

# NF $\kappa$ B Interacts with Serum Amyloid A3 Enhancer Factor to Synergistically Activate Mouse Serum Amyloid A3 Gene Transcription\*

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We had previously identified a distal regulatory element (DRE) in the mouse serum amyloid A3 (SAA3) promoter that functions as a cytokine-inducible transcription enhancer. Within this DRE, three functional elements interact with CCAAT/enhancer-binding protein (C/EBP) and SAA3 enhancer factor (SEF) transcription factors. In this study, we show that cotransfection of the SEF expression plasmid with an SAA3/luciferase reporter resulted in 3–5-fold activation of the SAA3 promoter. When SEF-transfected cells were further stimulated with conditioned medium or interleukin-1, SAA3 promoter activity was dramatically increased, suggesting that SEF may cooperate functionally with other interleukin-1-inducible transcription factors to synergistically up-regulate SAA3 gene transcription. Indeed, cotransfection of SEF and NF $\kappa$ Bp65 expression DNAs resulted in synergistic activation of the SAA3 promoter. Intriguingly, no consensus NF $\kappa$ B-binding site was found in the SAA3 promoter region; rather a putative NF $\kappa$ B-binding sequence with 3-base pair mismatches was identified in the DRE. When this sequence was used in an electrophoretic mobility shift assay, it interacted with NF $\kappa$ Bp50, albeit with binding affinities that were several hundredfold lower than that with the consensus NF $\kappa$ B probe. Functional cooperation between SEF and NF $\kappa$ B was further strengthened by the finding that SEF and NF $\kappa$ B formed stable cytokine-inducible protein-protein complexes. Finally, despite its weak binding, mutation of this NF $\kappa$ B-binding site nevertheless dramatically reduced both NF $\kappa$ Bp65- and cytokine-mediated induction of SAA3 promoter. Therefore, the molecular basis for the functional synergy between SEF and NF $\kappa$ B may, in part, be the ability of SEF to recruit NF $\kappa$ B through physical interactions that lead to enhancement or stabilization of NF $\kappa$ B binding to the SAA3 promoter element.

A prominent feature of the systemic response to acute inflammation, infection, and tissue injury is the rapid increase in the concentration of a number of plasma proteins collectively termed the acute phase proteins (1). Acute phase proteins can be divided into two groups. The type I acute phase proteins,

such as serum amyloid A (SAA)<sup>1</sup>, C-reactive protein, and complement C3, are induced by interleukin (IL)-1-like cytokines and can be further induced by IL-6-like cytokines. The type II acute phase proteins, including fibrinogen, haptoglobin, and  $\alpha_2$ -macroglobulin, are induced primarily by the IL-6-like cytokines (1).

Murine SAA genes belong to a small gene family consisting of four active genes (SAA1, SAA2, SAA3, and SAA5) and a pseudogene (2–4). The plasma concentrations of SAA rise from 0.5  $\mu$ g/ml to more than 1000  $\mu$ g/ml 24 h after injection of bacterial lipopolysaccharide (5). This large increase in hepatic SAA synthesis is primarily the consequence of increased transcription of SAA genes (6, 7) mediated by the proinflammatory cytokines IL-1, tumor necrosis factor, and IL-6 (1, 8). This dramatic induction has therefore been used as a model system for studying differential gene expression in response to a specific stimulus.

To dissect the molecular mechanisms of SAA gene regulation, we have studied the promoters of the rat SAA1 (9–13) and mouse SAA3 genes (8, 14–17). Our studies of the rat SAA1 promoter have shown the functional importance and cooperative interaction between NF $\kappa$ B and C/EBP proteins in cytokine-induced expression. Mutation of either transcription factor-binding site completely abolished SAA1 promoter activity. Studies on the mouse SAA3 promoter demonstrated that a 350-bp promoter fragment was necessary and sufficient to confer cytokine responsiveness (15). Two regulatory elements were identified in this 350-bp promoter fragment: a proximal response element that contains two adjacent C/EBP-binding sequences and enhances SAA3 gene expression in liver-derived cells (17) and a distal response element (DRE) that confers responsiveness to cytokine induction and has properties of an inducible transcription enhancer (16). The DRE consists of three functionally distinct elements: the A element, a weak binding site for C/EBP family proteins; the B element, which also interacts with C/EBP family proteins but with a much higher affinity; and the C element, which interacts with a constitutive nuclear factor termed SAA3 enhancer factor (SEF) (14, 16). Functional analyses revealed that all three elements are required for maximum SAA3 promoter activity (16).

We have recently purified SEF and shown by antibody supershift and amino acid sequence analysis that it is identical to the transcription factor LBP-1c/CP2/LSF (14). LBP-1c/CP2/LSF was initially identified as a cellular factor that binds at multiple sites in the human immunodeficiency virus type I

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<sup>1</sup> The abbreviations used are: SAA serum amyloid A; IL, interleukin; bp, base pair(s); DRE, distal response element; C/EBP, CCAAT/enhancer-binding protein; SEF, SAA3 enhancer factor; HIV-I, human immunodeficiency virus type I; EMSA, electrophoretic mobility shift assays; CM, conditioned medium.

(HIV-I) long terminal repeat (18, 19),  $\alpha$ -globin promoter (20), and SV40 major late promoter (21). It may function either as a transcription activator or a transcription repressor, depending on the promoter context of the gene it regulates and the transcription factors it interacts with. For example, LBP-1c/CP2/LSF stimulates transcription from the SV40 major late promoter (21, 22), whereas it cooperates with YY1 to repress HIV-1 long terminal repeat activity (23). Furthermore, inducers of cell growth can up-regulate the DNA binding activities of LBP-1c/CP2/LSF in human peripheral T lymphocytes, suggesting that it may participate in the regulation of growth-responsive genes (24). In rat pheochromocytoma PC12 cells, LBP-1c/CP2/LSF has been shown to physically interact with neural protein Fe65 (25), but the functional significance of such interaction is yet to be determined.

Binding of IL-1 or tumor necrosis factor to their receptors leads to potent activation of the transcription factors AP-1 and NF $\kappa$ B. Activated NF $\kappa$ B can then rapidly translocate into the nucleus and regulate the transcription of target genes (26, 27), including many effectors of the immune, inflammatory, and the acute phase responses. For example, the proinflammatory cytokines tumor necrosis factors- $\alpha$  and - $\beta$  and IL-1 are not only potent activators of NF $\kappa$ B but are themselves targets of NF $\kappa$ B regulation (28, 29). Other important genes regulated by NF $\kappa$ B include IL-6, IFN- $\beta$ , the chemokines IL-8 and Gro, which summon cells to sites of inflammation (30, 31), and cell surface adhesion proteins such as endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1 (32–35), and the intercellular cell adhesion molecule-1 (36). Many viruses also use NF $\kappa$ B to regulate their own expression. One example is that the expression of HIV-1 is critically dependent on the tandem NF $\kappa$ B sites in its long terminal repeats (37). In almost all cases, NF $\kappa$ B does not function alone. Instead, NF $\kappa$ B often physically associates with other DNA-binding factors and functions cooperatively to regulate transcription of their target genes.

In this study, we sought the molecular mechanisms by which SEF exerts its effect on SAA3 gene transcription in response to cytokine stimulation. We provide evidence that IL-1-induced activation of SAA3 gene transcription requires cooperative interactions between SEF and NF $\kappa$ B. The molecular basis for such functional synergy may be the ability of SEF to physically interact with NF $\kappa$ B and thus recruit NF $\kappa$ B to the active transcription complex.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Nuclear Extracts**—HepG2 cells were cultured in basal medium consisting of minimum essential medium (Life Technologies, Inc.) and Waymouth MAB (3:1 v/v) plus 10% fetal calf serum (38) and were passaged at confluence, approximately once a week.

HepG2 nuclear extracts were prepared essentially as described (39), and as modified by Singh and Aggarwal (40). Briefly, exponentially growing cells were washed twice with ice-cold 1 $\times$  phosphate-buffered saline and then recovered in 1 ml of phosphate-buffered saline. After centrifugation for 30 s in a microcentrifuge, the cells were resuspended in 1.2 ml of lysis buffer (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.4 mM NaVO<sub>4</sub>, and protease inhibitor mixture; Roche Molecular Biochemicals) and allowed to swell on ice for 20 min. Then 37.5  $\mu$ l of 10% Nonidet P-40 was added to the cell suspension, and the mixture was mixed vigorously for 10–15 s and centrifuged for 1 min in a microcentrifuge. The nuclear pellets were resuspended in 20–30  $\mu$ l of ice-cold extraction buffer (0.4 M NaCl, 20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, and a mixture of protease and phosphatase inhibitors), and the nuclear proteins were extracted by constant mixing at 4 °C for 30 min. Cell debris was then removed by centrifugation, and the nuclear extracts were either used immediately or stored in small aliquots at –70 °C. The protein concentrations of the nuclear extracts were determined by the Bradford method (41).

**Electrophoretic Mobility Shift Assays**—<sup>32</sup>P-Labeled C element (4  $\times$  10<sup>4</sup> cpm) containing a single SEF-binding site was incubated with recombinant NF $\kappa$ Bp50 or purified SEF at 4 °C for 30 min (16). After incubation, the reaction mixtures were loaded onto a 5% polyacrylamide gel (19:1 cross-linking ratio) in 1 $\times$  glycine buffer and subjected to electrophoresis at 200 V for 90 min at 4 °C. The gel was dried before autoradiography. For oligonucleotide competition experiments, wild-type or mutant oligonucleotides corresponding to LBP-1c- or NF $\kappa$ B-binding sites were used as specific competitors (see Table I).

**Plasmids and Oligonucleotides**—A DNA fragment containing 165 bp of the 5'-flanking region and 45 bp of the untranslated exon 1 region of mouse SAA3 promoter was inserted into the *Sma*I site of the pGL2-Basic vector (Promega) to generate the pSAA3/Luc(–165) construct. Two mutant constructs, pSAA3/Luc(–165)<sub>mSEF</sub> and pSAA3/Luc(–165)<sub>m $\kappa$ B</sub>, with point mutations in the SEF- and NF $\kappa$ B-binding sites, respectively, were generated by site-directed mutagenesis (Stratagene) with primers that contain mismatches to alter specific nucleotides. The primers with the mutated nucleotides shown in lowercase letters are as follows: mSEF(+), 5'-CTGGCCACATTTaTGGAAATGC-CTcATGGCGCAATCTGGGG-3'; mSEF(–), 5'-CCCCAGATTGCGCCATagAGGCATTTCcAtAAATGTGGCCAG-3'; m $\kappa$ B(+), 5'-GGCCACATTTCtAGAAATGaCTAGATGGCGCAATCTGGGG-3'; and m $\kappa$ B(–), 5'-CCCAGATTGCGCCATCTAGcATTTCtAGAAATGTGGCC-3'. We also generated two 5' deletion constructs, pSAA3/Luc(–93) and pSAA3/Luc(–63), and two internal deletion constructs, pSAA3/Luc(DRE-93) and pSAA3/Luc(DRE-63) (15). The human SEF cDNA was obtained by reverse transcription-polymerase chain reaction using total HeLa RNA as templates. The SEF cDNA was inserted into the *Xho*I site of a pCS2+MT vector (42), which contains six copies of the Myc epitope fused in frame at the N terminus of SEF cDNA. All constructs were verified by DNA sequencing.

**Conditioned Medium Preparation**—Conditioned medium (CM) was prepared from mixed lymphocyte cultures as described (43). Human peripheral blood mononuclear cells were isolated from multiple healthy donors by centrifugation through Ficoll-Hypaque (density, +1.077 g/cm) (Life Technologies, Inc.) for 30 min at 680  $\times$  g. Isolated cells were washed twice with RPMI 1640 and then cultured at 10<sup>6</sup> cells/ml of RPMI 1640 supplemented with 0.25% bovine serum albumin and 10  $\mu$ g/ml phytohemagglutinin (Life Technologies, Inc.). After incubation at 37 °C for 72 h, the CM was separated from the cells by centrifugation and filtration and then stored at –20 °C until use.

**Transient Transfection Assay**—HepG2 cells (5  $\times$  10<sup>5</sup>) were seeded in 2 ml of culture medium. After overnight incubation, cells were transfected with the indicated plasmid DNAs according to the FuGENE procedure (Roche Molecular Biochemicals). All plasmids used in the transfection studies were prepared by either CsCl gradient centrifugation or the QIAfilter Plasmid Maxi kit (Qiagen). For each transfection, 0.5  $\mu$ g of luciferase reporter was transfected into HepG2 cells with or without one or more expression plasmids. To assess cytokine responsiveness, transfected cells were treated with 50% CM or 10 ng/ml IL-1 for 24 h before they were harvested for cell lysate preparation. Cell extracts were assayed for protein content by the Bradford method (41), and the luciferase activity was quantitated according to the manufacturer's instructions.

**Coimmunoprecipitation and Western Blot Analysis**—HepG2 nuclear extracts (approximately 500  $\mu$ g) were preabsorbed with protein A-agarose-coupled beads (Santa Cruz) for 1 h at 4 °C. The preabsorbed beads were then pelleted and discarded. Anti-SEF antibody (~5  $\mu$ l) was then added to the supernatant and allowed to incubate with the nuclear extract for 2 h at 4 °C. Protein A-agarose-coupled beads were then added to the reaction mixture and incubated for another hour at 4 °C. The beads were pelleted and washed three times with 1 $\times$  phosphate-buffered saline. Immunoprecipitated proteins were then boiled in sample buffer and loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride membranes, and specific proteins were detected in TBST solution (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 0.05% Tween 20) containing 2% nonfat dry milk with specific anti-NF $\kappa$ Bp65 (1:5000) (Santa Cruz) or anti-SEF (1:1000) antibodies. Positions of NF $\kappa$ Bp65 and SEF were visualized with peroxidase-coupled second antibody by the ECL detection system (Amersham Pharmacia Biotech). For reprobing, membranes were stripped in 62 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM  $\beta$ -mercaptoethanol at 65 °C for 45 min.

#### RESULTS

**Identical DNA Binding Specificity between SEF and LBP-1c/CP2/LSF**—Our earlier protein sequencing and antibody

TABLE I

Oligonucleotide sequences from various gene promoters or enhancers used as competitors in EMSA

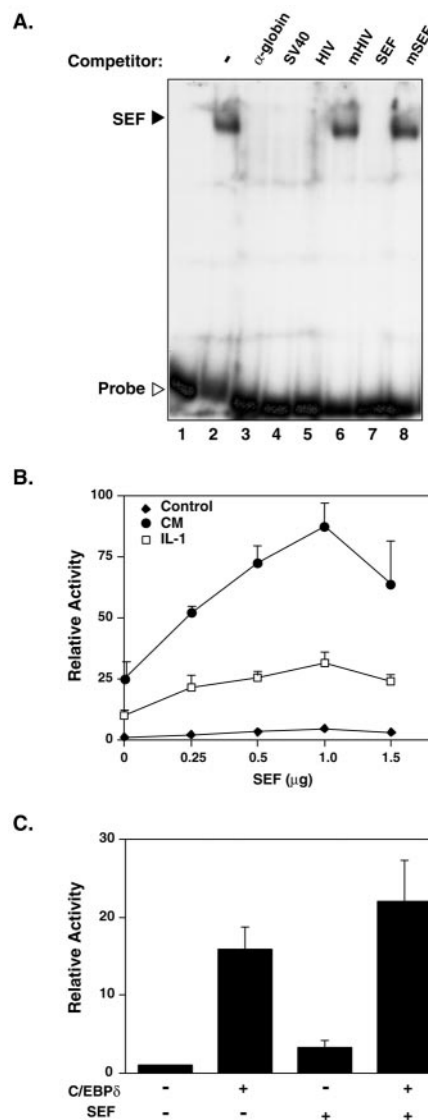
Oligonucleotide	Sequence <sup>a</sup>
SAA3-C element	5'-CGATCACATTTCTGGAAATGCCTAGAT-3'
SAA3-mC	5'-CGATCACATTTaTcaAAATtCCTaTAT-3'
$\alpha$ -Globin	5'-GAGCAAGCACAAACAGCCAA-3'
SV40 (LSF-280)	5'-ACAGCTGGTTCTTCCGCTCAGA-3'
HIV-S1W	5'-GTACTGGGTCTCTCTGGTTAG-3'
HIV-S1ml	5'-GTAagtTGTCTCTCTGGTTAG-3'

<sup>a</sup> The underlined sequences represent the half-sites of the putative SEF-binding sequences. The lowercase characters indicate the substituted bases.

supershift experiments strongly suggested that SEF is identical to or highly related to the transcription factor LBP-1c/CP2/LSF (14). To further examine whether SEF has the same DNA sequence binding specificities as those of LBP-1c/CP2/LSF, oligonucleotides corresponding to the known LBP-1c/CP2/LSF-binding sites were synthesized (Table I) and used as specific competitors in EMSA to inhibit SEF-DNA complex formation. As shown in Fig. 1A, the wild-type LBP-1c/CP2/LSF-binding sequences from the promoters of  $\alpha$ -globin, SV40, and HIV specifically inhibited SEF-DNA complex formation (lanes 3–5), whereas a mutated binding region from the HIV promoter was ineffective as a competitor (lane 6). As expected, wild-type but not mutant SEF-binding sequences specifically competed for complex formation (lanes 7 and 8). Their identical DNA sequence binding specificities further support our conclusion that SEF is identical to LBP-1c/CP2/LSF.

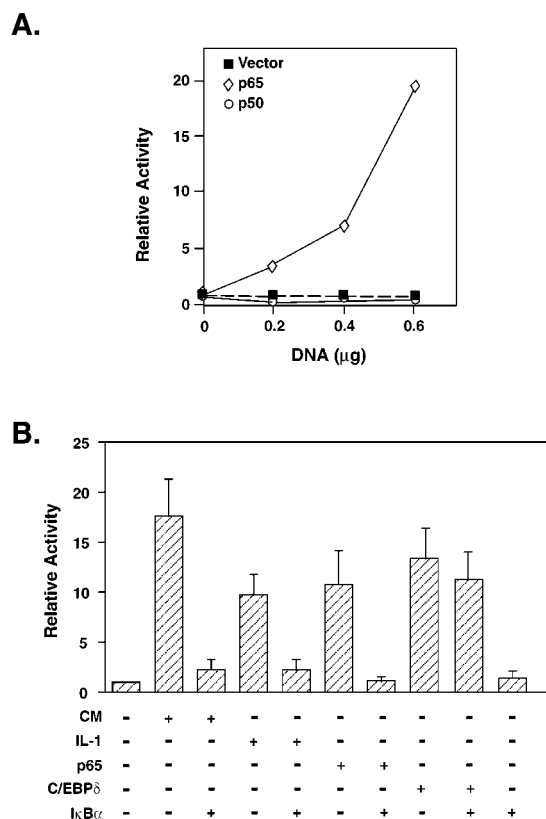
**SEF Enhances CM- and IL-1-induced SAA3 Promoter Activity**—To investigate the role of SEF in SAA3 gene transcription, the pSAA3/Luc(–165) reporter gene was transfected into HepG2 cells with increasing amounts of SEF expression plasmid. Overexpression of SEF activated SAA3 reporter gene expression in a dose-dependent manner, albeit only 3–5-fold. Intriguingly, stimulation of SEF-transfected cells with CM or IL-1 resulted in dramatic synergistic activation of reporter gene expression. In the absence of cotransfected SEF, CM, and IL-1 induced reporter gene activities by approximately 25- and 10-fold, respectively (Fig. 1B). In SEF-transfected cells, cytokine-induced reporter gene expression was even greater. At 1.0  $\mu$ g of SEF expression plasmid DNA, CM and IL-1 induced the pSAA3/Luc(–165) reporter gene by more than 80- and 25-fold, respectively. These results indicate that SEF plays an important role in the transcription of SAA3 promoter. More importantly, it suggests that SEF may cooperate with other cytokine-inducible transcription factors to confer synergistic activation on the SAA3 gene promoter.

**Overexpression of C/EBP $\delta$  with SEF Cannot Confer Synergistic Activation of the SAA3 Gene Promoter**—We had previously shown that the C/EBP family of transcription factors, C/EBP $\alpha$ ,  $\beta$ , and  $\delta$ , play a central role in SAA3 gene transcription (16). Although all can induce pSAA3/CAT reporter gene expression, C/EBP $\delta$  was the most potent transactivator. Because C/EBP $\delta$  is also induced by IL-1 (44), we tested whether C/EBP $\delta$  could be the transcription factor that cooperates with SEF and accounts for the synergistic activation of reporter gene expression. pSAA3/Luc(–165) reporter gene was transfected into HepG2 cells with SEF and C/EBP $\delta$  expression plasmids, individually or in combination. As shown in Fig. 1C, overexpression of C/EBP $\delta$  alone induced the SAA3 promoter by approximately 16-fold. Expression of SEF together with C/EBP $\delta$ , however, resulted in additive rather than synergistic activation of reporter gene activity. These results therefore suggest that C/EBP $\delta$  may not be the IL-1-induced transcription factor that cooperates with SEF to confer the observed synergistic activation of the SAA3 reporter gene.



**FIG. 1. DNA binding and functional properties of SEF.** A, comparison of DNA binding specificities between SEF and LBP-1c/CP2/LSF. <sup>32</sup>P-labeled C element was incubated with purified SEF in standard EMSAs. The SEF-DNA complexes were competed with a 100-fold molar excess of oligonucleotides of known LBP-1c/CP2/LSF-binding sites ( $\alpha$ -globin, SV40, and HIV promoters). As controls, the SEF-DNA complexes were competed with mutant HIV (*mHIV*) and wild-type (*SEF*) or mutant (*mSEF*) C element oligonucleotides. Positions of the SEF-DNA complexes and the free probe are indicated. B, SEF enhances cytokine-dependent activation of SAA3 promoter. HepG2 cells were cotransfected with 0.5  $\mu$ g of pSAA3/Luc(–165) and the indicated amounts of SEF expression plasmid. Transfected cells were then treated with medium alone (*Control*) or with CM or IL-1 (10 ng/ml). Results were normalized to the activities of the noncotransfected control cells, to which a value of 1.0 was assigned. C, SEF and C/EBP $\delta$  do not synergistically activate the SAA3 reporter. pSAA3/Luc(–165) reporter gene was cotransfected into HepG2 cells with SEF and C/EBP $\delta$  expression vectors, individually or together. Results were normalized to the activities of the cells transfected with the reporter gene, to which a value of 1.0 was assigned.

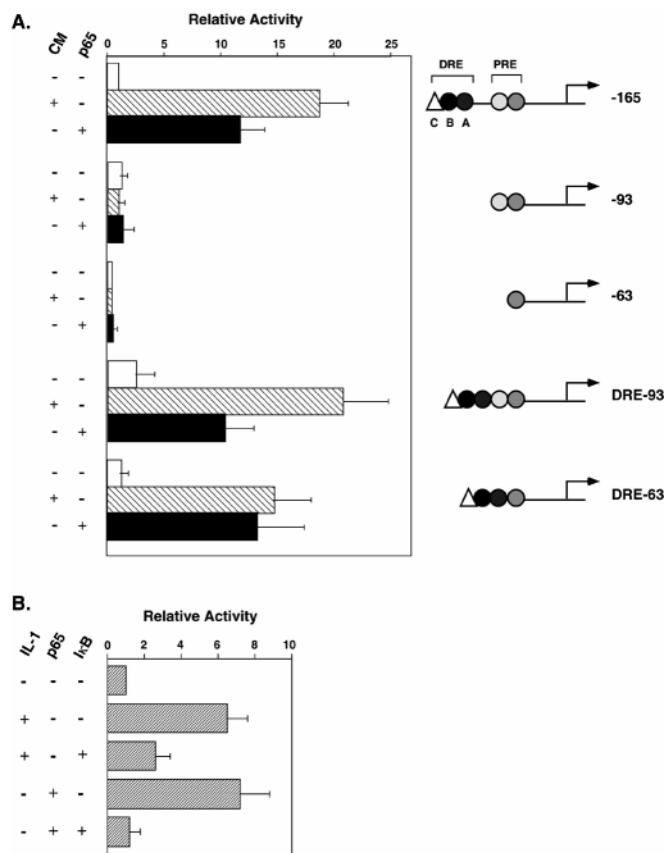
**NF $\kappa$ B Participates in the Cytokine-mediated Induction of SAA3 Promoter**—In addition to activating the C/EBP family of transcription factors, IL-1 is also a potent activator of AP-1 and NF $\kappa$ B (26, 27). Because NF $\kappa$ B has been shown to play a critical role in the regulation of human and rat SAA1 genes as well as the expression of other acute phase genes, we sought to examine whether it might also participate in regulating the SAA3 promoter. To test this possibility, we transfected pSAA3/Luc(–165) reporter DNA into HepG2 cells with the expression



**FIG. 2. NF $\kappa$ B mediates cytokine-dependent activation of SAA3 promoter.** A, NF $\kappa$ B transactivates the SAA3 promoter. pSAA3/Luc(-165) reporter gene was cotransfected into HepG2 cells with indicated amounts of vector DNA, NF $\kappa$ Bp65 or NF $\kappa$ Bp50 expression DNAs. B, I $\kappa$ B effectively inhibits cytokine-mediated induction of SAA3 reporter gene. Cells were transfected with SAA3 reporter gene together with indicated combinations of NF $\kappa$ Bp65, C/EBP $\delta$ , or I $\kappa$ B $\alpha$  expression vectors. Transfected cells were stimulated with CM or IL-1 approximately 16 h later. Results were normalized to the activities of the cells transfected with the reporter gene alone, to which a value of 1.0 was assigned.

vectors for either the p65 or the p50 subunit of NF $\kappa$ B. As expected, the vector control and the NF $\kappa$ Bp50 expression DNA, which lacks a functional transactivation domain, had no effect on reporter gene expression. In sharp contrast, cotransfection of NF $\kappa$ Bp65 expression DNA dramatically induced luciferase activity in a dose-dependent manner, and at 0.6  $\mu$ g of the expression DNA, reporter gene activity was induced by approximately 20-fold (Fig. 2A).

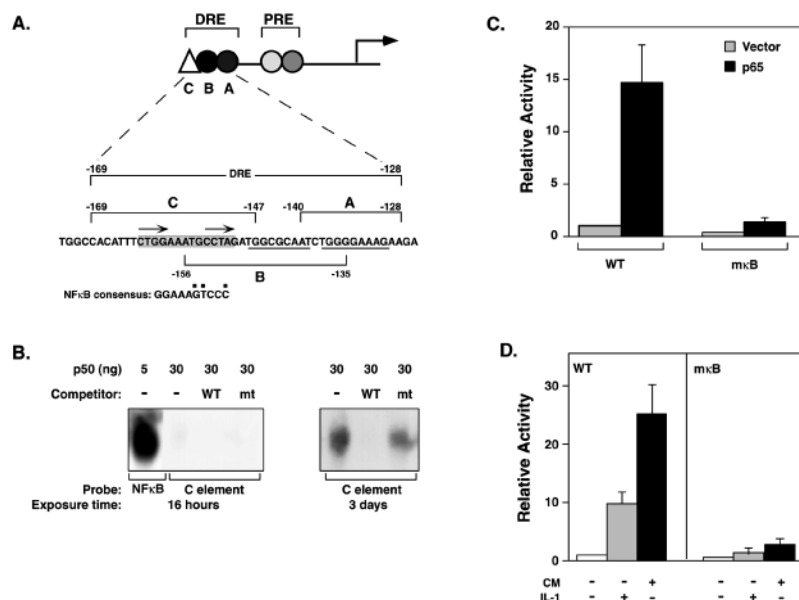
To ensure that the transactivation of pSAA3/Luc(-165) reporter gene by NF $\kappa$ Bp65 was a true reflection of the participation of NF $\kappa$ B in the regulation of SAA3 promoter activity and not merely the result of its overexpression, we examined whether cytokine-mediated induction of SAA3 promoter activities also requires NF $\kappa$ B by expressing its inhibitor I $\kappa$ B in the transfected cells. HepG2 cells were cotransfected with pSAA3/Luc(-165) reporter gene and either empty vector DNA or I $\kappa$ B $\alpha$  expression plasmid and then stimulated with CM or IL-1. As shown in Fig. 2B, in the absence of transfected I $\kappa$ B, CM and IL-1 stimulated the SAA3 promoter by approximately 18- and 10-fold, respectively. However, overexpression of I $\kappa$ B $\alpha$  resulted in greater than 85% inhibition of the cytokine-induced activation. This inhibition by I $\kappa$ B was deemed specific for NF $\kappa$ B activity because I $\kappa$ B had no inhibitory effects on C/EBP $\delta$ -mediated transactivation but completely blocked NF $\kappa$ Bp65-mediated activation (Fig. 2B). Taken together, these results are consistent with the notion that NF $\kappa$ B is one of the transcription



**FIG. 3. The DRE confers NF $\kappa$ B-mediated transactivation.** A, transactivation by NF $\kappa$ B requires DRE. Full-length and four deletion mutants of SAA3 promoter constructs were transfected into HepG2 cells with either NF $\kappa$ Bp65 expression DNA or vector control. The cells that were cotransfected with the vector control were then either treated with control medium or with CM for 16 h before being harvested to assay for reporter gene activities. The reporter gene constructs used were pSAA3/Luc(-165), pSAA3/Luc(-93), pSAA3/Luc(-63), pSAA3/Luc(DRE-93), and pSAA3/Luc(DRE-63). Results were normalized to the activities of the cells transfected with pSAA3/Luc(-165), to which a value of 1.0 was assigned. B, DRE confers NF $\kappa$ B responsiveness onto a heterologous promoter. HepG2 cells were transfected with pSV1(DRE) alone or with combinations of NF $\kappa$ Bp65 and I $\kappa$ B $\alpha$  expression plasmids as indicated. Some transfected cells were treated with basal medium or with IL-1. Results were normalized to the activities of the control cells, to which a value of 1.0 was assigned.

factors activated by IL-1 and that it participates in the regulation of SAA3 promoter activity.

**NF $\kappa$ B Mediates the Induction of the SAA3 Promoter through DRE**—We had shown previously that deletion of the DRE region from the SAA3 promoter completely abolished its cytokine responsiveness (16). To determine whether the DRE might also be necessary for NF $\kappa$ B-mediated activation of the SAA3 promoter, two 5' deletion constructs (pSAA3/Luc(-93) and pSAA3/Luc(-63)) and two internal deletion constructs (pSAA3/Luc(DRE-93) and pSAA3/Luc(DRE-63)) of the SAA3 promoter were tested for their responsiveness to transactivation by cotransfected NF $\kappa$ Bp65 and to CM stimulation. As expected, the wild-type pSAA3/Luc(-165) reporter was highly responsive to CM stimulation and to transactivation by NF $\kappa$ Bp65 (Fig. 3A). However, deletions to positions bp -93 and -63 rendered the promoters completely nonresponsive to both inducing agents. Insertion of the 66-bp DRE sequences in front of these two 5' deletion constructs restored their responsiveness to NF $\kappa$ B and CM, suggesting that transactivation of the SAA3 promoter by NF $\kappa$ B is mediated through the DRE. To further examine whether DRE was responsible for NF $\kappa$ B-mediated transactivation, we inserted one copy of the DRE se-



**FIG. 4. Identification of a weak NF $\kappa$ B-binding site in the C element and its role in NF $\kappa$ B- and cytokine-mediated activation of SAA3 promoter.** *A*, schematic diagram of the SAA3 promoter region and sequence of the DRE. The regions of DRE and A, B, and C elements are indicated by the brackets. The shaded sequence represents SEF-binding site with the short direct repeats indicated by the arrows. The C/EBP-binding sequences are underlined. The consensus NF $\kappa$ B sequence is shown with mismatched nucleotides indicated by the dots. *B*, NF $\kappa$ Bp50 binds weakly to the C element.  $^{32}$ P-labeled C element or NF $\kappa$ B consensus binding sequence was incubated with recombinant NF $\kappa$ Bp50 in EMSA. The protein-DNA complexes were competed with wild-type (WT) or mutant (mt) NF $\kappa$ B-binding sequences. Autoradiograms of two different exposures (16 h and 3 days) of the same gel are shown. *C*, mutation of NF $\kappa$ B-binding site abolished NF $\kappa$ Bp65-dependent activation. Wild-type pSAA3/Luc(-165) (WT) or mutant pSAA3/Luc(-165)<sub>m $\kappa$ B</sub> (m $\kappa$ B) reporter genes were cotransfected into HepG2 cells with vector DNA (Vector) or NF $\kappa$ Bp65 expression DNA (p65). Luciferase activities were normalized to the activity of the wild-type construct cotransfected with the vector DNA, to which a value of 1.0 was assigned. *D*, mutation of NF $\kappa$ B-binding site abolished cytokine-mediated activation. Wild-type (WT) and mutant (m $\kappa$ B) reporter genes were transfected into HepG2 cells and then stimulated with CM or IL-1 for 16 h.

quence in front of a minimal SV40 promoter to create the heterologous promoter construct, pSV1(DRE). When transfected into HepG2 cells, pSV1(DRE) could be induced by IL-1 and by the cotransfected NF $\kappa$ Bp65 (Fig. 3B). Consistent with the results obtained with the SAA3 promoter constructs, introduction of I $\kappa$ B also inhibited the responsiveness of the pSV1(DRE) construct to IL-1 and NF $\kappa$ Bp65. These results therefore strongly implicate the DRE region of the SAA3 promoter as the central regulatory region that confers NF $\kappa$ B-mediated transactivation.

**A Nonconsensus NF $\kappa$ B-binding Site in the C Element Is Required for NF $\kappa$ B-dependent Transactivation**—Transcription factors usually exert their effects on gene transcription by binding to the promoter or enhancer regions of their target genes. As DRE conferred NF $\kappa$ B-dependent activation of the SAA3 promoter, we searched the DRE sequence for potential NF $\kappa$ B-binding site(s). One such site was found within the C element of the DRE; however, it contained three mismatched nucleotides when compared with the consensus NF $\kappa$ B-binding sequence (Fig. 4A). To determine whether this putative NF $\kappa$ B-binding sequence could function as a binding site for NF $\kappa$ B, we incubated  $^{32}$ P-labeled C element with recombinant NF $\kappa$ Bp50, and the NF $\kappa$ Bp50-C element complexes formed were analyzed by EMSA. For comparison, a consensus NF $\kappa$ B-binding sequence was also radioactively labeled and used in EMSA with NF $\kappa$ Bp50. When the consensus NF $\kappa$ B-binding sequence was incubated with NF $\kappa$ Bp50, as little as 5 ng of NF $\kappa$ Bp50 was sufficient to form a strong DNA-protein complex. In sharp contrast, NF $\kappa$ Bp50 binding to the C element was barely detectable even when incubated with 30 ng of the recombinant protein (Fig. 4B). Nevertheless, after longer (3 days) exposure, a weak protein-DNA complex was detected. Furthermore, formation of this protein-DNA complex could be inhibited by the wild-type NF $\kappa$ B-binding consensus oligonucleotides but not by mutant oligonucleotides, indicating that NF $\kappa$ Bp50 can specifi-

cally interact with the C element despite its low affinity. When compared with that of the consensus sequence, interaction between NF $\kappa$ B and the C element was estimated to be several hundredfold weaker.

Such a weak NF $\kappa$ B-binding site in the DRE was somewhat surprising because, as shown earlier in our cotransfection experiments, NF $\kappa$ B is in fact a very potent transactivator on the SAA3 promoter. To determine whether this weak binding site is functionally important, a mutant reporter gene construct was generated in which the NF $\kappa$ B-binding site was mutated. Because this NF $\kappa$ B-binding site overlapped with that of SEF within the C element (Fig. 4A), we introduced a 2-bp mutation so that it affected only NF $\kappa$ B binding but not SEF binding, as determined by EMSA (data not shown). The resulting construct, pSAA3/Luc(-165)<sub>m $\kappa$ B</sub>, was transfected into HepG2. As shown in Fig. 4C, mutation of this weak NF $\kappa$ B-binding site in the DRE rendered the pSAA3/Luc(-165)<sub>m $\kappa$ B</sub> reporter nonresponsive to transactivation by the cotransfected NF $\kappa$ Bp65. Similarly, this mutant construct was also nonresponsive to CM and IL-1 stimulation (Fig. 4D). Taken together, these results strongly indicate that NF $\kappa$ B can bind, albeit very weakly, to a nonconsensus NF $\kappa$ B-binding site in the DRE and that this weak NF $\kappa$ B-binding site is nevertheless functionally important for NF $\kappa$ B- and cytokine-mediated activation of the SAA3 promoter.

**Functional Cooperation and Cytokine-induced Association between NF $\kappa$ B and SEF**—We showed earlier that SEF dramatically enhanced the CM- and IL-1-mediated induction of an SAA3 reporter gene (Fig. 1B), suggesting that SEF may functionally cooperate with one or more IL-1-inducible transcription factors to synergistically activate the SAA3 promoter. Further, we provided evidence that NF $\kappa$ B plays a key role in mediating the effects of IL-1 (Fig. 2). We therefore investigated whether SEF and NF $\kappa$ B can function cooperatively to activate the SAA3 promoter. Wild-type pSAA3/Luc(-165) reporter gene

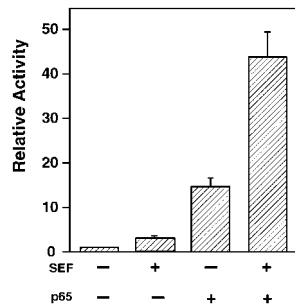


FIG. 5. **SEF and NF $\kappa$ B synergistically activated the SAA3 promoter.** Wild-type reporter construct was cotransfected into HepG2 cells with SEF and NF $\kappa$ Bp65 expression DNAs, individually or in combination. Results were normalized to the activities of the cells transfected with the reporter gene only, to which a value of 1.0 was assigned.

was transfected into HepG2 cells with SEF- or NF $\kappa$ Bp65 expression plasmids, individually or together. As shown in Fig. 5, cotransfection of the reporter gene with SEF- or NF $\kappa$ Bp65 expression DNAs increased the reporter gene activity by approximately 3- and 14-fold, respectively. However, when these two expression vectors were transfected together, the luciferase activity was increased by more than 40-fold, indicating transcriptional synergy between SEF and NF $\kappa$ B.

Because of the overlapping nature of their binding sites and their functional cooperation in SAA3 promoter activation, we investigated whether SEF and NF $\kappa$ B in fact physically interact with each other and thus provide an underlying molecular basis for their synergistic activation. Exponentially growing HepG2 cells were serum-starved for 16 h before they were stimulated with either IL-1 or CM for various time periods. After stimulation, cells were harvested and nuclear extracts were prepared and incubated with anti-SEF antibody to immunoprecipitate SEF and its associated proteins. The presence of NF $\kappa$ B in the immune complexes was then determined by Western blotting with anti-p65 antibodies. As shown in Fig. 6, in untreated HepG2 cells, a low level of NF $\kappa$ Bp65 was detected in the anti-SEF immunoprecipitates. However, within 5 min of IL-1 or CM stimulation, the amount of NF $\kappa$ Bp65 in the immunoprecipitates was increased substantially. The levels of NF $\kappa$ Bp65 in the immunoprecipitates were maintained at elevated levels even at 60 min after stimulation (Fig. 6). When these samples were probed with anti-SEF antibodies, the amount of SEF detected in each lane was approximately the same, indicating that differences in the levels of NF $\kappa$ Bp65 were not due to unequal loading or uneven immunoprecipitation with anti-SEF. To further demonstrate the specificity of the coimmunoprecipitation procedure, we used preimmune serum in the immunoprecipitation reactions and were not able to detect any NF $\kappa$ Bp65 (data not shown). These data therefore indicate that SEF can form protein-protein complexes, directly or indirectly, with NF $\kappa$ Bp65. Further, CM and IL-1 stimulation increases their association with a rapid kinetics. Thus, the transcriptional synergy between SEF and NF $\kappa$ Bp65 may be facilitated through their physical interactions.

**SEF-binding Site Is Critical for Cytokine-mediated Induction of SAA3 Promoter**—To assess its functional importance in conferring the cytokine response, we constructed a reporter gene in which the SEF-binding site was specifically mutated. The resulting construct pSAA3/Luc(-165)<sub>mSEF</sub> was then assayed for its responsiveness to cytokine stimulation. As shown in Fig. 7, the wild-type pSAA3/Luc(-165) construct showed an approximately 25- and 10-fold increase in luciferase activities when stimulated by CM and IL-1, respectively. In sharp contrast, the pSAA3/Luc(-165)<sub>mSEF</sub> construct was nonresponsive

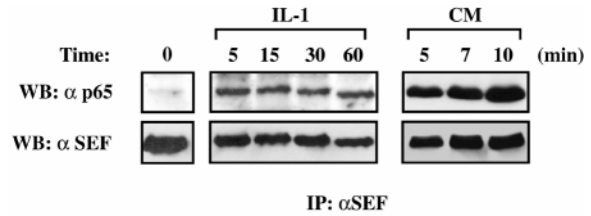


FIG. 6. **Cytokine-inducible association between SEF and NF $\kappa$ Bp65.** HepG2 cells were serum starved for 16 h before they were stimulated for the indicated time periods with IL-1 or CM and harvested for nuclear extract preparation. The nuclear extracts were first precleared with protein A-agarose before immunoprecipitated (IP) with anti-SEF antibodies. The immunoprecipitated proteins were then resolved on a 7.5% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membrane, and blotted sequentially with anti-p65 and anti-SEF antibodies. WB, Western blot.

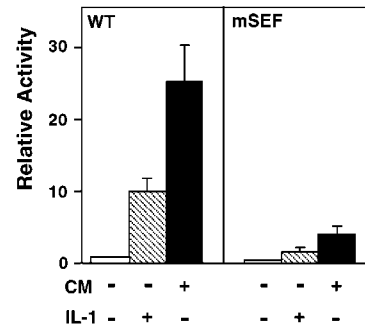


FIG. 7. **The SEF-binding site is required for cytokine induction.** HepG2 cells were transfected with wild-type pSAA3/Luc(-165) (WT) or mutant pSAA3/Luc(-165)<sub>mSEF</sub> (mSEF) reporter genes. Transfected cells were then stimulated with control medium, CM, or IL-1 for 16 h. Results were normalized to the activities of the cells transfected with the wild-type reporter gene, to which a value of 1.0 was assigned.

to cytokine stimulation. This result clearly demonstrates that SEF and its binding site play an important role in conferring maximum cytokine response on the SAA3 promoter.

#### DISCUSSION

We sought a molecular mechanism for cytokine-induced mouse SAA3 gene expression following acute inflammation or tissue damage by analyzing its regulatory elements in the 5' promoter regions and their interacting transcription factors. Our earlier studies identified a 66-bp DRE region that could confer cytokine responsiveness and had properties of an inducible transcriptional enhancer. Within the DRE, three functionally distinct regions, referred to as the A, B, and C elements, proved important for SAA3 promoter function. The A element, a weak C/EBP-binding site, appeared to affect the magnitude of SAA3 expression but not its responsiveness to cytokine stimulation. On the other hand, the B element, identified as a strong C/EBP-binding site, was crucial for the basal and cytokine-induced activities of the SAA3 promoter. The C element, which interacts with the constitutive transcription factor SEF, was important for both basal and cytokine-induced activation of SAA3 promoter (16).

In the present study, we analyzed further the function of SEF in SAA3 gene regulation. Surprisingly, whereas SEF by itself can only moderately activate the SAA3/Luc reporter, stimulation of SEF-transfected cells with IL-1 or CM resulted in dramatic synergistic activation of the reporter gene. We interpreted this result as a strong suggestion that SEF cooperates functionally with one or more cytokine-activated transcription factors to up-regulate SAA3 gene transcription. We had previously shown that C/EBP $\delta$ , an IL-1-inducible transcription factor, could transactivate the SAA3 promoter through the DRE. We therefore tested for functional coopera-

tion between SEF and C/EBP $\delta$ . However, no synergistic induction was observed.

As NF $\kappa$ B is one of the key transcription factors that mediates the IL-1 effects, we then examined whether NF $\kappa$ B could, by itself, activate the SAA3 promoter. Surprisingly, we found that it did. The fact that NF $\kappa$ B potently activated the SAA3 promoter was unexpected because sequence analysis of the SAA3 promoter did not reveal an obvious NF $\kappa$ B-binding site. However, our biochemical and functional results presented here argue for an important functional role of NF $\kappa$ B in conferring the transcriptional up-regulation of SAA3 in response to cytokine stimulation. First, cotransfection of NF $\kappa$ Bp65 with a SAA3/Luc reporter gene dramatically induced reporter gene expression (20-fold) in a dose-dependent manner. Second, CM- and IL-1-mediated activation of SAA3 promoter were completely inhibited by I $\kappa$ B $\alpha$ . These results are particularly significant because they show that NF $\kappa$ B not only can transactivate SAA3 promoter in an overexpression system, but, more important, they demonstrate that NF $\kappa$ B participates in normal physiological conditions, such as the cytokine-mediated transcription activation, to regulate SAA3 promoter. Finally, a nonconsensus NF $\kappa$ B-binding sequence was identified within the C element. Intriguingly, this putative NF $\kappa$ B-binding sequence overlapped with that of the SEF-binding site. This binding sequence showed very weak NF $\kappa$ B binding, several hundredfold lower than that of a consensus NF $\kappa$ B-binding sequence. It is noteworthy that despite its weak binding, mutation of this site nevertheless completely abolished NF $\kappa$ B-dependent and cytokine-induced activation of the SAA3 promoter. Thus, our results clearly demonstrate that NF $\kappa$ B plays a critical role in SAA3 gene regulation.

As a potent transcription activator, NF $\kappa$ B often does not act alone to regulate its target gene promoters. Instead, NF $\kappa$ B functions cooperatively with other DNA-binding factors to induce gene transcription. This functional cooperation usually involves physical interactions between these transcription factors. Some of the transcription factors that have been shown to physically interact with NF $\kappa$ B include AP-1 (45), Sp-1 (46, 47), C/EBP (48–50), SRF (51), Stat6 (52), and components of the basal transcription factors TBP and TFIIB (53). Other factors, such as HMGI/Y, have also been shown to interact directly with NF $\kappa$ B to induce transcription activation through the PDR-II region of the *IFN- $\beta$*  gene promoter (54–56). Physical interactions have also been shown to result in antagonistic effects on the expression of the target genes. For example, interactions between the glucocorticoid receptor and NF $\kappa$ B resulted in inhibition of gene activation (57–60).

In the case of the SAA3 promoter, we also observed strong synergy when NF $\kappa$ B and SEF were coexpressed in HepG2 cells with a SAA3/Luc reporter. The notion of functional cooperation between NF $\kappa$ B and SEF is further strengthened by the demonstration that these two factors can interact with each other. Moreover, their interaction is dependent entirely or is greatly enhanced upon cytokine stimulation. At present, we do not know whether the interaction between NF $\kappa$ B and SEF is through direct protein-protein interaction or whether it involves other protein mediators. Because NF $\kappa$ B is normally localized in the cytoplasm and translocated to the nucleus only after cytokine-dependent activation, this cytokine-dependent interaction may merely reflect their nuclear colocalization and may not require post-translational modification induced by the cytokines.

The underlying mechanism for their functional cooperation therefore appears to be facilitated by the ability of SEF to physically associate with NF $\kappa$ B. Studies have shown that protein-protein contacts between heterologous factors can stabilize

DNA binding or alter sequence specificity of binding. For example, sequence-specific binding of the homeodomain proteins Ubx, Hox, and Ftz-F1 depends on their stable interactions with other sequence-specific transcription factors (61–63). Because the NF $\kappa$ B site in the C element is estimated to be several hundredfold weaker than that of the consensus NF $\kappa$ B-binding sequence, it raises an intriguing possibility that SEF may participate to stabilize or enhance NF $\kappa$ B binding to this site. Thus, the striking functional synergy between SEF and NF $\kappa$ B may be facilitated by their ability to physically interact with each other and perhaps the ability of SEF to enhance or stabilize NF $\kappa$ B binding to its weak binding site in the DRE. It is interesting to note that in the regulatory regions of both SV40 and HIV-I, binding sites for SEF and NF $\kappa$ B have been described. Although it is not known whether these two factors also function cooperatively to regulate the expression of viral genes, it would be tempting to speculate that SEF may enhance the function of NF $\kappa$ B and thus contribute to the expression of viral genes.

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