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YY1 Represses Rat Serum Amyloid A1 Gene Transcription and Is Antagonized by NF-κB during Acute-Phase Response

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Serum amyloid A (SAA), one of the major acute-phase proteins, increases several hundredfold in concentration in plasma following acute inflammation, primarily as a result of a 200-fold increase in its transcriptional rate. Functional analysis of the rat SAA1 promoter has identified a 65-bp cytokine response unit (CRU; positions −135 to −71) that could confer cytokine responsiveness on a heterologous promoter. Within this CRU, two *cis*-regulatory elements, corresponding to NF-κB- and C/EBP-binding sites, were found to be functionally important and exerted synergistic effects on induced SAA1 expression. In this report, we show that a third transcription factor interacts with the CRU through a region located between the NF-κB- and C/EBP-binding sites. On the basis of its gel mobility shift patterns, ubiquitous binding activity, sequence specificity of DNA binding, zinc-dependent binding activity, and gel mobility supershift by specific antibodies, we concluded that this factor is identical to YY1. Methylation interference studies revealed that YY1 binding sequences overlapped with those of NF-κB, and gel mobility studies showed that NF-κB binding to the CRU was effectively inhibited by YY1. Consistent with its presumed antagonistic role to NF-κB, YY1 exerted a negative effect on SAA1 expression, whereas disruption of its binding in the promoter elevated basal and cytokine-induced activities. Furthermore, overexpression of YY1 *trans*-repressed SAA1 promoter activity. Thus, our results demonstrate that SAA1 expression is tightly regulated by an on-off switch of activators and repressors, presumably to ensure that it is expressed only under appropriate physiological conditions.

Transcription initiation in eukaryotic cells depends on the interaction of many transcription factors with an array of *cis*-regulatory elements and with each other (24, 37, 39). In response to cellular or environmental signals, these transactivators increase the rate of transcription initiation, presumably by interacting with and recruiting general transcription factors to the initiation complex (1, 13, 46, 61). These interactions together confer on a promoter its characteristic strength and specific pattern of expression (37, 39).

While transcription activation plays a central role in the upregulation of gene expression, factors that interfere with their function are also important for controlling these cellular responses. Similarly, active repression of transcription is required to suppress the expression of genes in tissues where they should be silent (45, 48). One of the mechanisms for such negative transcriptional control is selective repression by sequence-specific DNA-binding proteins. Such transcription repressors have been described recently (10, 19, 22, 23, 30, 33, 34, 36, 41, 43, 48, 57, 59) and were shown to negatively regulate transcription by a variety of mechanisms, including interfering with the binding of transactivators by competing for DNA-binding sites (19, 34, 57) and hindering bound activators from making proper contact with proteins in the transcription initiation complex (22, 41, 43). Additionally, the repressors may block the activity of the basal transcription complex directly (10, 48, 59). Consequently, the expression pattern of a target gene is ultimately determined by the combined effects of transactivators and repressors (19, 57).

One striking characteristic of most of the transcriptional repressors is that they can also function as transcription activators, depending on the promoter context (20; 23; 30 and references therein; 53). One of the transcription factors with such dual function is YY1 (55), variously called NF-E1, δ, UCRBP, CF1, and F-ACT1 (9, 18, 29, 42, 50). YY1, a zinc finger protein, is involved in the regulation of many genes, including immunoglobulin (κ light chain and μ heavy chain) (42), ribosomal protein (rpL30 and rpL32) (18), myogenesis-related (c-*myc* and α-actin) (29, 50), globin (γ and ε) (16, 44), and several viral (9, 55) genes. It exerts either positive or negative effects on transcription, depending on the promoter context and the intracellular environment (17, 18, 42, 50, 55). The functional diversity of YY1 is perhaps related to its structural complexity, since this *GLI*-Krüppel-related protein has both activator and repressor features encoded by different regions of the protein (51, 53, 55).

For studies of the interplay between transactivators and transrepressors on inducible gene control, the regulation of acute-phase genes by inflammatory mediators represents an excellent model (8, 27). While the acute-phase genes are normally expressed at low levels, they are dramatically and transiently induced in response to acute inflammation (8). The magnitude and transient nature of the induction suggest the potential involvement of activation and repression mechanisms in turning these genes on and off (8, 11). The level of serum amyloid A (SAA), one of the major acute-phase proteins, is increased up to 1,000-fold in response to inflammation and tissue damage (35, 49). This large increase in hepatic SAA synthesis is mainly a consequence of a 200-fold increase in SAA gene transcription (35).

To understand the molecular mechanisms of SAA gene regulation during inflammation, we have analyzed the 5′-flanking regions of the rat SAA1 gene. DNase I footprint and gel retardation assays have identified five *cis*-regulatory elements, including two C/EBP-binding sites and one NF-κB-binding site (31). Transient-transfection analysis of the pro-

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moter demonstrated that a 65-bp DNA fragment (positions −135 to −71) could confer cytokine responsiveness on a heterologous promoter in liver and nonliver cells (31, 32). This fragment thus functions as a non-cell-specific cytokine response unit (CRU). Within the CRU resided binding sites for C/EBP and NF-κB transcription factors. Site-specific mutations of either binding site completely abolished the promoter activity, suggesting the functional importance and synergistic interaction of these two nuclear factor-binding sites in SAA1 gene regulation (32).

In this report, we demonstrate that a third transcription factor also binds to the CRU and is identical to the ubiquitous transcription factor YY1 and that it functions as a repressor to repress SAA1 expression. We show that YY1 competes with NF-κB for overlapping binding sites. Site-specific mutations of the YY1-binding site resulted in derepression of the SAA1 promoter. Moreover, overexpression of YY1 further repressed both basal and cytokine-induced SAA1 expression. Thus, in the rat SAA1 promoter, YY1 functions as a repressor, not only contributing to SAA1's low basal expression, but also perhaps playing a role in the transiency of its expression in response to cytokine induction.

MATERIALS AND METHODS

Cell culture and conditioned medium. Hep3B cells were cultured in basal medium consisting of modified Eagle's medium and Waymouth MAB (3:1, vol/vol, pH 7.0) plus 2% fetal bovine serum and 8% equine serum. Cells were passaged at confluence approximately once a week by trypsinization. Conditioned medium (CM), which has been shown to contain inflammatory mediators capable of inducing acute-phase gene expression in liver-derived cells (8, 11), was prepared from activated mixed lymphocyte cultures as described by Huang et al. (21) and was used as a mixture with an equal volume of basal medium.

Plasmid constructs. pSAA1(−301) was constructed by inserting a DNA fragment of the rat SAA1 promoter containing 301 bp of the 5′-flanking sequence and 18 bp of the exon 1 untranslated region into the *Sma*I site of pSVoCAT vector (31). This DNA fragment was synthesized by PCR with a genomic clone containing the *Pst*I (−1.3 kb) to *Hin*dIII (+490 bp) restriction fragment as the template and oligonucleotides OL-100 (bp −301 to −285 of the coding strand) and OL-101 (bp +18 to +2 of the noncoding strand) as primers. pSAA1 (−117) was constructed similarly except that oligonucleotide OL-Is (bp −117 to −101) was used instead of OL-100 in the PCR. Two site-specific mutant constructs, pSAA1(−117mG$_2$) and pSAA1(−110mG$_2$), each with mutations that affect YY1 binding, were constructed by using oligonucleotides that contained the indicated mutations as primers in the PCRs. To construct a YY1 expression plasmid, human YY1 cDNA (55) was first digested with *Nco*I and *Eco*RI to eliminate the potential hairpin-loop structure at the 5′ untranslated region (centered around position +54 of the cDNA). The *Nco*I-*Eco*RI fragment was blunt-ended by Klenow polymerase and cloned into the *Sma*I site of pSVK3 expression plasmid (Pharmacia) in the sense (pSVK3/YY1) and antisense (pSVK3/YY1R) orientations. All constructs were examined by gel electrophoresis and verified by DNA sequencing.

Transient-transfection and CAT assays. Hep3B cells (10^6 cells per 100-mm dish) were incubated for 16 to 24 h at 37°C before being transfected with 20 μg of plasmid DNA as described before (31). To elicit the acute-phase response, cells were treated with 50% CM approximately 16 to 20 h after transfection. As controls, transfected cells were treated in parallel with the basal medium. Cells were harvested 18 to 24 h after treatment in 250 mM Tris-hydrochloride, pH 7.8. Cell extracts were prepared by four cycles of freezing (−70°C) and thawing (37°C). After centrifugation at 12,000 rpm in a microcentrifuge at 4°C for 15 min, the protein contents in extracts were measured by the Bradford assay (6), and the chloramphenicol acetyltransferase (CAT) activity was determined (14, 32) and quantified by measuring the radioactivity of [^{14}C]chloramphenicol spots corresponding to the acetylated and nonacetylated forms by liquid scintillation spectrometry or with an ImageQuant (Molecular Dynamics).

Nuclear extracts and whole-cell extracts. All steps for the preparation of nuclear and whole-cell extracts were carried out on ice or in the cold room. For nuclear extract preparations, cells were lysed in hypotonic buffer by homogenization with a Dounce homogenizer, and the extracts were prepared as described before (31, 54). For whole-cell extracts, cells were lysed for 5 min on ice in 4.6 volumes of Triton buffer (1% Triton X-100, 100 mM KCl, 50 mM Tris [pH 8.0], 0.1 mM EDTA, 1 mM dithiothreitol, 12.5 mM $MgCl_2$, 20% glycerol) with freshly added protease inhibitors (1 μg of leupeptin, 1 μg of pepstatin, and 1 μg of aprotinin per ml). The extracts were then clarified by centrifugation at 10,000 × *g* for 10 min and used immediately or stored in aliquots at −80°C. For the latter part of these experiments, we used a modified method for nuclear extract preparations that preserved YY1 protein intact. In this improved procedure, cells were first lysed in hypotonic buffer by homogenization as described before (54). The isolated nuclei were subsequently lysed in Triton buffer containing protease inhibitors as for the whole-cell extracts. Nuclear debris was removed by centrifugation, and nuclear extracts were then stored at −80°C.

Electrophoretic mobility shift assay and methylation interference analysis. For mobility shift assays, 2×10^4 cpm (0.2 to 1 ng) of ^{32}P-labeled DNA fragments and 2.5 μg of nuclear protein were mixed in the reaction buffer described by Stratagene (25). Following a 15-min incubation at room temperature, samples were loaded onto a 5.5% polyacrylamide gel (19:1 cross-linking ratio) in 1× Tris-glycine buffer (0.38 M glycine, 50 mM Tris, 2 mM EDTA) and subjected to electrophoresis at 200 V for 2 h at 4°C. The gel was then dried and autoradiographed. In oligonucleotide competition experiments, nuclear extracts were incubated with an excess amount of competitor for 5 min before the probe was added. Binding activities were quantified with an ImageQuant (Molecular Dynamics). When antibodies were included in the supershift experiments, nuclear extracts were incubated with the probe for 5 min before addition of preimmune or polyclonal antibodies. Reaction mixtures were incubated for an additional 10 min before being loaded onto the gel.

For the methylation interference experiments, the DNA probe was partially methylated with dimethyl sulfate before use in scaled-up mobility shift assays. The protein-bound and free DNAs were separated on gels, extracted, purified with Elutip microcolumns, and subsequently cleaved by piperidine (38). The samples were heated at 90°C for 2 min in 95% formamide before electrophoresis at 1,800 V on a 10% polyacrylamide sequencing gel.

Oligonucleotides and antibodies. The following oligonucleotides and their complements were used as competitors in gel retardation assays: wild-type NF-κB binding sequence, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega), and mutant NF-κB binding sequence, 5′-GATCCGCTCACTTTCCG-3′. The YY1 binding sequence from the chicken α-actin promoter (5′-CGTCGCCATATTTGGGTG-3′) and rabbit polyclonal antibodies against recombinant human YY1 were

kindly provided by Robert Schwartz and Te-Chung Lee (28, 29). Anti-p50 and anti-p65 antibodies were obtained from Santa Cruz Biotechnology and used in gel shift experiments as recommended by the supplier.

RESULTS

Ubiquitous CRU-binding factor identified as YY1. Our previous studies have suggested that NF-κB-like and C/EBP-like transcription factors interact with the 65-bp CRU (*Hin*fI [−135]-*Ava*II [−71] fragment) of the rat SAA1 promoter and are required for promoter function (Fig. 1A) (31, 32). Furthermore, oligonucleotide competition studies have shown that the cytokine-induced complexes can be specifically inhibited by NF-κB- or C/EBP-binding sequences. To determine whether some of these induced complexes were indeed due to NF-κB binding, a probe derived from the CRU that contains only the NF-κB-binding sequence was incubated with Hep3B nuclear extracts. As shown in Fig. 1B, two weak protein-DNA complexes were detected with the control extracts. Their intensities, however, were dramatically increased when the probe was incubated with extracts prepared from Hep3B cells that had been stimulated with interleukin-1 for 30 min. To further identify the protein components in these two complexes, antisera against the p50 and p65 subunits of NF-κB were used in supershift analyses. Regardless of the extracts used, addition of anti-p50 antiserum supershifted both complexes, whereas anti-p65 antibodies supershifted only the upper complex (Fig. 1B). These results indicated that the upper complex contains both the p50 and the p65 subunits of NF-κB (presumably in the form of a p50-p65 heterodimer), while the lower complex contains only the p50 subunit (p50-p50 homodimer). Thus, these studies demonstrate directly the binding of NF-κB to sequences present in the CRU of the SAA1 promoter. However, when the CRU was used as a probe in gel mobility shift assays, two additional DNA-protein complexes—a major complex (C_1) and a much weaker complex (C_2)—were detected (Fig. 1A). The formation of these complexes was not inhibited by C/EBP- and NF-κB-binding oligonucleotides (31).

To determine more precisely the DNA sequences involved in the formation of complexes C_1 and C_2, we performed methylation interference studies. Analysis of the C_1 complex showed that, on the coding strand, methylation of any one of the six guanidine residues from bp −96 to −86 interfered with protein binding. On the noncoding strand, two guanidines (positions −84 and −94) were found to interfere with DNA-protein complex formation when methylated (Fig. 1C). When the C_2 complex was analyzed, identical methylation interference patterns were observed for both the coding and noncoding strands (Fig. 1C). The fact that the proteins of the C_1 and C_2 complexes had identical DNA base contacts suggested that C_2 protein might be a degradation product of C_1 protein. Indeed, the ratios of these two complexes differed in the different nuclear extract preparations. In addition, the C_2 complex was more abundant than C_1 after repeated freezing and thawing of nuclear extracts (data not shown). It is noteworthy that this protein apparently bound to a region adjacent to the C/EBP-binding site that overlaps the NF-κB-binding site. Indeed, the methylation interference patterns showed that this protein shared not only binding regions with NF-κB, but also significant base contacts (Fig. 1C, bottom). Thus, this protein could interfere with NF-κB binding through competition for overlapping binding sites.

To examine the tissue distribution of the binding activity, nuclear extracts from rat kidney, lung, spleen, brain, and heart were prepared and tested in gel retardation assays. As shown in Fig. 1D, binding activities were detected in all tissues examined. Formation of the DNA-protein complexes was specifically inhibited by an excess amount of wild-type oligonucleotide oligo-G, which spans the region between the C/EBP- and NF-κB-binding sites, but not by the mutant oligonucleotide oligo-mG_3. Unexpectedly, each nuclear extract exhibited a distinct proteolysis pattern, reflecting different extents of proteolytic activity in these extracts and the high susceptibility of this protein to degradation. Special precautions were taken for all subsequent nuclear and whole-cell extract preparations to minimize proteolytic degradation.

Examination of the DNA sequences required to bind C_1 and C_2 revealed a close homology to that of the ubiquitous cellular factor YY1 (Fig. 2A) (9, 18, 42, 55). The C_1 sequence (CACCATGTCA) differs by only 1 bp from the YY1-binding consensus sequence (C/G)(G/T/A)CCATNTTN (17, 29, 50), and the core CCAT sequence is completely conserved (Fig. 2A). To determine whether the protein in the C_1 and C_2 complexes is actually YY1 or a YY1-related factor and whether YY1 can bind to CRU, several approaches were taken. First, the specificity of C_1 and C_2 binding was tested by oligonucleotide competition with a known YY1-binding sequence. As shown in Fig. 2B, the YY1-binding site as well as the wild-type oligo-G sequence specifically inhibited formation of the C_1 and C_2 complexes, while mutant oligo-mG_3 did not have any effect on complex formation. As expected, when the YY1-binding sequence was used as the probe, YY1 binding was also specifically blocked by an excess amount of oligo-G but not by oligo-mG_3 (data not shown). Thus, the similarity between the protein in the C_1 complex and YY1 extends beyond their binding sequence homology and suggests that YY1 forms the C_1 complex.

In addition to the C_1 and C_2 complexes, a more-slowly migrating complex, C_3, was detected. Formation of this complex was similarly inhibited by oligo-G and YY1-binding site oligonucleotides (Fig. 2B). Given that the protein in the C_1 complex was highly susceptible to degradation and that complex C_3 was not observed until the extract was prepared by the modified procedure, it is likely that the protein in the C_3 complex is related to the protein in the C_1 complex and may represent the intact form of the protein. Indeed, when the preparation time was shortened, the C_3 complex in some extracts became the most prominent complex (see below). In this regard, it shares with YY1 a high susceptibility to degradation by proteases (29, 60).

Since YY1 is a zinc finger-containing protein and requires zinc for its DNA-binding activity (29, 42), we tested the zinc requirement for the formation of complexes C_1, C_2, and C_3. The formation of all three complexes was inhibited when the zinc chelator 1,10-phenanthroline (52) was added to the gel shift reaction mixes, whereas the formation of DNA–NF-κB and DNA-C/EBP complexes was not affected (data not shown). This inhibitory effect could be partially relieved by the addition of 1 mM zinc but not by other divalent cations such as Ca^{2+} and Mg^{2+} (unpublished data). These results further suggest that all three complexes (C_1, C_2, and C_3) are formed by the same or a very similar protein and that their DNA-binding characteristics are similar to those of YY1.

To examine whether YY1 can bind the CRU, recombinant YY1 and YY1 synthesized in vitro were used in gel mobility shift experiments. Bacterially produced recombinant HisYY1 and in vitro-synthesized YY1 both bound to the CRU and formed complexes with the same mobility as the C_1, C_2, and C_3 complexes (data not shown). Formation of these complexes was specifically inhibited by addition of an excess of YY1-

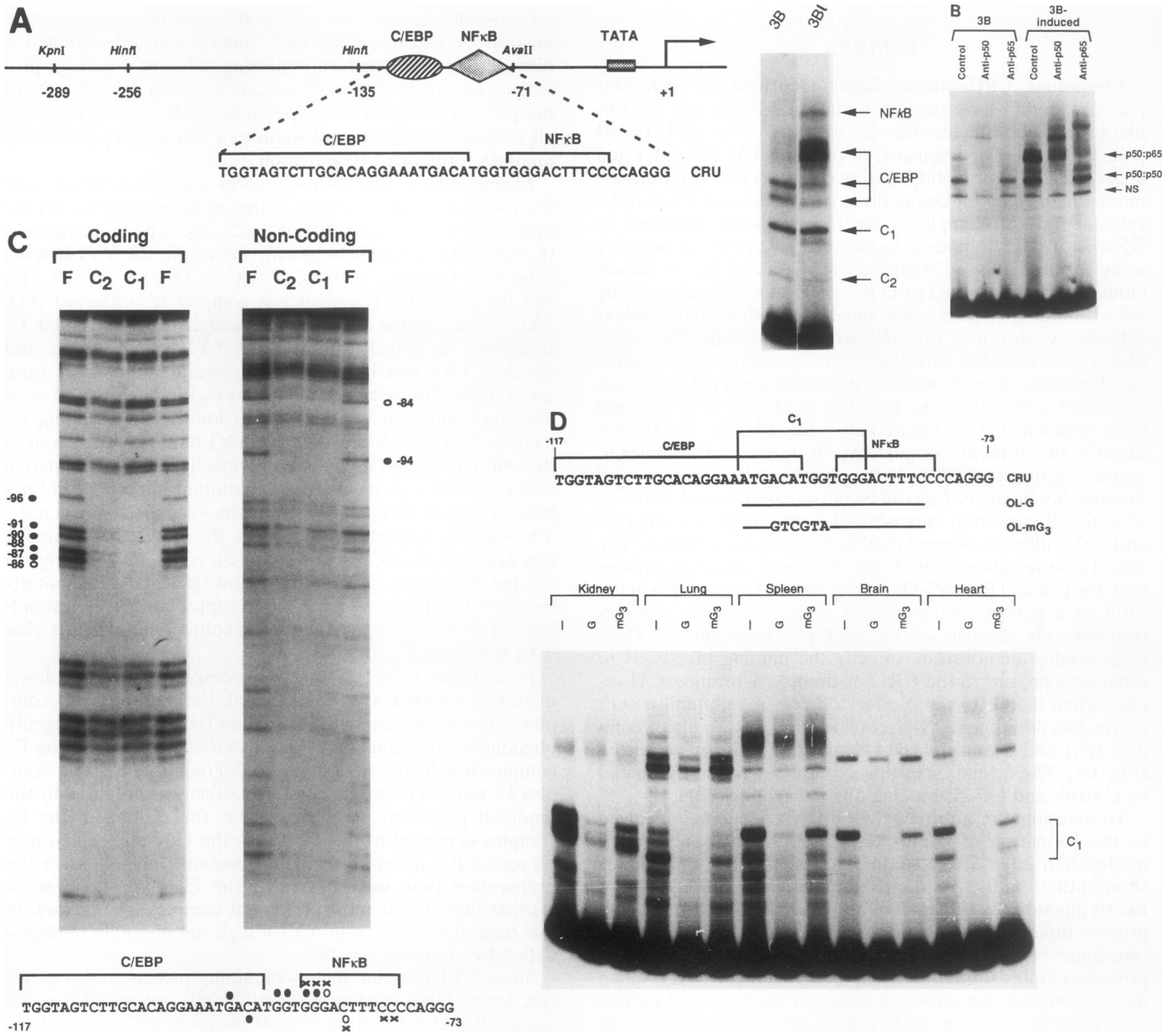

FIG. 1. Binding of a ubiquitous nuclear factor to the CRU. (A) Schematic of the 5′-flanking region of the rat SAA1 gene, with the nucleotide sequence spanning part of the CRU shown. The positions of NF-κB- and C/EBP-binding sites and the TATA motif are indicated. To the right of the schematic are shown gel mobility shift assays. End-labeled CRU (*Hin*fI [−135]-*Ava*II [−71]) was incubated with Hep3B nuclear extracts (2.5 μg) prepared from unstimulated (3B) and CM-stimulated (3BI) cells. The positions of specific DNA-protein complexes are indicated by arrows. (B) Antibody supershift. End-labeled NF-κB-binding sequence from the CRU was incubated with unstimulated (3B) and interleukin-1-induced (3B-induced) (100 U/ml for 30 min) Hep3B nuclear extracts in the presence of specific antisera against the p50 and p65 subunits of NF-κB. The positions of p50-p65 heterodimer and p50-p50 homodimer are indicated. NS, nonspecific complex. (C) Methylation interference analysis of complexes C_1 and C_2. The coding or the noncoding strand of CRU was radioactively labeled, partially methylated by dimethyl sulfoxide, and then used in scaled-up gel retardation assays with Hep3B nuclear extracts. The DNA-protein complexes C_1 and C_2 and the free DNA (F) bands were excised and treated as described in Materials and Methods. The positions of methylated guanidine residues that interfered with complex formation are indicated by solid circles. Open circles represent partial interference. The methylation interference patterns for complex C_1 and NF-κB (×) (2) are summarized at the bottom. Binding sites for NF-κB and C/EBP are indicated by brackets. (D) Complex C_1 detected in different tissues. The nucleotide sequences for oligo-G (OL-G) and oligo-mG_3 (OL-mG_3) are indicated at the top. Below is shown the gel retardation and oligonucleotide competition analyses, in which labeled CRU was incubated with nuclear extracts prepared from rat kidney, lung, spleen, brain, and heart. For competition, a 100-fold molar excess of unlabeled oligo-G (G) or mutant oligo-mG_3 (mG_3) was included in the binding reaction mixes.

A

Organism	Enhancer/Gene	Binding Sequence	Reference
Rat	SAA1	CACCATGTCA	This report
Adeno-associated virus	P5+1	CTCCATTTTG	(55)
	P5-60	CTACATTTTG	
Human	Ek3'	CTCCATCTTG	(42)
Mouse	rpL30	GGCCATCTTG	(18)
Mouse	μE1	GGCCATCTTG	(42)
Mouse/chicken	α-actin	CGCCATATTT	(28)
Mouse	myc-260	GACCATTTTC	(50)
	myc-390	CGCCATGTAC	
MuLV	UCR	CGCCATTTTG	(9)
Mouse	β-casein	AACCATTTTC	(47)
	Consensus	CGCCATNTTN GT A	

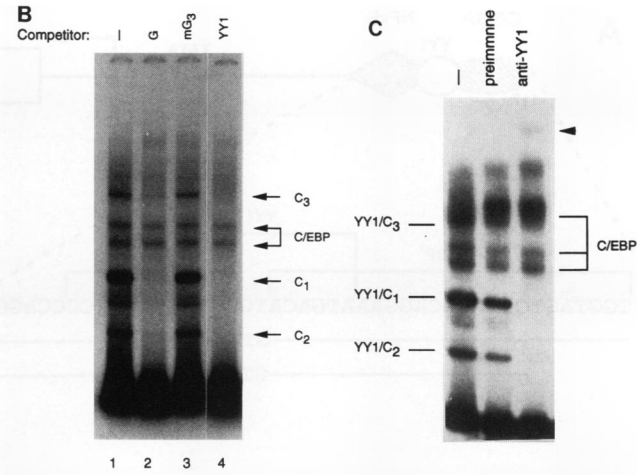

FIG. 2. YY1 participates in complex C_1 formation. (A) Sequence comparison of the DNA-binding site for complex C_1 in the rat SAA1 promoter with that of known YY1-binding sequences. The CCAT core sequence is highlighted. MuLV, murine leukemia virus; UCR, upstream conserved region. (B) Oligonucleotide competition. Labeled CRU was incubated with Hep3B nuclear extracts in gel retardation assays. Specific DNA-protein interactions were inhibited with a 100-fold molar excess of oligo-G (lane 2), oligo-mG_3 (lane 3), and YY1-binding sequence (lane 4) from the α-actin promoter (29). (C) Supershift of complexes C_1, C_2, and C_3 by anti-YY1 antibodies. Hep3B nuclear extracts were incubated with labeled CRU before the addition of preimmune antibodies (preimmune) or polyclonal anti-YY1 antibodies (anti-YY1). Specific DNA-protein complexes are indicated. The supershift caused by anti-YY1 antibodies is indicated by the arrowhead.

binding sequences, and thus they represent the intact (C_3) and the degraded (C_1 and C_2) forms of YY1.

Lastly, specific antibodies against YY1 were used in gel retardation assays to unequivocally confirm that the protein in complexes C_1, C_2, and C_3 is indeed YY1. As shown in Fig. 2C, while preimmune serum had no effect on DNA-protein complex formation, anti-YY1 antibodies specifically recognized and diminished the formation of complexes C_1, C_2, and C_3 from nuclear extracts. In addition, a supershifted complex was observed in the presence of anti-YY1 antibodies. In contrast, the DNA–NF-κB and DNA-C/EBP complexes were not affected by the antibodies. These results indicated that the proteins in complexes C_1, C_2, and C_3 and YY1 have the same antigenicity. From these results, we concluded that the proteins in these three complexes represent YY1 and degraded forms of YY1 and that they bind to the CRU in a highly sequence-specific manner.

YY1 functions as a repressor on SAA1 promoter. Because of the ability of YY1 to repress or activate transcription of many mammalian and viral genes (9, 15, 18, 29, 42, 55), we were intrigued with its effects on SAA1 transcription. To test the functional role of YY1, SAA1 promoter constructs containing site-specific mutations at the YY1-binding site were examined in transient-transfection assays. Three reporter constructs were generated: a wild-type construct, pSAA1(−117), and two mutant constructs, pSAA1(−117mG_2) and pSAA1(−110mG_2), which contained three base-pair mutations in the YY1-binding site (Fig. 3A). The effects of these mutations were first analyzed by binding assays. Wild-type and mutated CRU DNA fragments were used as probes in gel retardation assays. As shown in Fig. 3A, compared with the wild-type CRU, the mutated CRU lost YY1-binding activity completely. When transfected into Hep3B cells, the wild-type construct pSAA1 (−117) exhibited the expression pattern characteristic of the SAA1 promoter: very low activity under basal conditions but highly inducible by cytokine treatment (Fig. 3B) (31, 32). However, the pSAA1(−117mG_2) and pSAA1(−110mG_2) constructs consistently showed two- to fourfold the basal expression level of the wild-type construct. Furthermore, their induced activities were also increased, though to a lesser extent (Fig. 3B). Thus, mutations that prevented YY1 binding actually resulted in higher promoter activity. These results are consistent with a derepression model for the regulation of the SAA1 promoter: YY1 functions as a repressor on the SAA1 promoter, and the extent of repression correlates with DNA-binding activity.

To further confirm that YY1 exerts a negative effect on SAA1 expression, we tested directly whether overexpression of YY1 could repress in *trans* the SAA1 promoter in cotransfection assays. The YY1 expression plasmid was cotransfected into Hep3B cells with three different SAA1 reporter constructs containing an intact YY1-binding site, pSAA1(−301) or pSAA1 (−117), or a mutated YY1-binding site, pSAA1(−117mG_2). As shown in Fig. 4, compared with the vector control, overexpression of YY1 specifically diminished both basal and cytokine-induced CAT expression to 27 and 28% for the pSAA1 (−301) and pSAA1(−117) constructs, respectively. As an additional control, an expression plasmid containing YY1 sequence in the reverse orientation (YY1R) was included in the cotransfection assays and showed no effect on CAT expression (Fig. 4).

To examine whether YY1 exerts its repression through its binding site, the reporter construct containing the mutated binding site was tested. As shown in Fig. 4, the basal activity of pSAA1(−117mG_2) was not affected when it was cotransfected with the YY1 expression plasmid, indicating that the repression by YY1 occurred primarily through the YY1-binding site. Intriguingly, the cytokine-induced activity of pSAA1(−117mG_2) was reduced to 54% when it was cotransfected with the YY1 expression plasmid (Fig. 4) despite the absence of YY1-binding activity. Further sequence analysis did not reveal additional YY1-binding sites in the SAA1 promoter.

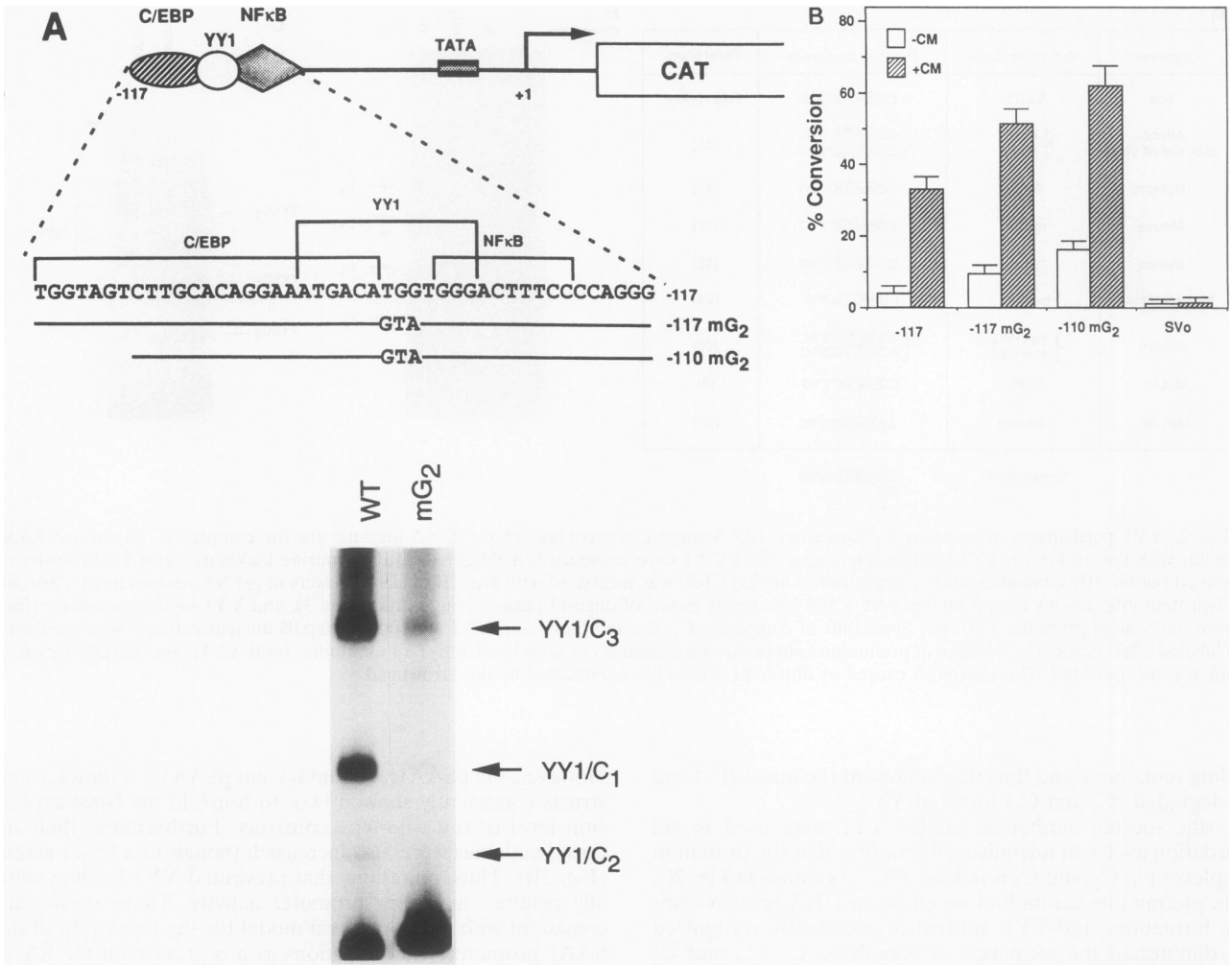

FIG. 3. Mutation of YY1-binding site derepresses SAA1 promoter activity. (A) Schematic of CAT constructs containing the wild-type or mutated YY1-binding site. The nucleotide sequences spanning the C/EBP-, YY1-, and NF-κB-binding sites are shown. Mutated nucleotides in two mutant constructs, pSAA1($-117mG_2$) and pSAA1($-110mG_2$), are indicated. Below the schematic are shown gel mobility shift assays with labeled CRU containing the wild-type or mutated YY1-binding site. Hep3B nuclear extracts (15 μg) prepared from nonstimulated cells by the modified procedure (Materials and Methods) were used. (B) Effect of YY1-binding site mutation on SAA1 promoter activity. Hep3B cells were transiently transfected with reporter constructs containing the wild-type or mutated YY1-binding site as described for panel A. The pSVoCAT vector (SVo) was included as a control. Approximately 24 h after transfection, cells were treated with basal medium (−CM) or 50% CM (+CM). Protein concentration and CAT activity were determined after 18 h of stimulation. CAT activities were quantified by liquid scintillation spectrometry. Error bars represent 1 standard deviation.

Apparently, induced SAA1 expression can be repressed in *trans* by YY1 through a mechanism independent of DNA binding.

NF-κB and YY1 compete for overlapping binding sites in the CRU. Transient-transfection studies suggested that YY1 functions as a repressor on the SAA1 promoter, exerting repressive effects not only on its basal transcription but also on cytokine-mediated transcription activation. Furthermore, methylation interference experiments showed that YY1 and NF-κB apparently have overlapping binding sites on the CRU (Fig. 1B). Taken together, these results raised the interesting possibility that the regulatory mechanism for SAA1 transcription involves a reversible blockage in which YY1 exerts its negative effects by interfering with NF-κB binding to the CRU. Conversely, transcription activation by NF-κB can be achieved by displacing YY1 from the CRU. Thus, the state of transcription repression or activation may depend on the relative binding efficiencies of YY1 and NF-κB. To test this hypothesis, gel retardation assays were performed under probe-limiting conditions to allow YY1 and NF-κB to compete for limited amounts of target DNA. As shown in Fig. 5A, when the amount of NF-κB p50 subunit was increased, its binding activity was correspondingly elevated to high levels, whereas YY1-binding activity was concomitantly reduced. The lack of intermediate-size or larger DNA-protein complexes argues against simultaneous binding of NF-κB and YY1 to the CRU. Instead, these results indicate that NF-κB and YY1 bind to the CRU in a mutually exclusive manner. To demonstrate that this competitive displacement is DNA template specific and also to exclude potential nonspecific protein-protein interactions, specific oligonucleotides were used in competition experiments (Fig. 5B). Addition of oligonucleotides containing the NF-κB-binding site specifically abolished formation of the p50-DNA complex and concomitantly increased the level of the YY1-

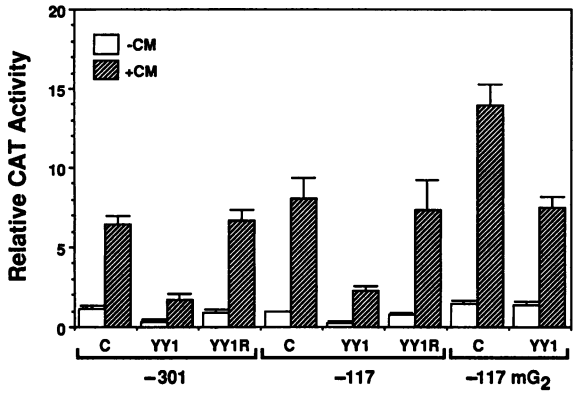

FIG. 4. Overexpression of YY1 represses basal and cytokine-induced SAA1 promoter activities. Reporter constructs pSAA1(−301), pSAA1(−117), and pSAA1(−117mG$_2$) were cotransfected with pSVK3 (C), pSVK3/YY1 (YY1), or pSVK3/YY1R (YY1R) into Hep3B cells as indicated. The molar ratio of cotransfected reporter and effector plasmids was 1:2. Transfected cells were incubated with basal medium (−CM) or 50% CM (+CM) and harvested 18 h later for the CAT assay. CAT activities were calculated relative to that of the pSAA1(−117) construct cotransfected with pSVK3, to which a value of 1.0 was assigned. Error bars represent 1 standard deviation.

DNA complex (Fig. 5B, compare lanes 2 and 3). Similarly, addition of YY1-binding oligonucleotides abolished formation of the YY1-DNA complex and increased the NF-κB–DNA complex (Fig. 5B, compare lanes 4 and 5). Thus, NF-κB and YY1 compete for overlapping binding sites, resulting in their mutually exclusive binding to the CRU.

YY1-binding activity is unaffected by cytokine treatment. Expression of SAA1 in liver cells is upregulated following induction by cytokines (31, 32). Levels of C/EBP and NF-κB are dramatically induced upon cytokine treatment and are required for SAA1 gene activation (31, 32). Because our data suggested that YY1 may antagonize the transactivating function of NF-κB, we sought to examine the effect of cytokines on YY1-binding activity. Hep3B whole-cell extracts from uninduced cultures and ones extracted at various times after cytokine treatment were prepared and used in gel mobility shift assays to determine changes in YY1-binding activities (Fig. 6A). NF-κB- and C/EBP-binding activities were also obtained for comparison. Unexpectedly, YY1-binding activity was not significantly changed following cytokine treatment for up to 48 h (Fig. 6A). In contrast, C/EBP- and NF-κB-binding activities were highly induced (Fig. 6A). In control cells, NF-κB-binding activity was detected at very low levels. However, cytokine treatment induced distinct biphasic kinetics of NF-κB induction. The first wave of induction was readily detectable within minutes and reached a maximum level in 30 min. The second wave peaked at a lower level in 4 h. This biphasic pattern of NF-κB induction has been reported for T cells stimulated by phorbol ester (40). Induction of C/EBP-binding activity, on the other hand, was more gradual, accumulating to its highest level at 6 h (Fig. 6A).

The gel retardation results for NF-κB, C/EBP, and YY1 following cytokine treatment were then quantified (Fig. 6B). Following cytokine stimulation, although the YY1-binding activity was unaffected, the ratios of NF-κB- and C/EBP- to YY1-binding activities were highly increased. At 30 min and 4 h posttreatment, the ratios of NF-κB- to YY1-binding activities were increased by 21- and 8-fold, respectively, while the ratios of C/EBP- to YY1-binding activities were elevated 6-fold.

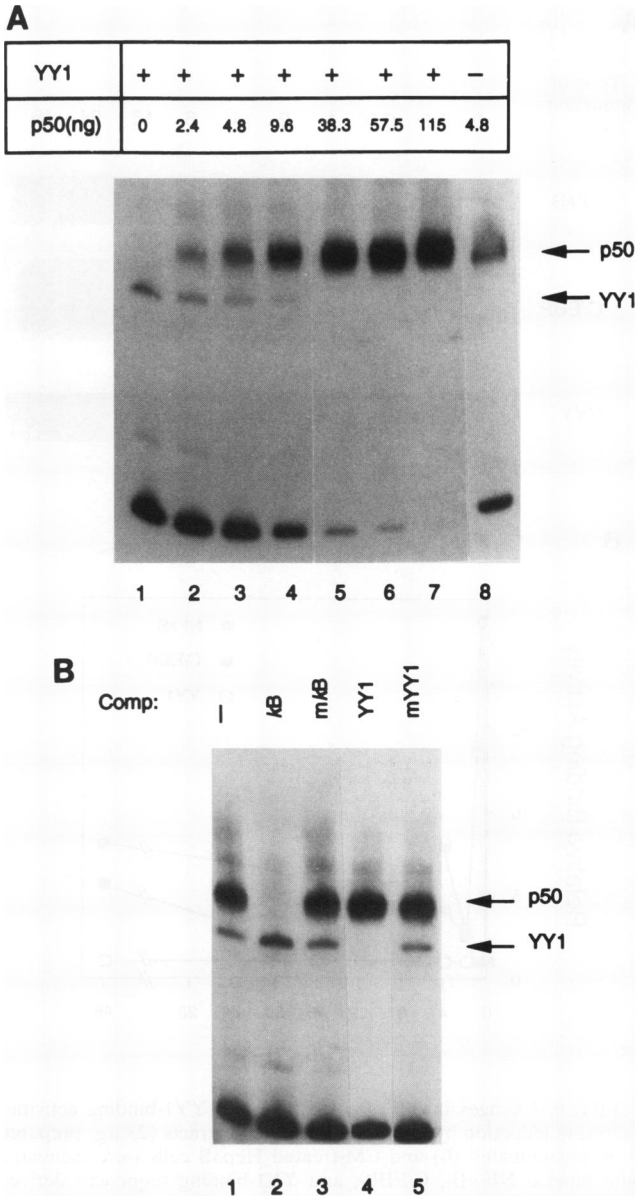

FIG. 5. Mutually exclusive binding of NF-κB and YY1 to the CRU. Gel retardation assays were performed with labeled CRU in probe-limiting conditions (3×10^3 cpm, 100 pg). (A) Displacement of YY1 from the CRU by NF-κB. The indicated amounts of recombinant NF-κB p50 subunit (Promega) were added to constant amounts (25 μg) of Hep3B whole-cell extracts, which served as the source of YY1 and DNA probes. The Hep3B whole-cell extract was omitted in lane 8. Specific protein-DNA complexes are indicated by arrows. (B) NF-κB and YY1 compete for binding to the CRU in a template-specific manner. NF-κB p50 (9.6 ng) and Hep3B whole-cell extract (25 μg) were incubated with labeled CRU in the absence (lane 1) or presence (lanes 2 to 5) of competitors. Oligonucleotides corresponding to NF-κB (κB), mutated NFκB (mκB), and YY1 (YY1) binding sites were used as competitors. Oligo-mG$_3$ was used as a mutated YY1-binding site competitor (mYY1).

Therefore, in the induced state, despite the continuous presence of YY1-binding activity, the highly induced transactivator NF-κB and C/EBP may nevertheless override the repressive effects of YY1.

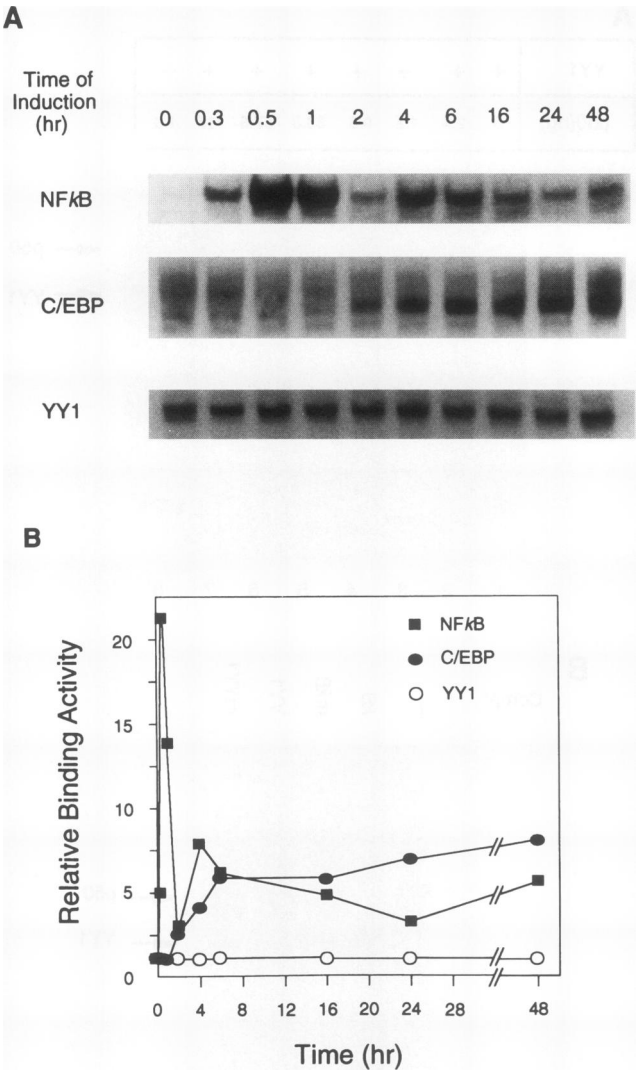

FIG. 6. Changes in NF-κB-, C/EBP-, and YY1-binding activities following induction by CM. (A) Whole-cell extracts (23 μg) prepared from unstimulated (0) and CM-treated Hep3B cells were incubated with labeled NF-κB-, C/EBP-, and YY1-binding sequences derived from the CRU. Gel retardation assays were performed as described in Materials and Methods. (B) Quantitation of NF-κB-, C/EBP-, and YY1-binding activities. Binding activities were quantified with an ImageQuant (Molecular Dynamics) and normalized to those of unstimulated cells, to which a value of 1.0 was assigned.

DISCUSSION

Previous studies on the rat SAA1 promoter have shown that the 65-bp CRU spanning positions −135 to −71 could confer cytokine responsiveness on a heterologous promoter. The induction of SAA1 by cytokines apparently depends on the synergistic interaction between NF-κB- and C/EBP-binding sites within the CRU. In this report, we identified a third distinct transcription factor that also interacts with the CRU in a sequence-specific manner. We demonstrated that this factor is YY1 by its binding site specificity, gel mobility, dependence on zinc for DNA-binding activity, ubiquity of expression, functional role, and immunological cross-reactivity. Methylation interference and protein competition experiments indicated that YY1 and NF-κB had overlapping binding sites and that the binding of one factor interfered with the binding of the other. Consistent with the notion that YY1 plays a repressive role in SAA1 expression, site-specific mutations that abolished YY1 binding elevated the basal and cytokine-induced activities of the SAA1 promoter. Moreover, overexpression of YY1 specifically reduced SAA1 expression in transient-cotransfection assays. Therefore, YY1 functions as a negative regulator, repressing the SAA1 promoter through competition with NF-κB for overlapping binding sites.

On the basis of these findings, we propose a model depicting the transcriptional repression and activation of the rat SAA1 gene in control cells and in response to cytokine induction (Fig. 7). Activation of the rat SAA1 promoter is achieved through combinatorial changes in the binding activities of the cytokine-induced NF-κB and C/EBP transactivators and the ubiquitously expressed YY1 repressor. In noninduced cells, NF-κB exists in a latent form and is retained in the cytoplasm (3, 4). On the other hand, YY1 is found in the nucleus, capable of binding to the CRU. Thus, YY1 exerts its repressive effects on the SAA1 promoter, contributing to the very low basal activity (Fig. 7A). The mechanism by which YY1 represses promoter activity is not clear. It may interfere with the binding of nonspecific activators to the promoter. Alternatively, it may interact with basic transcription machinery and prevent random transcription initiation. In response to cytokine stimulation, the binding activities of nuclear NF-κB and C/EBP are greatly elevated, while that of YY1 remains constant. Increased NF-κB- and C/EBP-binding activities displace YY1 from the CRU, converting the SAA1 promoter from a transcriptionally silent state to one that is highly active (Fig. 7B). At the end of the acute-phase response, NF-κB- and C/EBP-binding activities return to basal levels, allowing YY1 to reoccupy its binding site and shut off SAA1 transcription. The ability of YY1 to reoccupy the CRU may contribute to the transiency of SAA1 expression. Mutations that abolish YY1 binding result in derepression of the SAA1 promoter (Fig. 7C). Because NF-κB and C/EBP transactivators are not activated in nonstimulated cells, this derepression only elevates the promoter activity two- to fourfold. Subsequent stimulation with cytokines further increases promoter activity to levels that are higher than in the controls (Fig. 7D), presumably because of the absence of a YY1-binding site for YY1 to compete with NF-κB for DNA binding. Therefore, the expression of SAA1 is strictly regulated by the interplay between repressor and activators to ensure that it is expressed under appropriate physiological conditions.

YY1 is a multifunctional transcription factor that plays a key role in the expression of many mammalian and viral genes. It has been variously shown to exert positive, negative, or even no apparent effects, depending on the promoter (15, 17, 18, 42, 50, 55). Although the diverse effects of YY1 remain to be analyzed, our studies indicated that one of the mechanisms by which YY1 represses SAA1 transcription is through competition with the transcription activator NF-κB for overlapping binding sites. Interestingly, YY1 has also been shown to exert negative effects on other gene regulatory elements by competing for overlapping binding sites with transactivators. In the α-actin promoter, the YY1-binding sequence overlaps that of the serum response factor, resulting in their mutually exclusive binding to the serum response element. The serum response factor is required for muscle-specific transcription of the α-actin gene; thus, YY1 acts as a repressor for the α-actin promoter (15, 28, 29). Similarly, in the ε-globin gene silencer, YY1 attenuates ε-globin transcription by competing with the erythroid regulatory protein GATA-1 for overlapping sites, leading to the suppression of ε-globin gene expression during

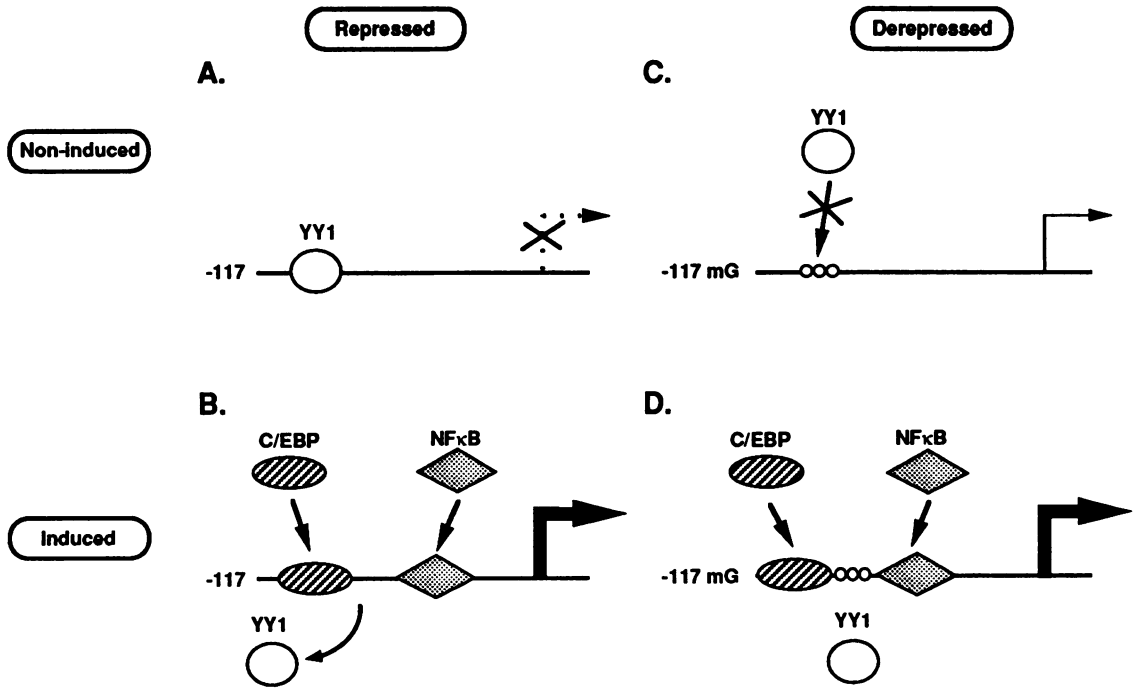

FIG. 7. Model for transcriptional repression and activation through the interplay of YY1, NF-κB, and C/EBP. A two-stage occupancy model (A and B) is proposed for the rat SAA1 promoter. (A) In noninduced cells, YY1 functions as a transcription repressor contributing to the low level of SAA1 basal expression. (B) Upon cytokine induction, elevated C/EBP- and NF-κB-binding activities not only displace YY1 or prevent YY1 from binding, but also transactivate the SAA1 promoter, thus converting the promoter from a transcriptionally silent state to one that is highly active. Mutation of the YY1-binding site results in the derepression of the SAA1 promoter in control cells (C) and even greater response in cytokine-stimulated cells (D). Potential protein-protein interactions of YY1, NF-κB, and C/EBP with each other or with transcription factors in the initiation complex are not shown.

the globin switch (44). Finally, Raught et al. (47), working on the hormonal regulation of β-casein gene transcription, reported that YY1 competes with a lactation-associated complex for overlapping binding sites and represses the β-casein promoter. Thus, competing with distinct transcription activators for overlapping *cis*-acting elements is one mechanism by which YY1 represses transcription. It is noteworthy that in contrast to SAA1 gene transcription, which is transiently induced in response to acute inflammation, the expression of the α-actin, ε-globin, and β-casein genes is developmentally regulated and has more long-term effects. Interestingly, our binding studies showed that the YY1-binding site in the CRU is a much weaker site than that in the serum response element of the α-actin promoter (unpublished results). The weak YY1-binding site in the CRU may allow inflammation-induced NF-κB to displace YY1 more readily, resulting in more efficient activation of the SAA1 promoter. Thus, a weak YY1-binding site in the SAA1 promoter may be a necessity for SAA1 induction, whereas a strong YY1-binding site is necessary for more long-term repression of the α-actin gene in myoblasts.

The results from our cotransfection studies also indicated that the interplay between YY1, NF-κB, and C/EBP modulates SAA1 gene expression. Overexpression of YY1 reduces basal SAA1 expression (Fig. 4), perhaps by decreasing random transcription or by interacting with components in the basic transcription machinery. In stimulated cells, overexpressed YY1 effectively lowers the NF-κB/YY1 ratio, thus reducing the level of induction. Intriguingly, a truncated YY1 containing intact DNA-binding domains was much less effective in repressing the SAA1 promoter (unpublished results). Therefore, DNA binding alone does not account for the full repressor activity; additional domains may be required for full suppression (18, 55). Such domains may influence YY1-DNA binding affinity. Alternatively, they may exert a repressive influence through interaction with transactivators or the general transcriptional machinery. Consistent with this proposal is that in our cotransfection studies, when the YY1-binding site was mutated, YY1 still exerted some negative effects on the SAA1 promoter, albeit to a lesser degree. This repression, however, is not observed for the basal expression, arguing against nonspecific squelching effects. Thus, YY1 may exert its repressive effects through interference with a cytokine-induced transcription factor(s). The most obvious candidates for this quenching effect are NF-κB and C/EBP, which are essential for the induction of the SAA1 gene. Alternatively, components of the transcription initiation complexes that are employed only for induced SAA1 transcription may also be targets for YY1. The finding that YY1 can function without direct binding to DNA was also observed for the c-*myc* promoter; YY1 activated the c-*myc* promoter despite the absence of YY1-binding sites (50). Thus, YY1-protein interactions are distinct possibilities; in vitro and in vivo protein association experiments should establish their existence (56, 61).

With regards to YY1 expression, Lee et al. (28, 29) reported that YY1-binding activity is abundant in replicating myoblasts and diminishes during myogenesis, correlating well with its repressive effects on the α-actin promoter and the pattern of α-actin expression. Thus, the binding activity of this ubiquitous factor may be modulated in response to developmental cues. However, in the immunoglobulin κ gene system, where YY1

serves as a repressor, YY1-binding activity is not altered at a developmental stage when the immunoglobulin gene is active (42). Similarly, our studies showed that YY1-binding activity is nearly constant regardless of the state of SAA1 transcription (Fig. 6A). Therefore, for SAA1 expression, it is the ratio of YY1 to the transactivators (NF-κB and C/EBP) that determines its transcriptional activity rather than the absolute level of YY1.

We show here that increasing amounts of NF-κB can displace YY1 from the CRU. However, given the transcriptional synergism between NF-κB and C/EBP (32, 58), it remains possible that in vivo, both NF-κB and C/EBP are required to efficiently displace YY1. This cooperative displacement may involve protein-protein interactions between these activators and the repressor. Interestingly, two recent studies (7, 26) demonstrated a functional and physical association between the ATF bZIP proteins and NF-κB. Thus, cross-family interaction between Rel family and bZIP family proteins, in which one transcription factor can modulate the activity of another by direct physical interaction, may constitute a novel mechanism for regulating the potent transcription factor NF-κB. Since NF-κB can respond to a variety of stimulating agents in many cell types and serve as a potent transcriptional activator for many inducible genes (3, 4), its interactions with C/EBP and YY1 may represent an additional strategy to ensure that the acute-phase mediators can cause the specific induction of SAA through NF-κB-activating pathways.

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