novo synthesis of SAF or a labile modifier protein of SAF, or both. Further characterization of SAF must await isolation of this factor at either the gene or the protein level. These studies are currently under way.

In view of the involvement of IL-6 in the activation of SAF and the possibility of its activation by a phosphorylation event, it appears that SAF is similar to the Stat family of transcription factors. The Stat proteins are latent transcription factors that are activated in response to extracellular signals by tyrosine and serine phosphorylation (53). Different ligands activate different members of the Stat family with distinct DNA-binding specificities in the same and in different cells. Cytoplasmic Stat proteins presumably activated by JAKs and mitogen-activated protein kinases translocate to the nucleus, where they bind to specific DNA-binding elements and stimulate gene transcription. Among Stat family members, Stat3/APRF and Stat5 are activated by IL-6 (46). Although, SAF has some general similarity with Stat proteins, particularly with regard to IL-6 inducibility and phosphorylation-mediated activation, it differs from them in several important features: (i) the binding element of Stat proteins with a core binding motif of TTNNNG GAA is distinct from the SAS element for SAF interaction described here (Fig. 5), (ii) antibodies against several Stat family members had no effect on SAF DNA-binding activity, and (iii) the molecular mass of SAF (55 kDa) is much less than that of the known Stat members. The mechanism of activation of SAF is not yet determined, but in light of its induction by an extracellular signal like IL-6, it is possible that activation of this protein involves a multiple-protein kinase cascade. In view of the activation of different members of Stat family by cytokines in many cell types, including HeLa, epidermoid carcinoma cell A431, Jurkat, hepatocyte, and lymphocyte cell lines, and in multiple sheep tissues, including spleen, kidney, lung, muscle,

and adrenal gland (1, 17, 46, 48, 52, 56), one could envision Stat involvement in SAA gene expression by forming a protein-protein complex with SAF. Such a possibility is remote, however, in light of the finding that anti-Stat antibodies have no effect on SAF binding to SAS (Fig. 7B).

One other interesting observation of this study is the apparent lack of C/EBP involvement in lung cells and synoviocytes. Our gel mobility shift assays demonstrated the absence of C/EBP-specific DNA-binding activities in lung cell and synoviocyte nuclear extracts (Fig. 2A) despite the presence of constitutive moderate level of C/EBP proteins in these extracts (Fig. 2C). The presence of C/EBP mRNAs and proteins in lung tissue has also been reported (9, 54). The same IL-6-induced lung cell and synoviocyte nuclear extracts showed increased levels of SAF-specific DNA-binding activity, which validates the good quality of these nuclear extracts. In view of these observations, it is possible that an inhibitor protein that can interfere with the C/EBP-specific DNA-binding activity is present in these extracts. In fact, the presence of a negative regulator of C/EBP-α and C/EBP-β proteins has been reported (44). This protein, termed as CHOP-10, has strong sequence similarity to C/EBP-like proteins within the bZIP region corresponding to the DNA-binding domain. Bacterially expressed CHOP-10 can inhibit the DNA-binding ability of C/EBP-α and C/EBP-β by forming heterodimers that cannot bind DNA. Although mRNA of CHOP-10 is present in many tissues, it is suggested that translational regulation or protein stability may be important in determining the actual level of this protein in a given tissue. Whether a CHOP-10-like molecule is also present in the lung and synoviocyte nuclear extracts needs further analysis. In the absence of functional involvement of this transcription factor, active participation of SAF appears essential for SAA expression in at least some nonhepatic cells.

In summary, we have identified a novel promoter element and a nuclear factor, or family of factors, crucial for the inducible expression of the SAA gene in several nonhepatic cells.

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