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Cooperative effects of C/EBP-like and NFκB-like binding sites on rat serum amyloid A1 gene expression in liver cells

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ABSTRACT

Serum amyloid A (SAA) is a major acute-phase protein synthesized and secreted mainly by the liver. In response to inflammation, its expression is increased by 1000-fold, primarily because of a 200-fold increase in the rates of SAA gene transcription. We have shown that when 304 bp of 5′ flanking region of the rat SAA1 gene is fused to a reporter gene, the chloramphenicol acetyltransferase (CAT) gene, CAT activity is induced in a cell-specific manner in response to conditioned media prepared from activated mixed lymphocyte cultures and recombinant interleukin-1. In this study, deletion of the SAA1 promoter to −120 bp with respect to the transcriptional start site did not diminish promoter activity; however, deletion to −94 bp renders the promoter completely inactive. Functional analysis have demonstrated that a 66-bp DNA fragment spanning −138 bp to −73 bp could confer cytokine responsiveness to a heterologous thymidine kinase promoter. Within this 66-bp responsive element resided an NFκB-like-binding site and a C/EBP-like-binding site. Although each binding site alone could confer responsiveness when stimulated with conditioned media and TPA, the response was much weaker than that observed when both sites were present. Moreover, site-specific mutations of either binding site completely abolished SAA1 promoter activity. Taken together, these results suggest a functional importance for and cooperative interaction of these two nuclear-factor binding sites in the cytokine-induced expression of the rat SAA1 gene.

INTRODUCTION

Acute systemic injury or inflammation in mammals initiates physiological processes termed the acute-phase response (1). One of the remarkable phenomena in this response is the accumulation of high levels of plasma proteins present at very low or undetectable levels before induction (1). Serum amyloid A (SAA), one of the major acute-phase proteins, is synthesized and secreted primarily by the liver (2,3). Its plasma concentration rises from 0.5 μg/ml to greater than 1 mg/ml 24 h after injection of bacterial lipopolysaccharide (4,5,6). This large increase in hepatic SAA synthesis is primarily a consequence of increased production of SAA mRNA at the transcriptional level (7). In cultures of primary hepatocytes and hepatoma cells, SAA gene expression can be induced by inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), which are produced by activated macrophages during the acute-phase response (1,8,9). Thus, the tissue-specific induction of SAA gene expression by inflammatory cytokines provides an informative model for studying cell-specific expression and differential gene expression in response to specific stimuli.

In mice, the SAA gene family consists of three genes (SAA1, 2, and 3) and a pseudogene (10). Two genes, SAA1 and SAA2, have 96% sequence homology over a region that includes the four exons, introns, and 5′- and 3′-flanking DNA sequences. The third SAA gene, SAA3, diverges substantially from the other two genes; it is up to 70% homologous in the translated exons but less than 25% homologous in the introns and the 5′- and 3′-flanking DNA sequences. During the acute-phase response, liver SAA mRNA increases approximately 1,000-fold, each SAA gene contributing an equal proportion (7). This large increase in SAA mRNA is mediated by a 200-fold increase in the rate of transcription from each of the three SAA genes (7).

Prolonged elevation of serum SAA, as in chronic inflammation, is often associated with the deposition of amyloid fibrils in various organs, leading to secondary amyloidosis (11,12). While the pathology of secondary amyloidosis has been well-studied in both humans and mice, amyloidosis has never been observed in rats, even after prolonged inflammation. In fact, even the SAA protein has not yet been observed in the rat. Thus, the rat appeared to be an exception in regard to SAA expression. However, recent reports demonstrated that rats express two distinct SAA-related mRNA species following inflammation (13). Furthermore, the pattern of SAA mRNA species expressed among various tissues and the level of induction by inflammatory agents are similar to those of mouse SAA mRNAs. Finally, characterization of a

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rat SAA genomic clone containing the promoter region revealed a high sequence homology to the mouse SAA1 gene (14). Thus, the SAA gene family is present and expressed in rats, and its expression is induced much like it is in mice.

To understand the molecular mechanisms of SAA gene regulation, we (14,15,16) and others (17) have analyzed the 5'-flanking regions of the SAA gene from human, mouse, and rat. Analysis of the mouse SAA3 promoter revealed two adjacent C/EBP-binding sites in the proximal region that enhanced basal expression in a liver cell-specific manner (16). In addition, a distal 68-bp region conferred responsiveness to both conditioned media (CM) prepared from activated mixed lymphocytes and recombinant IL-1 (15). In the 5'-flanking region of the human SAA1 gene, Edbrooke *et al.* (17) identified a phorbol ester response element with sequence identity to the NFκB-binding site. A NFκB-binding site was also found in the rat SAA1 gene promoter at approximately the same position relative to the transcriptional start site (14) as the human SAA1 promoter. In addition, four DNA regions interacting with nuclear factors were mapped in the rat SAA1 promoter immediately upstream from the NFκB-binding site. Two of these regions were identified as binding sites for C/EBP or C/EBP-related factors, with the distal binding site having an approximately 10-fold higher binding affinity (14).

In this study, we further studied these nuclear protein-binding sites in the rat SAA1 promoter by examining their role in the differential expression of a reporter gene in response to inflammatory cytokines. Functional analysis showed that both the NFκB-like-binding site and the proximal C/EBP-like-binding site conferred responsiveness to CM and 12-0-tetradecanoyl-phorbol-13-acetate (TPA); however, only the NFκB-like-binding site was responsive to IL-1 and TNF, while the C/EBP-like-binding site was nonresponsive. This result suggests that cytokines other than IL-1 and TNF in CM may function through this C/EBP-like-binding site. Site-specific mutation of either the proximal C/EBP-like-binding site or the NFκB-like-binding site abolished the SAA1 promoter activity completely, suggesting a functional importance and cooperative interaction of these two nuclear-factor binding sites in the cytokine-induced expression of the rat SAA1 gene.

MATERIALS AND METHODS

Cell lines

HepG2, Hep3B, and HeLa cells were cultured in basal medium consisting of modified Eagle's medium and Waymouth MAB (3:1, v/v, pH 7.0) plus 10% fetal calf serum (15,18) and were passaged at confluence approximately once a week by trypsinization.

Conditioned medium, cytokines, and phorbol ester

CM was prepared from activated mixed lymphocyte culture according to the method of Maizel *et al.* (19) as described by Huang *et al.* (15) and was used as a mixture with an equal volume of basal medium. Purified recombinant human cytokines IL-1α and TNF-α were studied separately by adding each cytokine to culture medium (mixture of equal volume of control medium and basal medium) to investigate the effect of each specific factor on the induction of acute-phase gene expression. IL-1 (specific activity, 1.2×10^8 U/mg) was generously provided by P.T. Lomedico (Hoffman-LaRoche, NJ). TNF-α was a gift from B.Aggarwal (M.D. Anderson Cancer Center, TX). A phorbol ester, TPA, purchased from Sigma (St. Louis, MO) was also added, at 50 ng/ml concentration, to test its effect on cytokine-induced expression.

Plasmid constructions

pSAA1/CAT (−304) was constructed by inserting a DNA fragment of the rat SAA1 gene containing 304 bp of 5' flanking sequence and 18 bp of the exon 1 untranslated region into the *Sma*I site of pSVoCAT vector (14). This DNA fragment was synthesized by the polymerase chain reaction using a genomic clone containing the *Pst*I (−1.3 kb) to *Hind*III (+490) restriction fragment as a template, and oligonucleotides OL-100 (−304 to −288 bp of the coding strand) and OL-101 (+18 to +2 bp of the noncoding strand) as primers. Three 5' deletion mutants, pSAA1/CAT (−198), pSAA1/CAT (−120), and pSAA1/CAT (−94) were constructed similarly, except oligonucleotides OL-III (−198 to −168 bp), OL-I (−120 to −96 bp), and OL-κB (−94 to −77 bp), respectively, were used instead of OL-100 in the polymerase chain reaction. Two site-specific mutants, pSAA1/CAT (−120 mI) and pSAA1/CAT (−120 mκB), with mutations at site I and the NFκB site, respectively, were constructed using OL-mI (−120 to −91 bp) and OL-ImκB (−120 to −76 bp) as primers in the polymerase chain reaction. Specific sites of mutation are indicated in Figure 6. All constructs were identified by gel electrophoresis and verified by DNA sequencing (20).

To test the function of each footprint region, double-stranded oligonucleotides corresponding to each of the four footprint regions and the NFκB site were synthesized and inserted into the *Bam*HI site of the pBLCAT vector (21). pTK/mI was constructed similarly by inserting OL-mI into the *Bam*HI site. Plasmid DNA containing each specific insert was verified by examining the size of the fragment after restriction digestion, and the orientation and copy number were determined by dideoxy sequencing using a primer derived from the TK promoter.

Transient transfection assay

Cells were seeded at a density of 5 to 10×10^5 cells per 100-mm culture dish and incubated at 37°C for 16 to 24 h before transfection with 20 μg of plasmid DNA by the Polybrene procedure (22). Plasmid DNA was co-precipitated with Polybrene (10 mg/ml) in HBS (23.5 mM HEPES, pH 7.05, 140 mM NaCl, 50 mM KCl, 0.7 mM Na_2HPO_4, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) in 1 ml for 20 min at room temperature. The precipitate was then added to the cells. Four to six hours after transfection, cells were washed with Hanks' balanced salt solution, subjected to a 25% glycerol shock for 2 min (23), and incubated for 16 to 20 h before treatment with CM or with purified recombinant cytokines. To elicit the acute-phase response, cells were treated with 50% CM, or with cytokine-containing medium, which consisted of culture medium and one of the human recombinant cytokines. As controls, transfected cells were treated in parallel with culture medium. Cells were harvested 18 to 24 h after treatment.

Chloramphenicol acetyltransferase (CAT) assay

Transfected cells were harvested approximately 36 to 40 h after transfection, washed with phosphate-buffered saline, and resuspended in 100 μl of 250 mM Tris-hydrochloride, pH 7.8. Cell lysates were prepared by freezing (−70°C) and thawing (37°C) for four cycles, and by centrifugation at 12,000 rpm in the microfuge at 4°C for 30 min. Supernatant fluids were

collected and assayed for protein content (24) and CAT activity (25,26). Our standard assay mixture contained 20 to 100 μg of protein, 0.1 μCi of [^{14}C]chloramphenicol (Amersham; specific activity, 50 to 57 mCi/mmol), and 1 mM acetyl coenzyme A in a final reaction volume of 120 μl. The reaction products were extracted with 1 ml of ethyl acetate and chromatographed on silica gel thin-layer chromatography plates using chloroform:methanol (95:5, v/v). CAT activities were quantitated in a liquid scintillation counter by measuring the radioactivity of [^{14}C]chloramphenicol spots corresponding to the acetylated and nonacetylated forms.

Nuclear extracts

Nuclear extracts from Hep3B cells were prepared as described by Shapiro *et al.* (27). Briefly, isolated nuclei were resuspended at a concentration of 10^9 nuclei in 6 ml of final nuclear resuspension buffer (9 volumes of nuclear resuspension buffer and 1 volume of saturated ammonium sulfate; nuclear resuspension buffer consists of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid [HEPES, pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM dithiothreitol [DTT], and 25% glycerol). The resulting suspension was mixed by gentle rocking at 4°C for 30 min. The chromatin was subsequently removed by sedimentation at 150,000×g for 90 min at 2°C. The supernatant was collected, and the proteins were precipitated by the addition of powdered ammonium sulfate (0.33 g/ml) and collected by centrifugation at 85,000×g in a SW28 rotor for 20 min at 2°C. Protein pellets were dissolved in 1 ml of nuclear dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, and 2 mM DTT) per 10^9 nuclei and dialyzed for 4 h at 4°C against 300 ml of nuclear dialysis buffer, with one change of buffer. The dialyzed nuclear extract was then clarified by centrifugation at 10,000×g for 10 min and frozen in small aliquots in dry ice and stored at −80°C.

Electrophoretic mobility shift assay

Reactions for the electrophoretic mobility shift assays were performed in a 20 μl reaction mixture consisting of 12 mM HEPES (pH 7.9), 60 mM KCl, 12% glycerol, 1.2 mM DTT, 0.12 mM EDTA, 4 μg of poly(dI-dC).poly (dI-dC), 1 to 2 μg of Hep3B nuclear protein, and 10^4 cpm (0.25 to 1 ng) of ^{32}P-labeled DNA fragments or double-stranded oligonucleotides. The reaction mixture was preincubated for 5 min at room temperature before the radioactive probe was added; then the reaction mixture was incubated for an additional 20 min at room temperature. Samples were then loaded onto a low-ionic-strength, 6% polyacrylamide gel (19:1 cross-linking ratio) in 0.25×TBE (1×TBE is 89 mM Tris, pH 7.8, 89 mM boric acid, 1 mM EDTA) and subjected to electrophoresis at 200 V for 2 h. The gel was then dried and autoradiographed.

RESULTS

Localization of a cytokine-responsive element in the rat SAA1 promoter

We have previously shown that a 322-bp fragment (spanning −304 to +18 bp) of the rat SAA1 gene was sufficient to confer cytokine-induced expression on the CAT reporter gene in a highly liver-cell-specific manner. While CAT activity in two hepatoma cell lines Hep3B and HepG2 can be induced approximately 20-fold by CM, similar treatment of HeLa cells had no effect on CAT activity (14). Furthermore, when individual recombinant cytokines and TPA were assayed for their ability to induce the expression of pSAA1/CAT (−304), we found that the magnitude of induction by IL-1 and TPA approached that for CM, while TNF, lymphotoxin, and IL-6 had essentially no stimulatory effect.

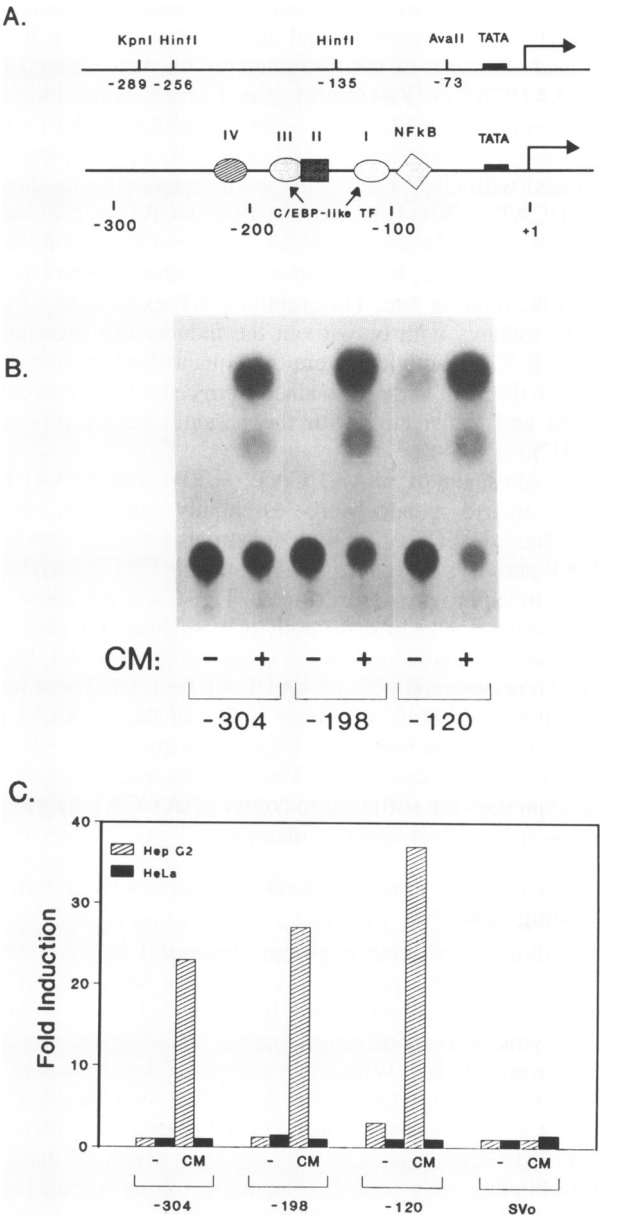

Figure 1. Deletion analysis of the SAA1/CAT fusion gene in cultured cells. Cells were transfected with 20 μg of pSAA1/CAT (−304), pSAA1/CAT (−198), and pSAA1/CAT (−120). pSVoCAT was used as control. Approximately 24 h after transfection, cells were treated with control medium (−) or 50% conditioned medium (CM). Protein concentration and CAT activity were determined after 18 h of stimulation. **(A)** Partial restriction map and schematic diagram of nuclear protein-binding activities interacting with the rat SAA1 promoter (14). Positions of the five specific binding sites (NFκB and footprint regions I-IV) and the TATA motif are indicated. Horizontal arrow indicates the direction of transcription. **(B)** CAT activity of each construct transfected into HepG2 cells. **(C)** Quantitation of CAT activity for each construct in HepG2 and HeLa cells. Results were calculated relative to the CAT activity in the control pSVoCAT− transfected cells, to which a value of 1.0 was assigned. Values represent the means of three independent transfection experiments.

Footprint analysis with the Hep3B nuclear proteins revealed four protected regions (regions I,II,III, and IV) (14). A schematic diagram of these regions is shown in Figure 1A. Two regions were identified by gel shift and oligonucleotide competition experiments as binding sites for C/EBP or C/EBP-related proteins (regions I and III). One additional element shares sequence identity with the NF$\varkappa$B-binding site. To assess the functional importance of these nuclear protein-binding sites, a series of CAT constructs were generated and their responsiveness to CM examined. Portions of the promoter region were deleted from the pSAA1/CAT (−304) hybrid gene. Constructs resulting from these deletions, pSAA1/CAT (−198) and pSAA1/CAT (−120), were transfected into HepG2 and HeLa cells, which in turn were stimulated with CM. The results were compared with those of pSAA1/CAT (−304) (Fig. 1B). In pSAA1/CAT (−198), region IV was deleted, whereas in pSAA1/CAT (−120) regions II, III, and IV were deleted, but the latter still retained region I and the NF$\varkappa$B-like-binding site. Differential CAT expression of these deletion mutants with or without the inducer are presented in Figure 1B. CAT activities were then quantitated and expressed relative to the CAT activity obtained from cells cultured in control medium and transfected with the parental vector pSVoCAT (Fig. 1C).

The expression of pSAA1/CAT (−304) and pSAA1/CAT (−198) hybrid genes were essentially identical. In the unstimulated HepG2 cells, both constructs had very low levels of CAT activity; however, treatment with CM induced CAT activity by approximately 20-fold. The CAT activities were specific to liver cells; no CAT activity was detected in HeLa cells regardless of the construct or the stimulation (Fig. 1C). Similar results were obtained with pSAA1/CAT (−120). These results demonstrate that the 5′ promoter region of the rat SAA1 gene contains at least one regulatory region proximal to bp −120 that confers responsiveness to CM. These results further indicate that these sequences are sufficient to confer SAA1/CAT hybrid gene expression in a liver-specific manner.

Cytokine responsiveness of NF$\varkappa$B-like and C/EBP-like (region I) binding sites

The finding of cytokine response element(s) in the proximal region of the SAA1 promoter is consistent with our previous results in which a 66-bp response fragment (−138 to −73) could confer cytokine responsiveness onto a heterologous thymidine kinase promoter (14). Within this 66-bp response fragment, two protein-binding sites have been identified: one interacts with NF$\varkappa$B or a NF$\varkappa$B-like protein and the other interacts with C/EBP or a C/EBP-related protein. To examine which of these two protein-binding sites contribute to the cytokine responsiveness of the 66-bp cytokine response element, oligonucleotides corresponding to each of these two regions were synthesized and inserted into the *Bam*HI site of pBLCAT vector. Resulting constructs containing single or multiple copies of the binding sites were transfected into HepG2 cells, and the responsiveness to TPA and IL-1 were examined. These constructs were also transfected into HeLa cells, but only the responsiveness to TPA was examined since HeLa cells are known to be refractory to IL-1 treatment. Constructs pTK/$\varkappa$B(1) and pTK/$\varkappa$B(5), containing a single copy and five copies of the NF$\varkappa$B-binding site, respectively, showed greatly elevated basal expression in HepG2 when compared with the parental vector (Fig. 2A). Treatment with TPA or IL-1 had a minimal effect on pTK/$\varkappa$B(1), but pTK/$\varkappa$B(5) was induced 3- to 4-fold in either HepG2 or HeLa cells (Fig. 2A). The low magnitude of induction is in part attributed to the high basal expression of this construct. These results are consistent with the findings of Ron *et al.* (28), in which

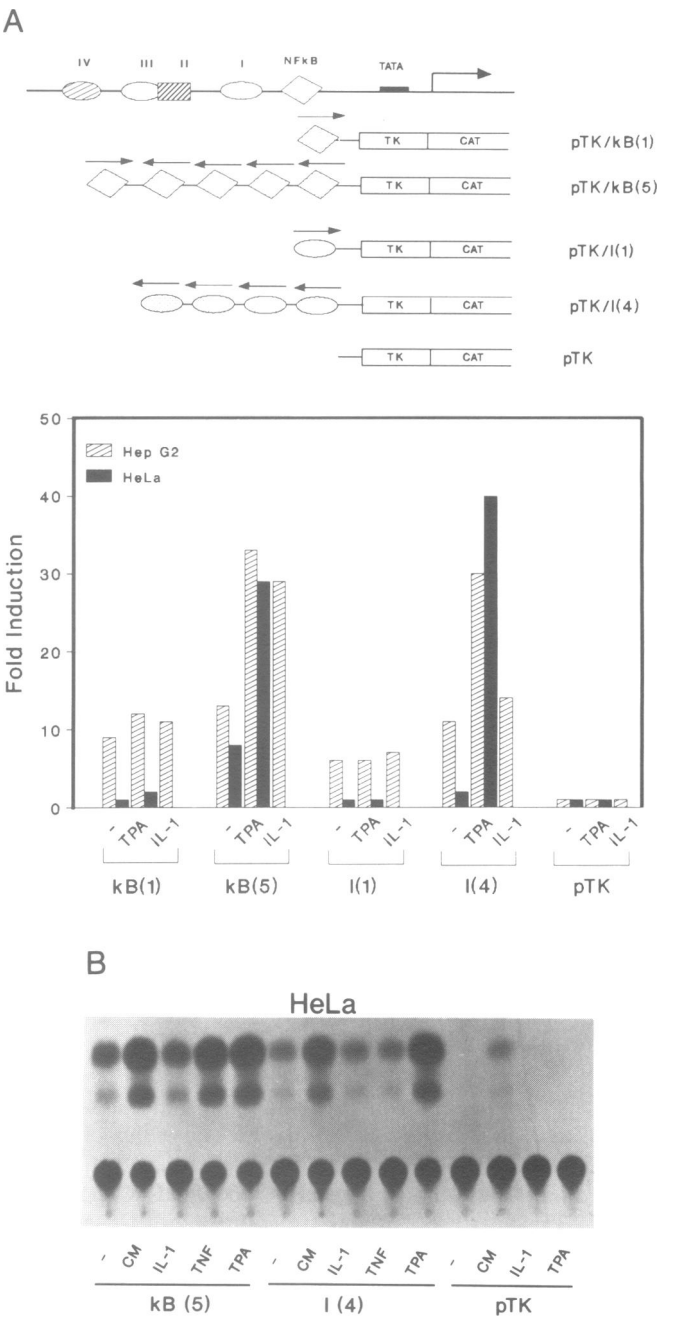

Figure 2. Functional analysis of the NF$\varkappa$B-like site and region I on a heterologous TK promoter. HepG2 and HeLa cells were transfected with 20 μg of pBLCAT vector (pTK) or TK/SAA1 constructs containing the NF$\varkappa$B-like site ($\varkappa$B) or footprint region I (I). Copy number and orientation of inserts in each construct are indicated in the parenthesis and by the arrows, respectively. Transfected cells were treated with control media (−), conditioned media (CM), IL-1 (100 U/ml), TNF (50 ng/ml), or TPA (50 ng/ml). Protein concentration and CAT activity were determined after 18 h of stimulation. **(A)** Schematic of constructs used and quantitation of the CAT activity for each construct in HepG2 and HeLa cells. Results were calculated relative to the control CAT activity for the pBLCAT vector in each cell line, to which a value of 1.0 was assigned. **(B)** Results of the CAT activity of each construct transfected into HeLa cells and stimulated with cytokines or TPA.

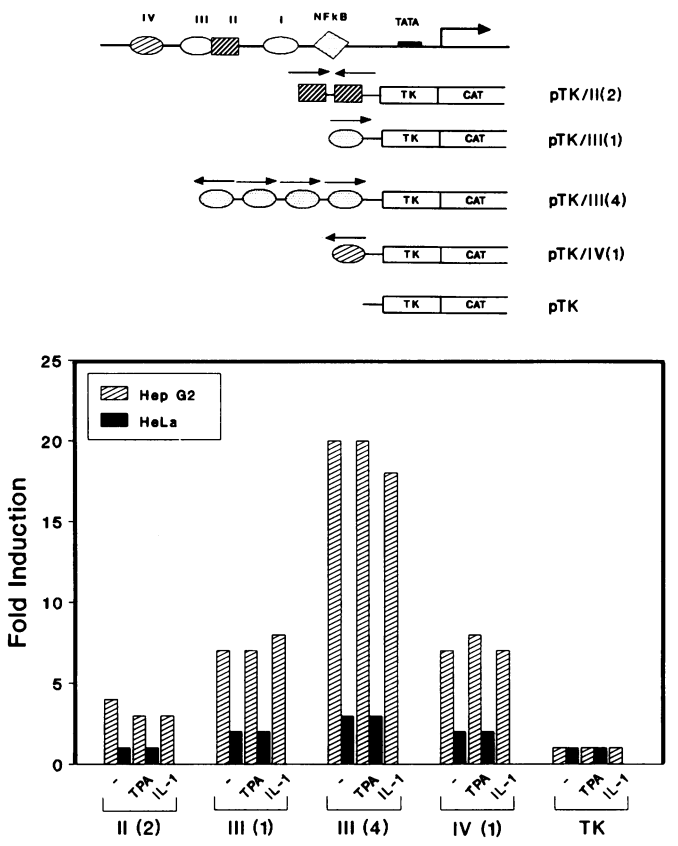

Figure 3. Functional analysis of footprint regions II, III, and IV on TK promoter. TK/SAA1 constructs containing footprint regions II, III, or IV of the rat SAA1 gene were transfected into HepG2 and HeLa cells. Copy number and orientation of inserts in each construct are indicated. Transfected cells were treated with control medium (−), TPA (50 ng/ml), or IL-1 (100 U/ml). Cells were harvested after 18 h of treatment, and CAT activity was determined and quantitated by counting in a scintillation counter. The CAT activity of the parental vector pBLCAT (TK) in HepG2 and HeLa cells without stimulation is given a relative value of 1.0.

addition of a NFκB-binding site from the angiotensinogen gene promoter was shown to elevate basal expression and confer responsiveness to stimulation by TPA and IL-1.

Constructs pTK/I(1) and pTK/I(4), containing one and four copies of footprint region I, respectively, were examined in a similar manner. Surprisingly, the results were similar to those of the pTK/κB constructs (Fig. 2A). Increased basal expression was observed in both pTK/I constructs, but only pTK/I(4) responded to stimulation by TPA. However, it is interesting to note that, unlike pTK/κB(5), the increased basal activity for pTK/I (4) was more evident in liver cells than in HeLa cells. Furthermore, while TPA treatment induced the expression of pTK/I(4) about 2- to 3-fold in HepG2 cells, and 15-fold in HeLa cells, addition of IL-1 did not have significant effect on CAT activity (Fig. 2A). These results clearly indicated that both NFκB-like and C/EBP-like (region I) binding sites, when placed in front of the TK promoter, not only significantly elevated basal CAT expression but also conferred responsiveness to TPA.

The finding that region I, in contrast with the NFκB-binding site, was responsive to TPA but not to IL-1 raises the possibility that this region may be responsive to other inflammatory mediators. To address this issue, transfected HeLa cells were treated with TNF and CM in addition to IL-1 and TPA. While IL-1 and TNF had no effect on CAT expression of pTK/I(4), CM significantly induced CAT expression to a level comparable to that obtained with TPA (Fig. 2B). Since CM also contains IL-6 known to induce the expression of several acute phase genes, we tested the effects of IL-6 on pTK/I(4) construct. The result showed that IL-6 also could not induce CAT expression of pTK/I(4) (data not shown). Therefore, region I is a responsive element that responds to particular cytokines in the CM, excluding IL-1, TNF, and IL-6. The fact that the NFκB-like binding site and the C/EBP-like region may be responsive to different cytokines raises an interesting possibility that these two binding sites are independent response elements that respond through separate mechanisms.

Since both protein binding sites tested were responsive to CM and TPA, we further examined constructs made from the other three footprint regions (II, III, and IV) for their ability to respond to stimulation. Of particular interest is footprint region III because it, like region I, was shown to interact with C/EBP or C/EBP-like proteins. Moreover, the region III-binding site had a >10-fold higher binding affinity than region I. Oligonucleotides corresponding to each of the three regions were inserted 5′ to the TK promoter and the resulting constructs were transfected into HepG2 and HeLa cells. In nonstimulated cells, all three regions conferred higher constitutive activity on the TK promoter (Fig. 3). This effect was more pronounced in HepG2 cells. Indeed, construct pTK/III(4), containing four copies of region III, showed as high as a 20-fold enhancement on the basal activity of the TK promoter in HepG2 cells when compared with the parental vector, a result reminiscent of our previous studies with the C/EBP-binding sites from the mouse SAA3 promoter (16). Stimulation with TPA or IL-1 did not further increase CAT activity. Taken together, these results indicated that while all three regions are able to mediate higher basal activity on the TK promoter, they are not responsive to stimulation by TPA or IL-1. Although both regions I and III conferred elevated basal expression in liver cells, only region I mediated increased CAT activity in response to CM and TPA, further indicating that these two C/EBP-like binding sites are functionally distinct.

Correlation of C/EBP-like protein binding with cytokine responsiveness

To examine whether additional nuclear factors may interact with region I and account for the cytokine responsiveness, a C/EBP-binding site oligomer from the albumin gene (16) was used as a competitor in a gel-shift experiment. As shown in Figure 4B, the protein-DNA complexes formed with the region I probe were efficiently competed by the probe itself and by the C/EBP-binding site from the albumin promoter, while the negative control NFκB-binding site oligomer did not compete. This result suggests that C/EBP or C/EBP-like proteins alone interact with the region I oligomer. To further determine whether the interaction with a C/EBP-like protein would correlate with the CM- and TPA-responsive activity, a mutated region I oligomer (OL-mI) was generated by introducing mutations at six positions within the binding site (Fig. 4A), and the effect of these mutations was analyzed by oligonucleotide competition assay. When the wild-type region I was used as a probe in the gel shift experiment, the protein-DNA complexes formed were specifically competed by OL-I but not competed by OL-ml (Fig. 4C). Furthermore, no protein-DNA complex was detected when OL-mI was used as a probe (data not shown), suggesting that the mutations abolished OL-mI's protein-binding ability.

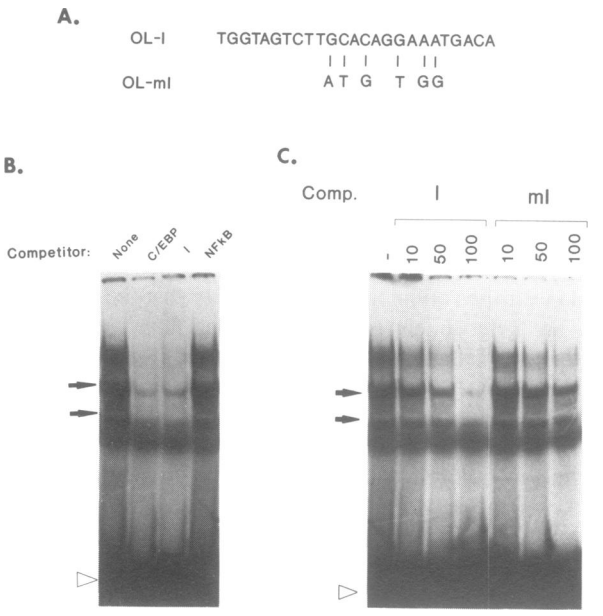

Figure 4. Effect of the mutations in region I on its binding activity. **(A)** Wild-type and mutated region I oligomers, OL-I and OL-mI, respectively, were synthesized as double-stranded oligomers and used in gel-shift assays. **(B)** OL-I oligomer was end labeled and incubated with nuclear extracts (1 μg) from Hep3B cells. DNA-protein complexes were then resolved in a native 6.5% polyacrylamide gel. Competitor oligonucleotide corresponding to the binding site of C/EBP (14) was used at a 100-fold molar excess with respect to the region I probe. The region I and NFκB oligomers were used as specific and nonspecific competitors, respectively. Positions of specific complexes formed are indicated by solid arrows; open arrows indicate positions of free probe. **(C)** Relative efficiency of competition by OL-I and OL-mI oligomers. Gel retardation assay was performed as in (B). Molar excess of the competitors is indicated.

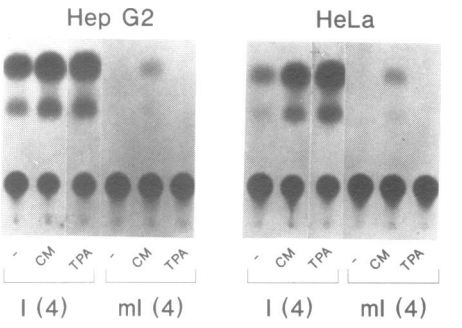

Figure 5. Functional analysis of the effect of mutations in region I. Two constructs, pTK/I(4) and pTK/mI(4), which contain four copies of the wild type and mutated region I sequence, respectively, of the rat SAA1 promoter fused immediately 5′ of the pBLCAT reporter gene were transfected into HepG2 and HeLa cells. Transfected cells were treated with control media (−), conditioned media (CM), or TPA (50 ng/ml). Cells were harvested 18 h later.

To examine whether the loss of the OL-mI oligomers protein-binding activity is correlated with an inability to respond to stimulation, the oligomer was inserted 5′ to the TK promoter and its function analyzed by transient transfection assay. While the CAT activity with the pTK/I(4) construct could be induced by both CM and TPA in HepG2 and HeLa cells, the pTK/mI(4) construct, which contains four copies of mutated region I, did not respond to CM and TPA stimulation, nor did it elevate the basal CAT activity in the control cells (Fig. 5). Taken together, these results show a close correlation between binding activity with a C/EBP-like protein and responsiveness to CM and TPA, suggesting that it was the C/EBP-like binding activity in region I that conferred cytokine responsiveness.

Both NFκB-like and C/EBP-like binding sites are required for promoter activity

Earlier results showed that the two responsive elements, C/EBP-like and NFκB-like sites, are only functional when multimerized (Fig. 2) and yet constructs pSAA1/CAT (−120) and pTK/SAA1 (−138/−73), containing one copy of each element were very responsive to stimulation by CM and TPA (Fig. 1) (14). This raises the possibility that full induction of the rat SAA1 gene is achieved through cooperative interactions between factors binding to these two responsive elements. To test this possibility, two constructs were generated, pSAA1/CAT (−120 mI) and pSAA1/CAT (−120 mκB), with site-specific mutations at region I and NFκB-like-binding site, respectively (Fig. 6). Results of the functional analysis in HepG2 cells of these two mutants were compared with the wild-type construct pSAA1/CAT (−120). Whereas CAT expression of pSAA1/CAT (−120) was induced approximately 20-fold by CM and IL-1, CAT activity was not detectable with pSAA1/CAT (−120 mI), and detected at very low levels with pSAA1/CAT (−120 mκB) following stimulation. This result clearly demonstrates that mutations of either element essentially abolished the inducible promoter activity, indicating that both elements were required for the full cytokine-induced expression of the rat SAA1 gene. It is worth noting that although region I alone was not responsive to IL-1, it was still required for the IL-1 responsiveness of the SAA1 promoter. The requirement of both responsive elements for SAA1 promoter function was further assessed by generating the construct pSAA1/CAT (−94) with the region I deleted, but the NFκB-binding site intact. As shown in Figure 6, this construct responded minimally to stimulation by CM and IL-1. This result further indicated that the NFκB response element alone was not functional in the absence of the region I-binding site and further supports the conclusion that the cytokine-induced expression of the rat SAA1 gene requires cooperative interaction between the NFκB-like and C/EBP-like proteins.

DISCUSSION

During acute inflammation, SAA expression in the liver is greatly increased in response to various inflammatory cytokines. Our previous studies have shown that constructs containing 304 bp of the rat SAA1 promoter region can confer cytokine-induced expression of a reporter gene in a liver-cell specific manner (14). DNase-I protection and gel shift assays have identified five DNA elements within this region that interact with nuclear proteins, including one site interacting with a NFκB-like protein and two sites interacting with C/EBP-related proteins. Our aim in this study was to further characterize these protein-binding sites and to assess their functional importance in the regulation of rat SAA1 gene expression. We showed previously that a 66-bp DNA fragment spanning positions −138 to −73 relative to the transcription start site could, when ligated to a heterologous promoter, confer increased CAT expression after cytokine stimulation. Two *cis*-elements, a NFκB-like and a C/EBP-like-binding sites, reside within this 66-bp DNA fragment. When

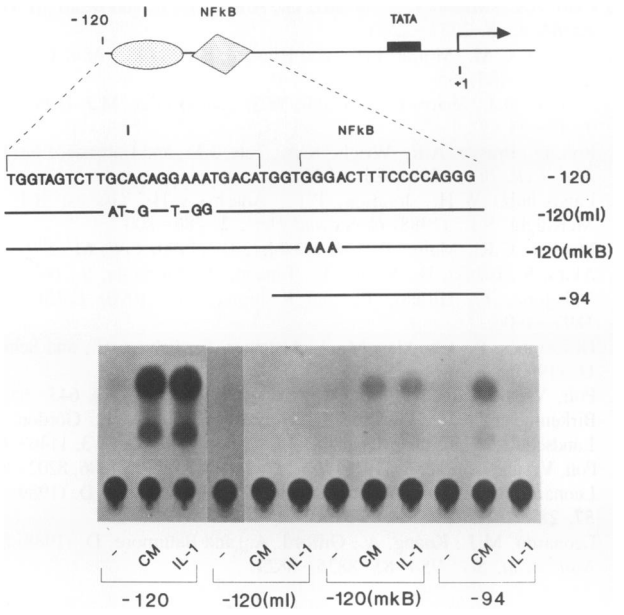

Figure 6. Functional analysis of the site-directed mutations in region I and NFκB-like binding site on rat SAA1 promoter activity. **Top**. Schematic of the immediate 5′ flanking region of the rat SAA1 gene with the nucleotide sequence spanning region I and NFκB-like binding sites shown. Nucleotides that were mutated are indicated. **Bottom.** Two deletion mutants, pSAA1/CAT (−120) and pSAA1/CAT (−94), which contained 120 and 94 bp, respectively, of the rat SAA1 5′ flanking sequences, and two site-directed mutants, pSAA1/CAT (−120 mI) and pSAA1/CAT (−120 mκB), which had mutations in region I and the NFκB-like site, respectively, were transfected into HepG2 cells. Transfected cells were treated with control media (−), conditioned media (CM), or IL-1 (100 U/ml). Cells were harvested 18 h later for CAT assay.

multimerized, each element could confer CM- and TPA-induced expression; however, the NFκB-like element also conferred responsiveness to IL-1 and TNF, while the C/EBP-like element did not. Using site-specific mutagenesis, we also demonstrated that both elements were necessary for the full functional activity of the SAA1 promoter: mutations that affect either response element abolished inducible promoter activity. These results strongly suggest that cooperative interactions between C/EBP- and NFκB-related proteins are necessary for the high rate of SAA gene transcription in response to inflammatory mediators.

Studies by Ramadori and colleagues showed a dose-dependent increase in murine hepatic SAA mRNA after a single injection of IL-1 (8). Their studies also showed that primary cultured murine hepatocytes responded to IL-1 in a dose- and time-dependent manner that could be inhibited by antibodies to IL-1. Using transient transfection assays, we have demonstrated that the expression of both mouse SAA3/CAT (15) and rat SAA1/CAT (14) hybrid genes can be greatly increased in a cell-specific manner by inflammatory cytokines, and that the major cytokine exerting these stimulatory effects in Hep3B and HepG2 cells is IL-1, whereas TNF had only moderate effects. Interestingly, the region I sequence conferred the ability to respond to TPA and CM, expressed as increased CAT activity. However, IL-1 had little effect on CAT activity under these conditions. Moreover, IL-6 and lymphotoxin had no effect, although they are known to have inducing activities on other acute phase genes. Thus, the region I element is responsive to particular cytokines in the CM other than IL-1, suggesting that cytokines besides IL-1 may also play a role in regulating SAA1 expression. At present, we are unclear which cytokine may act via this responsive element.

Region I not only presents an intriguing possibility of identifying other cytokines that regulate SAA1 gene expression, but in combination with region III also allows an interesting comparison between two apparently similar binding sites with different functions. Our earlier studies have demonstrated that both region I and region III sequences can interact with C/EBP or C/EBP-related proteins, with region III having an approximately 10-fold higher affinity for these proteins than region I (14). Functional assays show that while both regions could elevate basal activity of a heterologous TK promoter, region III was more efficient than region I, consistent with its higher C/EBP-binding affinity. With respect to cytokine responsiveness, however, region I was responsive to CM and TPA, whereas region III was not. Consistent with these results are the findings that mutations in the region I sequence completely abolished the cytokine responsiveness of the SAA1 promoter, while deletion of the region III sequence had no comparable effect.

Functional differences between regions I and III might be explained by their interactions with different members of the C/EBP-related proteins. Indeed, a family of C/EBP-related proteins has been described, including C/EBP (29), DBP (30), NF-IL-6 (31), and LAP/IL-6 DBP (32,33). Among these, C/EBP and DBP are abundant in normal livers and function as liver-enriched transcription factors. Both transcription factors can stimulate the transcription of chimeric genes containing the albumin D site in both in vivo and in vitro assays (30,34). In contrast, NF-IL-6 is normally expressed at very low levels in liver, but can be induced to high levels by IL-1. Furthermore, the expression of NF-IL-6 is not restricted to liver. Therefore, instead of being a liver-specific transcription factor, NF-IL-6 may serve as a non-cell-specific transcription factor involved specifically in regulating the expression of IL-1 responsive genes. Another member of the C/EBP family, LAP/IL-6 DBP, was cloned by its binding to the IL-6 responsive elements of several acute-phase genes (35). It displays high homology with NF-IL-6 and may, therefore, be the rat homologue of human NF-IL-6. LAP/IL-6 DBP is also highly homologous to C/EBP in the basic DNA-binding and the leucine zipper domains. As a result, it can form heterodimers with C/EBP and bind to DNA with the same sequence specificity as the respective homodimers. NF-IL-6 and LAP/IL-6 DBP-binding sites have been found in lipopoly-saccharide-inducible cytokine genes including IL-6, IL-8, TNF, and G-CSF (31,35), and several acute-phase genes including haptoglobin, hemopexin, and C-reactive protein (35). Therefore, these nuclear factors play a key role in the regulation of gene expression in response to inflammation.

When multimerized, the C/EBP-like region I site and the NFκB-like site could confer CM-and TPA-induced expression on a heterologous promoter; however, only the NFκB-like site, was responsive to stimulation by IL-1 and TNF. Site-specific mutation of region I completely abolished the CM- and IL-1-induced expression, demonstrating that while the region I element is not IL-1 responsive, it is still required for the IL-1-induced expression of the SAA1 promoter. Similarly, mutation of the NFκB-like binding site also rendered the SAA1 promoter relatively inactive. Taken together, these results strongly suggest that cytokine-induced expression of the rat SAA1 gene requires the cooperative interaction between nuclear factors at the NFκB- and the C/EBP-like binding sites. Analogous

regulatory elements have been reported for the β-interferon gene. Viral induction of the β-interferon gene is mediated by two virus-inducible activators, NFκB and PRDI, and the release of a repressor (36). The two activators bind distinct DNA sites, but must act together to respond to induction. As single copies, neither site is responsive.

Studies in a number of systems have shown that NFκB can be activated by different stimulating agents in a variety of cell types and that it serves as a transcriptional regulator in the expression of many inducible genes (37). Given the ubiquity of systems using NFκB as a transcriptional regulator, a level of regulation in addition to NFκB activation is needed to ensure that each NFκB-activating signal gives rise to a specific response. This may be achieved by its interaction with other transcription regulators specific for that response. Our studies with the rat SAA1 gene regulation along with those of the β-interferon gene regulation are in accord with this hypothesis. In both cases, the response to a stimulatory signal is achieved by the cooperative interaction between NFκB and a second transcription activator.

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REFERENCES

1. Fey, G.H., and Fuller, G.M. (1987) *Mol. Biol. Med.*, **4**, 323–338.
2. Meek, R.L., and Benditt, E.P. (1986) *J. Exp. Med.*, **164**, 2006–2017.
3. Rienhoff, Jr., H.Y., Huang, J.H., Li, X., and Liao, W.S.L. (1990) *Mol. Biol. Med.*, **7**, 287–298.
4. Benson, M.D., and Aldo-Benson, M. (1979) *J. Immunol.*, **122**, 2077–2082.
5. Benson, M.D., and Kleiner, E. (1980) *J. Immunol.*, **124**, 495–499.
6. Morrow, J.F., Stearman, R.S., Peltzman, C.B., and Potter, D.A. (1981) *Proc. Natl. Acad. Sci., USA*, **78**, 4718–4722.
7. Lowell, C.A., Stearman, R.S., and Morrow, J.F. (1986) *J. Biol. Chem.*, **261**, 8453–8461.
8. Ramadori, G., Sipe, J.D., Dinarello, C.A., Mizel, S.B., and Colten, H.R. (1985) *J. Exp. Med.*, **162**, 930–942.
9. Woo, P., Sipe, J., Dinarello, C.A., and Colten, H.R. (1987) *J. Biol. Chem.*, **262**, 15790–15795.
10. Lowell, C.A., Potter, D.A., Stearman, R.S., and Morrow, J.F. (1986) *J. Biol. Chem.*, **261**, 8442–8452.
11. Gorevic, P.D., and Franklin, E. (1981) *Ann. Rev. Med.*, **32**, 261–271.
12. Rosenthal, C.J. Franklin, E.C., Frangione, B., and Greenspan, J. (1976) *J. Immunol.*, **116**, 1415–1418.
13. Meek, R.L., and Benditt, E.P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1890–1894.
14. Li, X., and Liao, W.S.L. (1991) *J. Biol. Chem.*, **266**, 15192–15201.
15. Huang, J.H., Reinhoff, Jr., H.Y., and Liao, W.S.L. (1990) *Mol. Cell. Biol.*, **10**, 3619–3625.
16. Li, X., Huang, J.H., Rienhoff, Jr., H.Y., and Liao, W.S.L. (1990) *Mol. Cell. Biol.*, **10**, 6624–6631.
17. Edbrooke, M.R., Burt, D.W., Cheshire, J.K., and Woo, P. (1989) *Mol. Cell. Biol.*, **6**, 3173–3179.
18. Darlington, G.J., Wilson, D.R., and Lachman, L.B. (1986) J. Cell. Biol., **103**, 787–792.
19. Maizel, A.L., Mehta, S.R., Hauft, S., Franzini, D., Lachman, L.D., and Ford, R.J. (1981) *J. Immunol.*, **127**, 1058–1064.
20. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
21. Klein-Hitpass, L., Ryffel, G.U., Heitlinger, E., and Cato, A.C.B. (1988) *Nucleic Acids Res.*, **16**, 2647–2663.
22. Kawai, S., and Nishizawa, M. (1984) *Mol. Cell. Biol.*, **4**, 1172–1174.
23. Parker, B.A., and Stark, G.R. (1979) *J. Virol.*, **31**, 360–369.
24. Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
25. Cato, A.C., Miksicek, R., Schutz, G., Arnemann, J., and Beato, M. (1986) *EMBO J.*, **5**, 2237–2240.
26. Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
27. Shapiro, D.J., Sharp, P.A., Wahli, W.W., and Keller, M.J. (1988) *DNA*, **7**, 47–55.
28. Ron, D., Brasier, A.R., Wright, K.A., Tate, J.E., and Habener, J.F. (1990) *Mol. Cell. Biol.*, **10**, 1023–1032.
29. Landschulz, W.H., Johnson, P.F., Adashi, E.H., Graves, B.J., and McKnight, S.L. (1988) *Genes and Dev.*, **2**, 786–800.
30. Mueller, C.R., Maire, P., and Schibler, U. (1990) *Cell*, **61**, 279–291.
31. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) *EMBO J.*, **9**, 1897–1906.
32. Descombes, P., Chojkier, M., Lichtsteiner, S., Falveg, E., and Schibler, U. (1990) *Genes Dev.*, **4**, 1541–1551.
33. Poli, V., Mancini, F.P., and Cortese, R. (1990) *Cell*, **63**, 643–653.
34. Birkenmeier, E.H., Gwynn, B., Howard, S., Jerry, J., Gordon, J.I., Landschulz, W.H., and McKnight, S.L. (1989) *Genes Dev.*, **3**, 1146–1156.
35. Poli, V., and Cortese, R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8202–8206.
36. Leonardo, M.J., Fan, C.M., Maniatis, T., and Baltimore, D. (1989) *Cell*, **57**, 287–294.
37. Leonardo, M.J., Kuang, A., Gifford, A., and Baltimore, D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8825–8829.